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The role of epigenetics in the development of childhood asthma

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Abstract

Introduction: The development of childhood asthma is caused by a combination of genetic factors and environmental exposures. Epigenetics describes mechanisms of (heritable) regulation of gene expression that occur without changes in DNA sequence. Epigenetics is strongly related to ageing, is cell-type specific, and includes DNA methylation, non-coding RNAs and histone modifications.

Areas covered: This review summarises recent epigenetic studies of childhood asthma in humans, which mostly involve studies of DNA methylation published in the recent five years. Environmental exposures, in particular cigarette smoking, have significant impact on epigenetic changes, but few of these epigenetic signals are also associated with asthma. Several asthma-associated genetic variants relate to DNA methylation. Epigenetic signals can be better understood by studying their correlation with gene expression, which revealed higher presence and activation of blood eosinophils in asthma. Strong associations of nasal methylation signatures and atopic asthma were identified, which were replicable across different populations.

Expert opinion: Epigenetic markers have been strongly associated with asthma, and might serve as biomarker of asthma. The causal and longitudinal relationships between epigenetics and disease, and between environmental exposures and epigenetic changes need to be further investigated. Efforts should be made to understand cell-type specific epigenetic mechanisms in asthma.

Key words: asthma, cell types, DNA methylation, environmental exposures, epigenetics

Article Highlights

- Various epigenetic markers in blood and airway epithelium are associated with asthma have been identified.
- DNA methylation may serve as a biomarker of asthma.
- Environmental exposures impact the development of asthma and is associated with epigenetic changes, but it's not clear now if epigenetic regulation mediates the effect of environmental exposures on asthma development.
- Several asthma-associated genetic variants regulate epigenetic signatures, yet their causal role in asthma has not been established.
- Future studies should address the development of tissue and cell-type specific, longitudinal epigenetic patterns, and perform integrative multi-omic analysis to deepen our understanding of asthma development in childhood.

1. Introduction

Asthma is a chronic inflammatory airway disorder, leading to symptoms including repeated episodes of shortness of breath, wheezing, coughing and chest tightness [1-2]. According to the WHO, about 235 million people currently suffer from asthma and asthma is the most common chronic disease among children. The prevalence of asthma in children keeps rising in both developed and developing countries over the last half century [3]. The majority of asthma starts in early childhood. The etiology of childhood-onset asthma may be different from adult-onset asthma. Childhood-onset asthma is usually associated with family history of allergic disease, allergic sensitization, early-life viral infections and microbiome, and showed higher prevalence in boys; while adult-onset asthma is more strongly associated with smoking, obesity and occupational exposures [4]. Besides, the genetic architecture of childhood-onset asthma is partly different from adult-onset asthma [5]. This review will mainly focus on childhood asthma.

The development of asthma results from the interaction of genetic factors with environmental exposures at different stages of development, in particular in early life [6]. The contribution of genetic factors to asthma risk has been demonstrated by twin studies, and the heritability was estimated between 50% and 90% [7]. The genome-wide association analysis (GWAS) has been widely used to identify genetic variants that contribute to disease. The first comprehensive GWAS of asthma reported by Moffatt *et al.* identified genetic variants at chromosome 17q21 regulating *ORMDL3* expression that contribute to the risk of childhood asthma [8]. This study has been followed up by larger meta-analyses, that collectively describe over 130 loci associated with asthma at genome wide significance. For example, a recent meta-analysis of GWAS performed by the Trans-National Asthma Genetic Consortium (TAGC) consortium, which consisted of 23,948 asthma cases and 118,538 controls, identified 18 loci associated with asthma, but explained only 3.5% of the variance in asthma liability [9].

The increase in prevalence reflects that environmental exposures play an important role in the development of asthma. A study in Amish and Hutterite schoolchildren revealed that children exposed to an environment rich in microbes had a low prevalence of asthma [10]. Besides microbial exposure, many other different environmental factors, from *in utero*, early life to later life, have been reported to affect the development of asthma, including (environmental) cigarette smoking, air pollution, pets, molds, diet and dust mites [3, 11-12]. Since environmental factors, especially early in life, may have long lasting effects through epigenetic mechanisms, epigenetic studies of asthma have sparked a lot of interest.

Epigenetic regulation is determined by genetic factors and also responsive to environmental exposures, and may help to build a link between these two factors. Epigenetics, literally means ‘above the genetics’, usually describes mechanisms of heritable regulation of gene expression without a change of the DNA sequence itself. Epigenetic marks can be

heritable from one cell generation to the next after cell division, or from one organism generation to the next [13]. Epigenetic processes are in general changeable, cell-type and tissue specific and can occur in response to the environment, aging, disease and other factors [14]. There are three main types of epigenetic regulatory mechanisms: DNA methylation, histone modifications and non-coding RNAs, such as microRNAs (Figure 1).

DNA methylation occurs when a methyl group (CH_3) is added to a cytosine nucleotide by DNA methyltransferases (DNMTs), resulting in 5-methyl-cytosine (5mC). This is usually done when a cytosine located next to a guanine in the 5' to 3' direction, which is called CpG (cytosine-phosphate-guanine) site [15]. The CpG occurs at a higher frequency in regions known as CpG islands, which are often found in promoter regions and generally remain unmethylated [16]. DNA methylation is often associated with gene repression and may relate to the development of disease [17]. Methylation of CpG islands in promoter regions may contribute to gene silencing, as has been shown in cancer [18]. Over 80% CpG dinucleotide sites are located outside of CpG islands, and CpG sites within gene bodies also plays an important role in the complex regulation of gene expression and alternative splicing [19].

Histones are octameric proteins that organize chromatin. A histone modification is a covalent post-translational modification of the N-terminal tail of histone proteins, including acetylation, methylation, phosphorylation and ubiquitylation [20]. Histone modifications are important in transcriptional regulation, DNA repair and replication, alternative splicing and chromosome condensation [21]. Histone acetylation is the process by which lysine residues on histone tails are acetylated via histone acetyltransferases. Higher histone acetylation levels increase the accessibility of DNA to the transcriptional machinery leading to higher gene expression [22]. Histone methylation usually occurs at lysine or arginine residues of histones mediated by histone methyltransferases. The influence of histone methylation on gene expression depends on where is the amino acid residue located and how many methyl groups

are added [22]. There is close biological relationship and interaction between histone modification and DNA methylation [20].

Diverse classes of small and non-coding RNAs have shown to be key regulators of gene expression, genome stability and defence against foreign genetic elements, contributing to another layer of epigenetic regulation [23]. Micro RNAs (miRNAs), one of small non-coding RNAs, are single-stranded RNAs with around 19-24 nucleotides, targeted on 3' untranslated mRNA region leading to degradation or translational inhibition [24-25]. RNA regulation also shows interaction with both DNA methylation and histone modification [23].

Recently, epigenetic studies have emerged as an important field in childhood asthma research. In this review, publications were retrieved after searching PubMed using the key words “child”, “asthma” and “epigenetics or DNA methylation or histone or micro RNA” in the past 5 years (as of September 2019). We will summarize the recent studies in humans and discuss future perspectives of this field and mainly focus on the best studied epigenetic phenomenon in asthma so far: DNA methylation.

2. Recent studies of epigenetics and asthma development

2.1 Studies of DNA methylation and asthma

The associations between DNA methylation and diseases can be identified by epigenome-wide association study (EWAS). The global human DNA methylation profile is usually measured by DNA methylation arrays, including the Infinium Human Methylation 450K BeadChip which covers over 450,000 CpGs spread across the whole genome, and the Infinium MethylationEPIC BeadChip which covers around 850,000 CpGs. DNA methylation at a single CpG can be measured by targeted pyrosequencing, which can also be used in validating CpGs identified by EWAS. In EWAS, where hundreds of thousands of CpGs are being evaluated, multiple testing issues should be addressed to reduce false positive findings.

A simple and strict way to adjust for the number of tests is the Bonferroni correction (the significance level is divided by the number of tests), while false discovery rate (FDR) is a more widely used and less conservative method, which controls for the expected ratio of false findings [26].

DNA methylation changes may derive from different sources including aging, regulation by genetic variation, environmental exposures, cell type effects and cell type activation (Figure 2), which will be discussed in detail later in this review. Some of these factors might potentially confound the association between DNA methylation and asthma, and should be adjusted for during the analyses. Traditionally, age and sex are adjusted for by adding these to the regression models as covariates. Besides these, inhaled or nasal corticosteroids (ICS or nasalCS) usage as well as smoking behaviour may affect DNA methylation and should be considered for adjustment. Moreover, DNA methylation is cell type specific, and most of EWASs are performed in tissues consisting of a mixture of different cell types. This cell-type heterogeneity could be a major confounder in EWAS [27], but conversely, epigenetics could also be used as a sensitive marker of cell type composition. The Houseman's method has been widely used to estimate cell type composition of white blood cells using DNA methylation signatures which can be used for adjustment [28]. Moreover, in EWASs of airway structural cells, where no published cell specific DNA methylation reference datasets are available, correcting for cell type heterogeneity by other methods such as Principal Component Analyses or Surrogate Variable Analysis should be considered.

Recently, many EWASs identified differentially methylated CpG sites and CpG regions associated with asthma in whole-blood, buccal cells, bronchial and nasal epithelial samples, which may help to understand the role of DNA methylation in asthma pathogenesis. Study details of DNA methylation studies of asthma mentioned in this review are summarized in Table 1.

A recent and well-powered EWAS of whole-blood DNA methylation and childhood asthma within Mechanisms of the Development of ALLergy (MeDALL) project, that consisted of 1548 children (392 cases and 1156 controls) in total from four different European birth cohorts. Using a cross-sectional design, this study identified that asthma across childhood were consistently associated with reduced DNA methylation levels at 14 CpG sites, but methylation levels of 14 CpGs in cord blood at birth didn't show significant association with early childhood asthma, indicating these asthma-associated DNA methylation signals developed after birth [29]. All 14 CpG sites were replicated in seven cohorts using a different laboratory method. This study went on to show that these 14 CpG sites were also strongly associated with asthma in purified eosinophils. This indicated that DNA methylation differences in asthmatics compared to healthy children were mainly driven by eosinophils, which stressed the importance of eosinophil epigenetic profile in childhood asthma. Genes correlated with the 14 CpG sites were not only related to increased activity of eosinophils but also related to cytotoxic T cells, which may relate to the response to viral infections or air pollution.

A subsequent, even larger EWAS meta-analysis by the Pregnancy and Childhood Epigenetics (PACE) consortium identified differential DNA methylation in blood associated with asthma in newborns and children [30]. PACE is a worldwide consortium that investigates the early life environmental impacts on human disease using epigenetics [31]. The PACE asthma study combined a prospective design (newborn blood DNA methylation in relation to subsequent asthma development in childhood) with a cross-sectional design. In the prospective analysis in eight cohorts, nine significant CpGs and 35 regions ($FDR < 0.05$) were identified to be associated with childhood asthma in newborns (668 cases and 2,904 controls), indicating that newborn blood DNA methylation may predict risk of asthma in later life. None of the nine CpGs had been previously associated with asthma nor showed overlap with

the findings of the cross-sectional design, and the different CpGs identified in cord blood and in later life may indicate that DNA methylation at birth could reflect intra-uterine exposures and mechanisms that are important in the inception of disease rather than in persistence of asthma. However, these findings need further replication and validation. In the cross-sectional meta-analysis of asthma and whole blood DNA methylation in children aged 7 to 17 years (631 cases and 2,231 controls) from nine cohorts, 179 CpGs and 36 regions (FDR<0.05) were associated with childhood asthma. These CpGs were also replicated in purified eosinophils, confirming the findings from the MeDALL study. Several CpGs were annotated to genes previously associated with asthma, for example *IL5RA*, which is the target for an approved drug for severe asthma patients.

Similarly, the association between peripheral blood DNA methylation and asthma was evaluated in the ALSPAC cohort at two time points (7.5 and 16.5 years) [32]. At 7.5 years, 302 CpGs were associated with current asthma and genes annotated to these CpGs were enriched for pathways related to movement of cellular/subcellular components, locomotion, interleukin-4 production and eosinophil migration. However, none of the CpGs remained significant after adjusting for eosinophil and neutrophil cell count estimates and the associations between DNA methylation and asthma were driven by high eosinophil cell counts in asthma patients, which stresses the importance of cell type heterogeneity in the analysis of whole blood DNA methylation. At 16.5 years, only 2 CpGs, which annotated to *AP2A2* and *IL5RA* genes, were associated with current asthma, but some CpGs identified at 7.5 years (e.g. cg04983687 in the *ZFPM1* gene) remained nominally significant in two longitudinal associations (current asthma at 16.5 years ~ methylation at 7.5 years, and current asthma at 7.5 years ~ methylation at 16.5 years) with the same direction of effect.

Next to blood cells, which contain important cells relevant for immune responses in asthma, including T helper 2 (Th2) cells and eosinophils, epigenetic studies of the airway

epithelium also attract interest. The airway epithelium is the primary interface with the environment and the first line of defence against inhaled harmful substances and aeroallergens [33]. However, bronchial epithelial samples are difficult to collect, specifically in children. Proxy tissues of bronchial epithelial cells with DNA methylomes that are comparable to bronchial epithelium are searched for. For example, buccal cells have been studied as proxies for airway epithelial cells. A study performed in buccal cells from 37 monozygotic twin pairs identified nominally significant differentially methylated positions that associated with childhood asthma, and the longitudinal studies indicated that DNA methylation differences associated with persistent asthma from childhood to early adulthood were different from those associated with asthma which remits. However this study didn't identify probes that reached genome-wide significant threshold, which may be due to the small sample size [34]. A hierarchical clustering study showed that nasal epithelium was more representative of bronchial epithelium than blood DNA methylation, and was a better proxy tissue than blood and buccal cells [35]. Yang *et al.* performed an EWAS of nasal DNA methylation and atopic asthma in 72 African-American children (36 cases and 36 controls), which identified 186 differentially methylated genes (including both CpG sites and regions). These CpGs were annotated to genes related to asthma and atopy, as well as to extracellular matrix, immunity, cell adhesion, epigenetic regulation, airway obstruction, obesity and autophagy [36]. This study provided a basis for nasal DNA methylation studies in larger populations.

Recently, EWAS of DNA methylation in nasal epithelium and atopy (i.e. presence of serum specific IgE to aeroallergens) or atopic asthma were performed in a study of 483 Puerto Rican children and 8664 CpG sites were significantly associated with atopy and atopic asthma [37], showing strong signals of DNA methylation in nasal epithelium. Twenty-eight out of the top 30 CpG sites associated with atopy were replicated in the US Inner City study

and the Dutch Prevention and Incidence of Asthma and Mite Allergy (PIAMA) birth cohort. Some of the associated CpGs were annotated to genes related to epithelial barrier function (*CDHR3* and *CDH26*) and immune responses (*FBXL7*, *NTRK1*, and *SLC9A3*). Moreover, a 30-CpG panel was designed to classify children according to atopy or atopic asthma and was also well replicated in the PIAMA and Inner City Asthma cohorts, although the ethnicity of the three study populations was markedly different: Puerto Rican, European and predominantly African American. These findings showed that the nasal DNA methylation may serve as an important biomarker that is applicable across different ethnicities to identify children that have atopy or atopic asthma. Similarly, another recent EWAS of asthma and airway inflammation in nasal epithelium was performed in a population of 547 children, and 285 CpGs associated with asthma as well as 1,235 CpGs and 7 regions associated with allergic asthma were identified. Some of the regions were annotated to gene that previously associated with asthma and IgE in blood (*ACOT7*, *SLC25A25*) and genes related to Th2 activation and increase in eosinophils (*EPX*, *IL4*, *IL13*), also suggesting DNA methylation may be a sensitive biomarker for asthma and allergic diseases [38].

Although these studies may differ in study design, study population and analytical methods, there are several consistent signals of DNA methylation associated with asthma. For example, CpGs or regions annotated to the *interleukin-5 receptor alpha gene (ILR5A)* (in PACE study [30], UK study [32]) and the *SLC25A25* (in MeDALL study [29], PACE study [30]) gene encoding a mitochondrial transport protein, were identified by different studies in blood. CpGs or regions annotated to the *GJA4* gene (US study in African American children [36], study in Puerto Rican children [37], US study [38]), which encodes a component of gap junctions and *Methyltransferase like 1 gene (METTL1)* gene (in US study in African American children [36], study in Puerto Rican children [37], US study [38]) showed up in different nasal EWASs. Besides, some CpGs also showed consistency in different tissues, for

example, CpGs or regions annotated to *ACOT7*, encoding an enzyme that hydrolyzes long chain fatty chain acids and is potentially involved in Prostaglandin synthesis (in PACE study [30], US study [38]), *Eosinophil Peroxidase (EPX)* (in PACE study [30], UK study[32], US study[38]) and *EVL* (in PACE study [30], US study[38]) gene thought to be involved in cytoskeleton remodelling and cell polarity, were identified in EWASs of both blood and nasal brushes cells.

2.2 Studies of histone modification and asthma

Histone modification, a classical epigenetic regulation that modifies the accessibility of chromatin, also has a relation with asthma. Histone modifications are likely to play a role in asthma development by influencing differentiation and maturation of immune cells including those involved in asthma such as CD4⁺ T-helper cells, for example, H3K4me3/H3K27me3 were reported to be associated with T helper cell differentiation and IL-5 expression [39]. A genome-wide histone modification study in isolated T cells from healthy (n=12) and asthmatic (n=12) subjects identified 200 enhancer regions in three cell types (naïve T cell, Th1 cell and Th2 cell) that showed differential enrichment in demethylation of the lysine residue at 4th position on the N-terminal tail of histone 3 (H3K4me2), a marker of gene activation in promoter and enhancer regions [40]. Most of the regions (163 regions) were specific to Th2 memory cell population, only small numbers specific to naïve T cell (29 regions) and Th1 cells (11 regions). Th2 cell-specific enhancers that obtained the H3K4me2 mark were highly enriched for asthma-associated SNPs. These findings supported a pathogenic role of Th2 cells in asthma. Another study of histone acetylation in isolated CD4⁺ T cells from 14 children with allergic asthma and 18 healthy controls identified higher histone 3 acetylation levels at the *IL13* and *FOXP3* locus in asthmatic children than in controls. Histone acetylation is an epigenetic marker for transcriptional activation, and in this

study, the association between *IL13* H3 acetylation and higher protein level of IL13 showed potential regulatory role of *IL13* acetylation on protein secretion [41].

Some other studies also identified associations between histone modifications and asthma in airway epithelial cells. Wawrzyniak *et al.* identified higher expression levels of histone deacetylases (HDACs) 1 and 9 in primary human bronchial epithelial cells from asthmatic patients (n=18) than controls (n=9), and *IL-4* and *IL-13* significantly increased the expression of HDACs [42]. The results showed that the inhibition of HDAC activity had protective effect on epithelial barrier integrity by increasing tight junctions expression, which may reduce epithelial barrier leakiness in asthma patients. Global histone acetylation and methylation status in airway epithelial cells was measured in 5 asthmatics and 5 healthy controls [43]. Higher level of the acetylation of lysine 18 on histone 3 (H3K18ac) and trimethylation of lysine 9 on histone 3 (H3K9me3) was identified in the airway epithelium in asthma patients than healthy controls, and increased association of H3K18ac around the transcription start site (TSS) of *ΔNp63* (one alternative promoter of the gene encoding P63), *EGFR*, and *STAT6* was found in asthmatics. However, modification on histone acetylation of these genes by HDAC inhibitor didn't significantly increased the expression of these genes in airway epithelial cells of healthy controls, which couldn't confirm the regulation role of the histone acetylation on expression of these genes.

2.3 Studies of non-coding RNA and asthma development

Non-coding RNA, such as microRNA (miRNA) is relevant in post-transcriptional control and regulation of pathological processes in disease. Many miRNAs have been identified to be associated with asthma in various tissues including blood cells, airway epithelial cells and smooth muscle, and some miRNAs are considered to be potential biomarkers and promising therapy targets for asthma [44-45]. As reviewed previously, some asthma-associated

miRNAs may target genes related to immune function (such as miR-155, miR-210, miR-21 and miR-19a), and some may function on genes related to airway function (such as miR-140-3p, miR-708 and miR-10) [44]. For example, a recent study of miRNA expression profiles in IL-22- and IL-17-positive T cells sorted from human PBMCs showed higher expression levels of miR-323-3p in asthmatics and a reverse correlation between miR-323-3p levels and IL-22 production, suggesting a negative feedback mechanism of miR-323-3p that controls the production of IL-22 in IL-22/IL-17-producing T cells in asthma [46].

Serum miR-21 was identified to be associated with childhood asthma or allergic inflammation and may serve as a biomarker for asthma in children [47–49]. For example, a study performed in 95 asthmatic children and 80 healthy children identified serum miR-21 level was higher in asthmatics compared with controls, and higher level was also found in asthmatics without ICS or in steroid resistant patients compared to steroid sensitive children, which indicate miR-21 may also help to distinguish ICS sensitive and ICS resistant asthma patients [48]. Similarly, a recent miRNA study performed in 27 asthmatic children and 21 healthy controls also revealed the increased level of plasma miR-21 and miR-146a in asthmatics compared with controls. Both miR-21 and miR-146a were associated with increased eosinophils percentage suggesting these two miRNAs to be potential biomarkers for eosinophilic endotype of asthma [49].

Another study performed within 100 asthmatic children and 100 healthy controls identified increased levels of miR-155 and decreased level of Let-7a in peripheral blood of asthmatics than controls. The relative levels of miR-155 and Let-7a also performed well in differentiating severe asthma from mild and moderate asthma [50]. MiR-155 was reported to play an important role in regulating type 2 innate lymphoid cells and IL-33 signaling of allergic airway inflammation in murine models [51]. Let-7 miRNA family was reported to target on IL13 and showed anti-inflammatory role in an allergic asthma model in mice [52].

These findings suggested that miRNA might become a non-invasive biomarker in diagnosis of asthma in children and also help to understand the molecular mechanism of endotypes in asthma.

3. Environmental epigenetics in asthma development

Environmental exposures, especially in early life, play an important role in the development of asthma. Some environmental exposures, such as air pollution, cigarette smoking, and early life respiratory viral infections are risk factors for the development of asthma, whereas others have protective effects, including exposure to pets, prolonged breast feeding and a higher diversity of the microbiome in early life [12, 53–57]. Besides, some *in utero* exposures such as maternal nutrition, maternal disease (e.g. asthma and obesity) during pregnancy also have an impact on childhood asthma development [58–60].

Several studies showed overlapping epigenetic associations between environmental exposures and asthma, indicating that the impact of environmental exposures on asthma development might be mediated through epigenetic regulation (Table 2).

3.1 Cigarette smoking

Both passive and active exposure to tobacco smoke may reduce lung function and increase the risk of developing asthma and other lung diseases, and also showed large effects on DNA methylation of cord blood or whole blood DNA. The largest EWAS meta-analysis of newborn blood DNA methylation and maternal smoking in pregnancy by the PACE consortium (n=6,685) identified 2,965 CpGs associated with maternal smoking, including two CpGs in the genes encoding *ESR1* and *IL-32*, which were genes previously reported to be associated with asthma [61]. Variants in *ESR1* gene were previously associated with lung function decline in asthma patients especially in females [62]. *IL-32* is a proinflammatory

cytokine and its levels were higher in serum of asthma patients than controls [63]. Bauer *et al.* showed the DNA methylation signal in relation to maternal smoking during pregnancy was persistent in both mothers and children up to 4 years after birth. They also identified differential methylation was enriched in “commuting” enhancers (an enhancer located in one gene but act on other distal genes), and that the loss of DNA methylation in commuting enhancer of *JNK2* was linked to the development of asthma [64]. *JNK2*-regulated suppressive activity of naturally occurring regulatory T cells was related to airway inflammation and impaired lung function [65]. Another EWAS of active smoking in small airway epithelial cells identified 204 unique genes that were differentially methylated in smokers compared to non-smokers, including *CYP26A1* which was reported to be highly methylated in small airway epithelial cells of allergic asthma patients [66]. However, the strongest pathways that are differentially methylated upon smoke exposure are detoxification pathways that include genes such as *AHRR*, which have no clear relation to asthma. Thus, these epigenetic marks mostly reflect the host response to the toxic substances that are part of tobacco smoke.

Studies also showed the influence of cigarette smoking on the expression of miRNA [67]. For example, lower level of miR-21 in peripheral blood was identified in smokers compared with non-smokers, and the lower level of miR-21 was also associated with increased level of IFN- γ in smokers [68]. Some active smoking-associated miRNA also have been related to COPD [69], but not asthma.

3.2 Air pollution

Traffic and power generated air pollution can cause the exacerbation of asthma and also contribute to new-onset asthma [70]. Exposure to air pollution, such as nitrogen dioxide (NO₂) and particulate matter (PM) could affect DNA methylation, including genes associated with asthma [71–73]. Recently, a large EWAS meta-analysis of prenatal air pollution and newborn

blood DNA methylation identified six CpGs associated with prenatal PM₁₀ (particulate matter 10 micrometers or less in diameter) and 14 with PM_{2.5} (particulate matter 2.5 micrometers or less in diameter) exposure (FDR<0.05), and two CpGs associated with PM₁₀ were annotated to gene *FAM13A* and *NOTCH4* which were previously reported to be related to asthma and lung function [74]. Some studies also focused on airway epithelium, which is the primary interface with inhaled air, that may include air pollution. One study of the epigenetic impact of PM_{2.5} on human bronchial epithelial BEAS-2B cell line identified 5501 CpGs that were differentially methylated in PM_{2.5}-exposed BEAS-2B cells, and 45 CpG loci were annotated to 43 miRNAs, including two miRNAs (miR-126 and miR-485) previously related to asthma [75]. In another observational study, Sominen *et al.* identified lower methylation level in *TET1* gene in nasal airway epithelium was associated with childhood asthma and traffic-related air pollution [76]. In population studies, the epigenetic effects of air pollution appear less strong than cigarette smoking, which may also relate to difficulties in exposure assessment of air pollution at a personal level. Future innovations, such as measuring personal exposures by wearable technology, may improve air pollution assessments.

3.3 Microbiome and viral infections

The microbiome in our environment may affect the development of the immune system in childhood and thus relate to susceptibility of allergic disease including asthma [77]. Exposure to an environment rich in microbes and endotoxins, such as on classical farms, was associated with reduced risk of asthma [10, 78]. The microbiota might influence host immune system through multiple mechanisms including epigenetic regulation [79]. For example, Obata *et al.* identified that microbial colonization induced expression of DNA methylation adaptor Uhrf1 in regulatory T cells (Treg cells) in mice [80]. Treg cells play an important role in reducing inflammatory responses and airway hyper-responsiveness, and can be

characterized by *FOXP3* expression [81]. Another study focusing on maternal farm exposure identified demethylation of *FOXP3* in cord blood cells of offspring with mother exposed to farm milk [82].

Apart from bacteria, virus infection also influences the development of asthma. For example, respiratory syncytial virus (RSV) infection in early life was associated with an increased risk of early childhood wheezing and asthma development [83-84]. DNA methylation signals were identified to be associated with rhinovirus-associated atopic asthma in whole blood of children, and the strongest association was identified in the promoter region of *SMAD3* gene, which is involved in the regulation of immune responses [85]. Studies also have shown that histone methylation status in immune cells can be affected by respiratory viral infection, for instance, the upregulation of H3K4 Demethylase KDM5B was identified in marrow-derived DCs induced by RSV infection [86-87]. RSV infection also influences the expression of miRNAs. Inchley *et al.* identified seven miRNAs were downregulated and five miRNAs were upregulated in nasal epithelium from RSV-positive infants compared to healthy controls, and many of the miRNAs (e.g. miR-29c, miR-27b, miR-34b, miR-34c, let-7d and miR-203a) were previously associated with asthma [88]. The roles of these epigenetic markers on the mechanisms underlying the association between RSV infection in early life and later development of asthma still need further studies.

3.4 *In utero* exposures

Apart from the environmental risk factors for asthma mentioned above, some other *in utero* exposures may also be associated with offspring asthma development, for that *in utero* exposures might affect lung growth and immune system development of the offspring [89]. For example, a previous systematic review summarized the studies of association between nutrition intake during pregnancy and asthma, and concluded that maternal intake of vitamin

D, vitamin E, and zinc had protective effect on childhood wheeze, although not on asthma [58]. Maternal obesity was reported to be associated with increased risk of childhood asthma, and the association might be explained by epigenetic regulation [59]. However, few studies have investigated the role of epigenetics in the association between these *in utero* exposures and childhood asthma. One study showed that maternal asthma influenced the epigenetic modifications of their offspring and could contribute to childhood asthma [60]. The authors identified the methylation level in *SMAD3* promoter in cord blood mononuclear cells was increased in asthmatic children with asthmatic mothers, and was associated with childhood asthma. Methylation level of *SMAD3* in newborns with asthmatic mothers was positively correlated with an innate inflammatory mediator IL-1 β level in newborns, indicating the influence of asthmatic mothers on childhood asthma was related to epigenetic regulations in immune regulatory and pro-inflammatory function. Differentially methylated region (DMR) in promoter-regulatory region of *PM20D1* gene was identified to be associated with wheezing in saliva collected from infant at the age of 6-18 months, and the associations were stronger in children with atopic mothers [90]. Although the function of *PM20D1* gene, which is involved in the synthesis of N-acyl amino acids from free fatty acids and free amino acids, was not well explained in asthma, *PM20D1* gene was identified to be lower methylated in peripheral blood from infants born to asthmatic mothers than to healthy mothers [91].

3.5 Environmental allergens

Environmental allergens from house dust mite (HDM), furred pets, mice, and cockroaches during early life may also influence the development of allergic disease like asthma in later life [55]. Exposure to high levels of HDM during early life were associated with higher risk of asthma, and exposure to mouse and cockroach allergen were reported to be correlated with allergic disease prevalence and severity [55]. Studies on pets allergens didn't show a

consistent conclusion, some studies identified protective effect of exposure to pets in early life, while some studies identified opposite effect or no significant difference [92-93].

Limited studies focused on epigenetics changes in relation to environmental allergens. Zhang *et al.* identified changes in methylome and hydroxymethylome after HDM challenges in human airway bronchial epithelial cells, and these epigenetic changes are located in genes related to oxidative stress response, epithelial function and immune responses [94]. Lower mouse allergen level was identified to be associated with reduced *FOXP3* DNA promoter methylation in buccal cells from asthmatic children, and methylation of *FOXP3* gene was previously associated with asthma [95]. miR-155 was identified to be increased in cockroach extract treated human bronchial epithelial cells, and also increased in plasma of asthmatics with cockroach allergy compared with those without cockroach allergy [96]. This study showed that miR-155 might play an important role in cockroach allergen-induced oxidative stress in asthma through regulating *Cyclooxygenase-2* expression.

Although many studies investigated the association of epigenetic regulation and environment, and the correlation between asthma and epigenetic regulation, only few epigenetic signatures were identified to be associated with both environmental exposure and asthma. Besides, there are still several limitations in current studies, that include the difficulty to move from an association to a causation: an environmental factor may relate to epigenetics modifications and to asthma, but this does not necessarily imply that the asthma effect is mediated through epigenetics. Longitudinal data and robust causal inference methods are needed to address this issue. Current studies are mainly from small cohorts or animal experiments, comprehensive studies and integrative analysis on the interaction of environment and epigenetic regulation in asthma development in large cohort studies are still lacking. Studies are also needed to

explore the potential role of epigenetics in the relationship between these *in utero* exposures and childhood asthma.

4. Interpretation of epigenetic regulation in asthma studies

4.1 Epigenetic changes regulated by genetic variants

The variation in epigenetic markers between healthy subjects and asthma patients can be influenced by underlying genetic differences. Genetic variants such as SNPs (Single Nucleotide Polymorphisms) may regulate epigenetic changes. For example, the effect of SNPs on DNA methylation can be identified by meQTL (methylation quantitative trait loci) analysis, which compares the methylation levels of a given CpG site for each genotype group. The BIOS consortium investigated the association of SNPs and DNA methylation in 3,841 individuals within a limited distance (*cis*-meQTL). These authors identified *cis*-meQTL effects for 34.4% tested CpGs (n=405,709) in whole blood DNA. However, the proportion of methylation variance explained by SNPs is limited. Moreover, one-third of SNPs, which previously associated with complex traits and disease, affect CpGs *in trans* (long distance between SNP and CpG), and these CpGs identified by *trans*-meQTL analysis showed enrichment around TSSs of genes and depletion in heterochromatin, which may also affect gene transcription [97].

Several studies showed the potential regulation of asthma-associated SNPs on nearby DNA methylation. SNPs in Interleukin-1 receptor-like 1 (*IL1RL1*), which encodes a Toll-like/IL-1 receptor expressed on inflammatory cells, as well as a soluble isoform IL1RL1-a, were also associated with methylation of four *IL1RL1* CpGs in whole blood. However, *IL1RL1* methylation was not related to IL1RL1-a protein expression [98]. Studies in airway epithelial cells identified that the asthma-associated SNP rs1837253, near the *TSLP* gene, was associated with four CpGs located in *TSLP* gene [99]. This gene encodes an epithelial cell

cytokine which is involved in the inflammatory response in asthma. A chitinase-like protein YKL-40, encoded by *CHI3L1* gene showed upregulation in blood samples of asthma patients and was previously reported to be under genetic regulation. A recent study from Guerra *et al.* indicated that DNA methylation of *CHI3L1* gene can partly mediate the effect of *CHI3L1* SNPs on circulating YKL-40 levels in blood by mediation analysis [100]. YKL-40 was shown to play a role in Th2 adaptive immune responses in mice [101]. Similarly, local DNA methylation mediated part of the effect of *cis* asthma-associated SNPs, in 17q21 locus, on the expression level of *GSDMB* and *ORMDL3* gene in whole blood and CD4⁺ T cells, which was identified by causal inference testing by Kothari et al [102].

Thus, several asthma-associated SNPs have been shown to regulate DNA methylation, and some of these methylation sites were shown to be associated with gene expression levels. However, it remains to be determined if there exist a causal pathway from SNP to methylation to gene expression to asthma.

Apart from DNA methylation, histone modification and non-coding RNA may also be under the regulation of genetics, and associated with complex diseases [103-104]. A study performed in mice showed that microRNAs associated with allergic airway disease were regulated by genetic loci. For example, rs264778660 located in miR-342-3p might influence the maturation and expression of miR-342-3p [105] and this miRNA was previously associated with human lung function [106].

4.2 Relationships between epigenetic regulation and gene expression

DNA methylation has a close relationship with gene expression, and this correlation can be assessed by eQTM (expression quantitative trait methylation) analysis. In cancer research, when the CpG island located on the promoter region of a gene, high methylation (hypermethylation) typically leads to transcriptional silencing of tumour suppressor genes.

However, about half of the CpG islands are located in gene body or intergenic region rather than promoters, which also play a role in regulation of gene expression such as regulating non-coding RNAs [15]. The relationship between DNA methylation and gene expression in complex disease can be more complex than that in cancer. For example, DNA methylation is not always negatively correlated with gene expression, and DNA methylation changes may be a consequence rather than cause of changes in gene expression.

An eQTM study performed by BIOS consortium in blood identified 12,809 unique CpGs in correlation with 3,842 unique genes in *cis*, and only 69.2% of these eQTMs showed typical negative correlation. eQTMs which showed negative correlation were usually enriched in active regulation region of a gene such as active TSSs, while positive correlations were mainly enriched in repressed regions (regions that related to repression of transcription, for example, repressed polycomb regions) [97].

Another question in eQTM analysis is: are the changes of DNA methylation the cause or consequence of gene expression changes? Whereas the classical dogma is that gene methylation regulates gene expression, recent studies evidenced that DNA methylation changes may happen after changes in gene expression. Pacis *et al.* identified lower DNA methylation level in dendritic cells post *Mycobacterium tuberculosis* infection, but the detectable losses in methylation occurred after the changes in gene expression, indicating the demethylation was a downstream consequence of transcriptional activation [107]. The results also suggested that DNA demethylation in response to infection could play a role in facilitating the binding of methylation-sensitive TFs (Transcription Factors) at enhancers and may relate to innate immune memory.

eQTM analysis were widely used to help understand the function of CpGs associated with asthma or other allergic diseases. However, most of the studies were limited to association analysis with few studies that revealed the causal relationship between epigenetic changes

and gene expression changes in context of asthma. Xu *et al.* identified that asthma-associated CpGs significantly associated with gene expression in whole blood, and identified the activation of eosinophils and cytotoxic T cells in childhood asthma by clustering of methylation signals with gene expression profiles [29]. This study indicated that DNA methylation patterns can reveal activated cell subsets in blood, such as eosinophils in asthma. Nicodemus-Johnson *et al.* identified that methylation level changes of CpGs induced by IL13 in airway epithelial cells were correlated with nearby gene expression, and those CpG-gene pairs were enriched for genes previously associated with asthma [108]. This group also identified correlations of gene networks with specific characteristics of asthma, such as asthma severity, exhaled NO, use of ICS and eosinophilia, by integrating CpG methylation with expression of closest genes, which helped to understand mechanisms of asthma endotypes [109]. Forno *et al.* identified 1570 CpG-gene pairs which showed the association between atopy-associated CpGs and gene expression in nasal epithelial cells, and the mediation analysis showed that 40% of top 30 eQTM genes showed significant mediation in the pathway between methylation and atopy [37].

4.3 Epigenetics in aging and cell-type specific epigenetics

Epigenetic markers play a crucial role in changes of cellular functions observed during aging, for example, mammalian aging is more commonly associated with decreased DNA methylation [110]. Xu *et al.* identified 14,150 age-differential methylation sites (a-DMSs) in blood cells from children at birth and at 4–5 years old (538 pairs), and children at 4 and 8 years old (726 pairs), and four a-DMSs were associated with childhood asthma, which still need replication; they also identified the association between maternal smoking during pregnancy and decreasing DNA methylation level from birth to 4/5 years old, indicating that

the effect of maternal smoking during pregnancy on aging might be mediated by decreasing methylation levels [111].

Epigenetic markers are also involved in the process of cell differentiation, and epigenetic signatures relate strongly to the function of differentiated cell types [112]. A clear example is the T helper cell differentiation, that decreased level of DNA methylation at genetic enhancers in the Th2 locus and increased histone acetylation at Th2 cytokine loci were identified during Th2 differentiation [113].

Disease-related changes in epigenetic profiles of tissues with mixed cell types may be due to the epigenetic changes within a cell type, or due to disease associated changes in cell type composition. For example, asthmatic patients tend to have more goblet cells in their bronchial epithelium than healthy people [114]. The cell-type specific epigenetic changes can be assessed by using sorted or purified cells. Liang *et al.* identified the partial associations of IgE-associated loci with eosinophil numbers, and that IgE-associated CpGs were also differentially methylated in isolated eosinophils from subjects with and without high IgE levels [115]. The asthma-associated loci identified in whole blood by Xu *et al.*, also showed strongly association with asthma in purified eosinophils, indicating that DNA methylation differences between asthmatics and controls in whole blood were mainly driven by eosinophils [29].

Many bioinformatic methods have been established to help detect cell-type specific methylation status identified by EWAS. eFORGE can be used to identify cell-type specific signals in heterogenous EWAS data as well as cell-composition effect [116], and CellDMC can detect the specific cell types driving differential methylation [117].

Sequencing at single-cell level may also enable researchers to approach this issue. scRNAseq (single-cell RNA sequencing) has been successfully applied in identifying cell-type specific gene expression profile and showed its potential in identifying new cell states

associated with disease. Since the eQTM analysis can link gene expression to DNA methylation, it might be helpful to understand cell-type specific DNA methylation through eQTM and scRNAseq. New single-cell techniques have become available in the single-cell epigenetics field, such as single-cell transposase-accessible chromatin (scATAC-seq), which works robustly in both fresh and cryopreserved cells [118]. Other methods such as single-cell bisulfite sequencing (scBS) [119] and reduced-representation bisulfite sequencing (scRRBS) [120], were newly developed to help to explore DNA methylation profile more directly in different cell types. However, there are still some limitations of these single-cell DNA methylation techniques, such as technological limitations, high cost and lack of well-established analytical methods, which need to be overcome in the future.

5. Expert opinion

Epigenetic markers, including DNA methylation, histone modification and regulation of non-coding RNAs have been identified to be associated with childhood asthma, and might serve as a biomarker of asthma. Remarkably strong associations of DNA methylation with atopic asthma have been found, in particular in blood eosinophils and nasal cells. These findings indicate potential diagnostic utility of DNA methylation patterns in childhood asthma.

Most epigenetic studies of asthma up to now are cross-sectional studies, and could not infer whether the epigenetic profiles identified are cause or consequence of asthma. Thus, at current it cannot be inferred of these epigenetic profiles should be target of future asthma treatments. However, DNA methylation may serve as a good biomarker of eosinophilic asthma, which could be explored in relation to predicting response to targeted therapy using monoclonal antibodies, such as anti IL5. Information if DNA methylation or other epigenetic modifications are causally implicated in asthma is urgently needed. Longitudinal studies are

needed to evaluate the dynamic changes of epigenetic markers during the development of asthma, and need to address if cell specific epigenetic signatures could be used in early diagnosis of childhood asthma.

One hypothesis about epigenetics is that environmental factors can influence the development of disease through epigenetics. However, it is still not clear whether epigenetics mediates these environmental effects. Although some studies showed epigenetic markers associated both asthma and environmental exposures, the causal relationship have not been explained fully. More information is needed on the longitudinal epigenetic regulation by environmental factors relevant for asthma, such as smoking, viral infections, microbiome and air pollutants, and how this affects gene expression in asthma development.

It is important to consider which tissue to focus on in epigenetic studies of asthma. Recent studies identified strong DNA methylation signals associated with atopic asthma in nasal epithelium. Nasal epithelium is easily accessible especially in children and may in some cases serve as a good proxy for bronchial epithelium. Epigenetics is also cell-type specific, but only a few studies tried to address this issue and were mostly focused on blood cells. The cell-type specific epigenetics in asthma, especially in the lower airways, have not been well studied yet.

The cell-type specific epigenetic methods should be considered in future studies in relevant tissues of asthma such as innate and adaptive immune cells as well as airway epithelial cells, and need to address if cell specific epigenetic signatures could be used in early diagnosis of childhood asthma. This will overcome the cell type heterogeneity problem that now hampers interpretation of epigenetic studies of mixed cell populations, and increase our understanding of the development of childhood asthma.

Next to the study of asthma phenotype, several previous studies showed epigenetic marks such as DNA methylation and miRNA that also associated with specific characteristics of

asthma such as severity of asthma, sensitivity to ICS, and exhaled NO[48, 50, 109], which suggested that epigenetic marks may also be associated with specific asthma endotypes and that the epigenetics may assist in understanding the clinical heterogeneity of asthma in the future.

Epigenetics can be regulated by genetics and correlate with gene expression, and different epigenetic levels may also correlate with each other, so the integration of multi-omics data will be helpful to interpret the how the epigenetics works in asthma development, and to improve the understanding of asthma pathogenesis.

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*** An important study showed that demethylation was a downstream consequence of transcriptional activation in dendritic cells post bacterial infection.**

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Figure Legend:

Figure 1. Types of epigenetic regulation, including histone modification, DNA methylation and regulation of non-coding RNA.

Figure 2. Potential sources that could change DNA methylation in relation to asthma.

Table 1 Characteristics of asthma DNA methylation studies mentioned in this review

Study	Study design	Age	Ancestry	Sample size (discovery and replication)	Methylation platform	Tissue	Asthma definition	Number of significant CpGs (or DMRs)	Significance threshold	Examples of candidate genes
Xu (2018) [29]	Cross-sectional	4-5 years old, and 8 years old	European	Discovery: (1) 4-5 years: 207 cases and 610 controls; (2) 8 year: 185 cases and 546 controls. Replication: 247 cases and 2949 controls	Illumina 450K and Agena iPlex	Whole blood	Two of three criteria present: doctor diagnosis of asthma ever; use of asthma medication in the past 12 months; or wheezing or breathing difficulties in the past 12 months.	27 CpGs were identified, 14 were replicated.	$P < 1.14 \times 10^{-7}$ (Bonferroni correction)	<i>LMAN2</i> ; <i>STX3</i> ; <i>LPIN1</i> ; <i>DICER1</i>
Reese (2019) [30]	Prospective (newborn DNA methylation and childhood asthma) and cross-sectional (childhood methylation and childhood asthma)	7-17 years old	European (13 cohorts); mixed ancestry (3 cohorts), African American (1 cohort)	Discovery: (1) Prospective: 668 cases and 2,904 controls; (2) Cross sectional: 631 cases and 2231 controls	Illumina 450K	Whole blood	A doctor's diagnosis of asthma and the report of at least 1 of the following: (1) current asthma, (2) asthma in the last year, or (3) asthma medication use in the last year.	(1) Prospective part: 9 CpGs and 35 regions; (2) Cross sectional part: 179 CpGs and 36 regions.	FDR<0.05	1) Prospective part: <i>CLNS1A</i> , <i>Mir_548</i> ; <i>GPATCH2</i> ; <i>LOC100129858</i> ; <i>AK091866</i> ; <i>SUB1</i> ; <i>WDR20</i> 2) Cross sectional part: <i>ACOT7</i> ; <i>IL5RA</i> ; <i>KCNH2</i> ; <i>RUNX1</i> ; <i>ZFPM1</i>
Arathimos	Cross-sectional	7.5	European	Discovery: (1) 7.5	Illumina	Periph	Current asthma (7.5	(1) Current	FDR<0.05	<i>ZFPM1</i> ; <i>AP2A2</i> ;

(2017) [32]	(7.5 years) and longitudinal (7.5 and 16.5 years)	years and 16.5 years		years: 149 cases and 632 controls; (2) 16.5 years: 194 cases and 554 controls (asthma ever) and 184 cases and 427 controls (current asthma)	450K	eral blood	and 16.5 years): doctor's diagnosis of asthma ever and at least one of following present (1) asthma in past 12 months; (2) asthma medication the last 12 months or (3) wheezing or whistling on the chest in the past 12 months	asthma at 7.5 years: 302 CpGs; (2) Current asthma at 16.5 years: 2 CpGs. (3) DNA methylation at 7.5 years ~ asthma at 16.5 years: 4 CpGs; (4) DNA methylation at 16.5 years ~ asthma at 7.5 years: 28 CpGs		<i>IL5RA</i>
Murphy (2015) [34]	Longitudinal	5 years, 10 years and 18 years	European	Discovery: 37 monozygotic twin pairs	Illumina 450K	Buccal cells	1) woken by an attack of shortness of breath at any time in the last 12 months; 2) an attack of asthma in the last 12 months; 3) currently asthma medicine (inhalers, aerosols or tablets)	No significant sites after Bonferroni correction, top-ranked nominal significant sites were provided	--	<i>HGSNAT, HLX</i>
Yang (2017) [36]	Cross-sectional	10-12 years	African American	Discovery: 36 cases and 36 controls. Replication: n=30	Illumina 450K	Nasal brush cells	(1) a physician diagnosis of asthma; (2) persistent or uncontrolled asthma; (3) physiologic evidence of asthma ($FEV_1 < 85\%$ or $FEV_1/FVC < 85\%$; and bronchodilator responsiveness ($\geq 12\%$) or $PC_{20} < 8$	119 DMRs and 118 CpGs	FDR<0.05	<i>ALOX15; HLA-DPA1; GJA4; POSTN; LDLRAD3; ATXN7L1; METTL1; CXCR6; CCL5; CTSC</i>

							mg/ml of methacholine); and (4) positive skin prick test to at least one of a panel of indoor aeroallergens (dust mite, cockroach, mold, cat, dog, rat, mouse).			
Forno (2018) [37]	Cross-sectional	9-20 years	Hispanic or Latino ancestry	Discovery: (1) atopy: 312 cases and 171 controls; (2) atopic asthma: 169 cases and 104 controls. Replication: 72 African American children; 432 Dutch children	Illumina 450K	Nasal brush cells	Atopy was defined as: at least one positive IgE (≥ 0.35 IU/mL) to five aeroallergens (house dust mite, cockroach, cat dander, dog dander and mouse urinary protein); asthma was defined as a physician's diagnosis and at least one episode of wheeze in the past year.	(1) Atopy: 8664 CpGs were identified, 28 of top 30 CpGs were replicated; (2) atopic asthma: top 30 CpGs shown, and 28 of top 30 CpGs were replicated.	FDR<0.01	<i>CDHR3</i> ; <i>CDH26</i> ; <i>FBXL7</i> ; <i>NTRK1</i> ; <i>SLC9A3</i>
Cardenas (2019) [38]	Cross-sectional	12.9±0.65 years	Mixed ancestry	Discovery: 65 cases and 463 controls; Replication: in two cohorts, n1=72, n2=483	Illumina EPIC	Nasal swab cells	Current asthma was defined as mother's report of a doctor's diagnosis of asthma since birth reported on the early teen questionnaire plus report of wheeze or asthma medication in the past year at early teen follow-up (comparison group had no asthma diagnosis, no wheeze, or no	(1) current asthma: 285 CpGs were identified, 58 were replicated (2) allergic asthma: 1235 CpGs and 7 DMRs, 185 CpGs were replicated	FDR<0.05	<i>ACOT7</i> ; <i>EPX</i> ; <i>EVL</i> ; <i>NTRK1</i>

							asthma medication use).			
DeVries (2017) [60]	Nested case-control	9 years	Mixed	Discovery: 18 cases and 18 control Replication: in 2 studies, n1=30, n2=28	Methylated CpG Island Recovery Assay (MIRA)-chip	Cord blood mononuclear cells	Physician-diagnosed, with symptoms or medication use for asthma in the past year reported at least once on the age 2, 3, 5 or 9-year questionnaires	589 DMRs	FDR<0.01	<i>SMAD3</i>
Lund (2018) [85]	Longitudinal	13 months to 5-year	European	Discovery: (1) rhinovirus + asthma + (n = 16), (2) rhinovirus + asthma - (n = 16), (3) rhinovirus - asthma - (n = 16).	Reduced representation bisulfite sequencing (RRBS)	Whole blood	Current asthma at 4-years visit: present one or more criteria in the past 12 months: doctor-diagnosed asthma, regular use of doctor prescribed corticosteroid asthma therapy, use of oral corticosteroids for asthma exacerbations, acute asthma attack relieved by repeated use of bronchodilator.	(1) 17 regions (rhinovirus + atopic asthma + (n = 11) vs rhinovirus + asthma - (n = 16)); (2) 13 regions (rhinovirus + nonatopic asthma + (n = 5) vs rhinovirus + asthma - (n = 10))	FDR<=0.05	<i>SMAD3</i> ; <i>DDO/METTL24</i>
Nicodemus-Johnson (2016) [109]	Cross-sectional	39 ± 13years	African American and European American	Discovery: 74 cases and 41 controls	Illumina 450K	Endobronchial airway epithelial cells	Doctor's diagnosed asthma and current asthma medication use	40,892 CpGs	FDR<0.05	<i>ORMDL3</i> ; <i>CCL26</i>

Table 2 Epigenetic studies of environmental factors for asthma mentioned in this review				
Environmental factor		Risk/Protective	Epigenetic studies	Examples of candidate genes
Cigarette smoking	Maternal smoking	Risk	DNAm: [61]; [64]	<i>ESR1; IL-32</i>
	Second hand smoking	Risk	--	--
	Active smoking	Risk	DNAm: [66] miRNA: [68]	<i>CYP26A1; AHRR;</i> <i>miR-21</i>
Air pollution		Risk	DNAm: [71-74]; [76]	<i>FAM13A; NOTCH4; TET1</i>
Microbiome		Protective	--	--
Respiratory viral infection		Risk	DNAm: [85] Hism: [86]; [87] miRNA: [88]	<i>SMAD3;</i> <i>H3K4 Demethylase KDM5B;</i> <i>miR-29c; let-7b</i>
<i>In utero</i> exposures	Maternal asthma	Risk	DNAm: [60]; [90]; [91]	<i>SMAD3; PM20D1</i>
	Maternal obesity	Risk	--	--
	Maternal nutrition intake	Protective (some nutrients)	--	--
Environmental allergens	House dust mite	Risk	DNAm: [94]	<i>CMTM1; DNAH10</i>
	Furred pets	Risk/Protective	--	--
	Pests (mice, cockroaches)	Risk	DNAm: [95] miRNA: [96]	<i>FOXP3;</i> <i>miR-155</i>
Breast feeding		Protective	--	--
DNAm: DNA methylation; miRNA: micro RNA; Hism: histone modification				

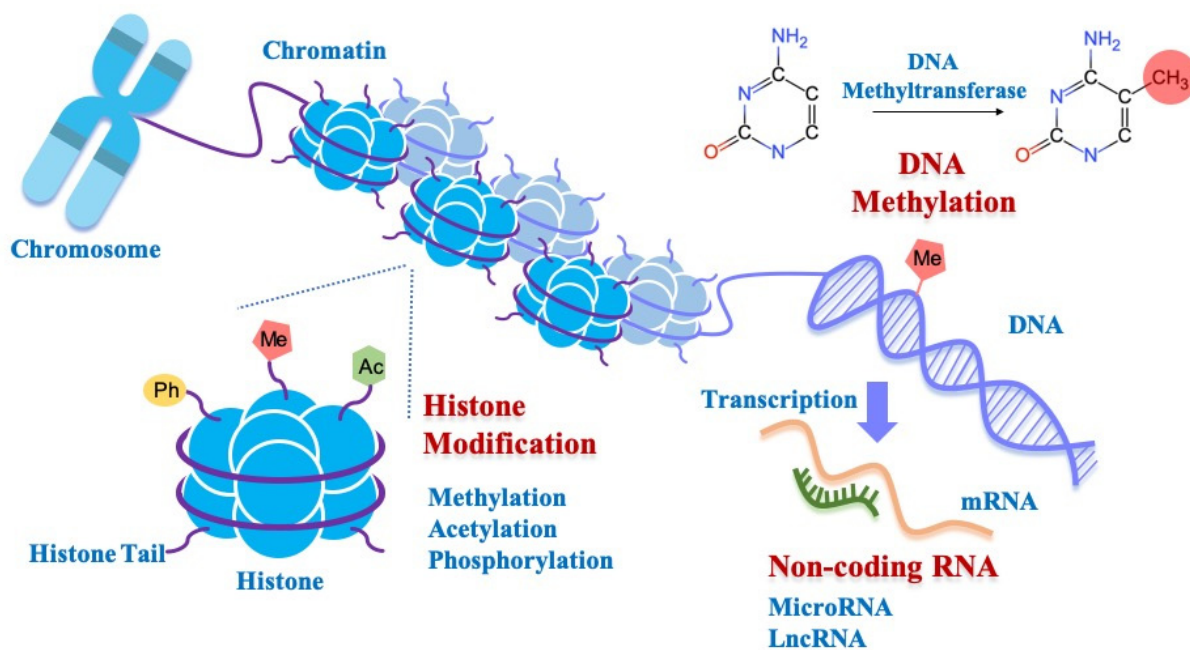


Figure 1

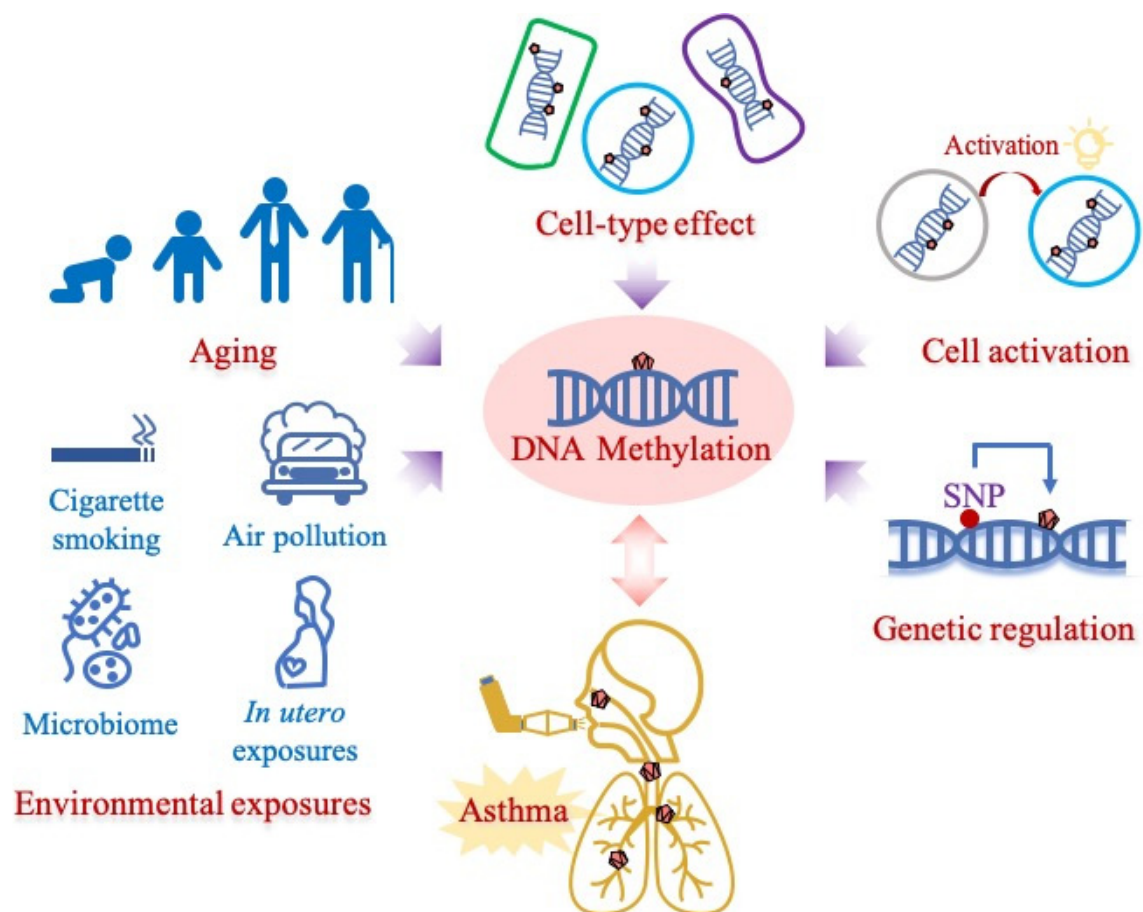


Figure 2