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Epigenomics and allergic disease

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Abstract

Allergic disease development is affected by both genes and the environment, and epigenetic mechanisms are hypothesized to mediate these environmental effects. In this article, we discuss the link between the environment, DNA methylation and allergic disease, as well as questions of causality inherent to analyses of DNA methylation. From the practical side, we describe characteristics of allergic phenotypes and contrast different epidemiologic study designs used in epigenetic research. We examine methodological considerations, how best to conduct preprocessing and analysis of DNA methylation data sets, and the latest methods, technologies and discoveries in this rapidly advancing field. DNA methylation and other epigenetic marks are firmly entwined with allergic disease, a link that may hold the basis for future allergic disease diagnosis and treatment.

Keywords

allergy; asthma; DNA methylation; environment; epigenetic

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DNA methylation & allergic disease

Allergic disease

'Allergic disease' is used to describe the spectrum of clinical conditions characterized by allergic hypersensitivity [1]. The term covers a long list of diseases, with the most prevalent being asthma, allergic rhinitis, eczema, food allergy and anaphylaxis. The global prevalence of allergic diseases is increasing, constituting a major healthcare burden [2,3]. Allergic diseases have multifactorial causes, with genetic factors, environmental exposure and their interactions playing important roles [4].

Genetics of allergic diseases

Family history is the most consistently associated risk factor of allergic diseases and the increased concordance of allergic disease in monozygotic twins compared with dizygotic twins suggests that genetics plays an important role in allergic susceptibility. Many genome-wide association studies have been conducted for asthma [5] and other allergic phenotypes, including total IgE, atopic dermatitis (eczema), hay fever and sensitization [6]. Hundreds of genes have been identified in these and candidate gene studies; however, most of the genes confer only small increments in risk and do not account for the observed heritability of the phenotypes [7]. Many reasons have been proposed for this 'missing heritability', such as not yet detected common variants with small effects on phenotype, rare variants with larger effects, structural variants such as copy number variation [8], noncoding inheritance and environmental influences [5]. In addition, transgenerational inheritance of epigenetic factors is considered to contribute to the missing heritability in complex diseases [9].

Environmental exposures associated with allergic disease correlate with DNA methylation

A large number of diseases are now recognized as having an epigenetic component [10], including allergic diseases [11,12]. Recent research has revealed that several key asthma and allergy susceptibility genes are epigenetically regulated; for example, transcription of *STAT6* [13] and *FOXP3*, a regulator of Treg activity [14], are regulated by DNA methylation (DNA-M) and transcription of the cytokine IL-13 is regulated by histone acetylation [15]. There are also typical DNA-M patterns associated with allergic disease status [16–19] and, critically, the epigenetic regulator *Dnmt1* is downregulated in asthma [20], suggesting that DNA-M is dysregulated in allergic disease. A range of environmental exposures linked to allergy have been shown to correlate with DNA-M, offering a potential mechanism through which the environment is related to allergic disease development.

Farming environment

Perhaps the best-known environmental exposure associated with allergic disease is exposure to a farm environment. A recent meta-analysis of 39 studies found a 25% lower prevalence of childhood asthma with farm exposure [21], and even *in utero* farm exposure is protective against hay fever, asthma and eczema [22]. The number of different animals the mother is exposed to seems to be linearly associated with the expression of innate immune receptors [23], and the variety of environmental microorganisms a child is exposed to correlates inversely with risk of asthma [24]. The farm effect on allergic disease is thought to be mediated by epigenetics: DNA-M in cord blood differs between farmers' and nonfarmers' children at the asthma-associated genes *ORMDL1*, *STAT6*, *RAD50* and *IL-13* [25]. DNA-M in the placenta has also been reported to be altered at the *CD14* promoter if the mother was living on a farm [26]. Pet exposure has been shown to influence the development of allergic disease [27] and correlates with DNA-M at the *CD14* locus [28].

Air pollution

Exposure to ambient air pollution, including diesel particles, has well-known detrimental effects on allergic disease [29]. Exposure to air pollution increases DNA-M in *FOXP3*, which correlates with childhood wheeze and asthma [30], suggesting that this is the molecular mechanism by which air pollution worsens asthma severity. Children living in areas of high air pollution differ in blood cell DNA-M at thousands of other loci throughout the genome, some of which show >10% change in the DNA-M level [31]. Exposure to the ambient particulate pollutant black carbon also alters DNA-M in blood [32]. Maternal polycyclic aromatic hydrocarbon exposure correlates with both altered DNA-M at *ACSL3* and reported asthma before the age of 5 years [33]. Diisocyanate-induced occupational asthma is associated with increased DNA-M within the *IFN*-γ promoter [34]. Results of a more complex analysis showed that air pollutant exposures are associated with severe asthma only in children with high levels of *ADRB2* methylation [35].

Respiratory viral infection

Rhinovirus infections are associated with infant atopic dermatitis and asthma [36], and both viral and bacterial infections with childhood wheeze [37]. Rhinovirus infections differentially alter genome-wide DNA-M in the nasal epithelial cells of asthmatics and nonasthmatics, including methylation differences in the immune-associated genes *CXCR4* and *HLA-H* [38], suggesting that the effects of rhinovirus infection on allergic disease development could also be mediated epigenetically.

Developmental environment

Various elements of the developmental environment are associated with both allergic disease and DNA-M. High birth weight [39], a fast rate of weight- and height-gain after birth, height and a larger head circumference have been reported to be linked with asthma [40,41], suggesting that fetal growth is a risk factor for allergic diseases. Whether fetal alcohol exposure has an effect on allergic disease development is contentious [42–44], possibly being confounded by other environmental stimuli. Maternal obesity before or during pregnancy has been reported to increase risk of childhood asthma [45]. Maternal prepregnancy obesity modulates the association between DNA-M at the *IGF2* promoter and IGF-2 protein levels in plasma [46]. Interestingly, paternal obesity also influences the offspring's DNA-M at *IGF2* [47]. DNA-M around the glucocorticoid receptor gene is altered by maternal stress [48] and maternal depressed/anxious mood in the third trimester [49].

Gender & age

Allergic disease incidence varies with gender and age [50–52], and associations between factors such as these and allergic disease could also be mediated by DNA-M. For example, Naumova *et al.* examined the asthma-associated 17q12-21 region and found sex-associated DNA-M within *ZPBP2* [53]. Indeed, methylation of imprinted genes and sex chromosomes differs between men and women, but there are also subtle gender-associated DNA-M patterns throughout the rest of the genome [54].

Smoking

In utero exposure to cigarette smoke has been associated with several adverse health conditions in offspring [55]. Maternal smoking during pregnancy is associated with allergic diseases including asthma, wheezing, eczema and rhinitis, and allergic sensitization [56–58]. Smoking also has transgenerational effects on allergic disease: the risk of asthma is increased in children whose maternal grandmother smoked during their mother's fetal period, even if they themselves were not directly exposed to gestational smoking [59].

DNA-M is thought to underlie such non-Mendelian inheritance: genome-wide DNA-M is all but erased each generation; although in addition to imprinted genes, a small number of transgenerationally inherited DNA-M marks are retained.

Gestational smoking has been associated with differential DNA-M in both epigenome-wide and gene-specific studies. Suter *et al.* demonstrated that the promoter region of *CYP1A1*, responsible for the metabolism of nicotine and other carcinogenic compounds found in cigarette smoke, was hypomethylated in placental samples from mothers who smoked compared with nonsmoking controls [60]. In a subsequent epigenome-wide investigation, differential methylation was detected at 1024 CpG sites in the placentas of smokers and nonsmokers, with 38 CpG sites differing by a methylation level ≥10% [61]. The same study suggested that changes in DNA-M due to gestational smoking correlate with, and alter, gene expression. Breton *et al.* showed that DNA-M levels in buccal cells of kindergarten and first-grade students differed between those exposed and not exposed to tobacco smoke *in utero* [62]. More comprehensively, Joubert *et al.* used Illumina®'s (CA, USA) 450K array to measure DNA-M levels at >484,000 CpGs across the genome in the cord blood of infants from a Norwegian birth cohort [63]. They identified 26 CpGs in ten genes functionally related to gestational smoking. In a subset of the Isle of Wight birth cohort with epigenome-wide profiling (245 girls aged 18 years), we identified 18 CpGs (mapped to five genes) associated with *in utero* exposure to gestational smoking [Karmaus W *et al.* Maternal smoking during pregnancy is associated with DNA methylation in female offspring at age 18 – results of an epigenome-wide scan (2013), Manuscript in preparation]. A total of 14 CpG sites within five genes were in agreement between our results and Joubert *et al.*, further highlighting the stability of DNA-M associated with gestational smoking between birth and 18 years of age. Hence, gestational smoking can lead to prominent changes in the epigenome that could lead to adverse health conditions. In a targeted gene-approach, Wang *et al.* investigated the methylation of the *TSLP* gene [64]. Exposure to gestational smoking was related to differential methylation of the *TSLP* gene measured in cord blood cells, which in turn was associated with increased risk of subsequent childhood eczema at 2 years of age. Given the substantial effect of gestational smoking on DNA-M and the strong impact of gestational smoking on allergic disease manifestation, we anticipate a large array of future studies with DNA-M as a mediator between gestational smoking and allergic disorders.

Nutritional exposures

Nutrition is considered to alter DNA-M and thus influence allergic disease outcomes, especially maternal nutrition during pregnancy, as gestation is a key developmental period.

Intrauterine nutrition—It has been found that maternal consumption of apples and fish during pregnancy has protective effects against childhood asthma and eczema [65,66], maternal fish oil supplementation during pregnancy is protective for asthma at 16 years of age [67], and fish consumption in infancy is protective against rhinitis and eczema at 12 years old [68]. Higher consumption of the lipids α-linolenic acid and docosahexaenoic acid [69] and dairy products [70] during pregnancy reduces risk of infant wheeze at 16–24 months, whereas n-6 polyunsaturated fatty acids and linoleic acid increase the risk of infant eczema [69]. However, *in utero* supplementation with methyl donors (i.e., folate) has been reported to increase the risk of infant wheeze [71] and asthma at 3 years [72,73]. In addition, in children bearing the C677T mutation in *MTHFR*, which determines folate and homocysteine status, higher folate levels are associated with an increased risk of eczema after 6 years of age [74]. Levels of trace nutrients have been linked with the development of allergic disease. For example, higher levels of selenium, iron, vitamin E and zinc in the mother's blood during early pregnancy and in the umbilical cord are inversely associated

with childhood wheeze and eczema [75–77], and vitamin A (retinoic acid) treatment has been found to increase allergic response in mice [78].

Postnatal nutrition—Breastfeeding is considered to be protective against childhood asthma and atopy [79,80], although the evidence is not conclusive [81,82]. The effects of breastfeeding on DNA-M profiles [31] and allergic disease may be caused by immune factors [83] and/or fatty acid profiles [84]. Increased consumption of antioxidants has also been hypothesized to underlie the increasing prevalence of atopic diseases in western countries [85]. Whole diet patterns such as the Mediterranean diet are associated with allergic disease [86]; however, diet pattern effects may be confounded [87].

Nutrition & DNA-M—Nutritional exposures described above may well exert their effects on allergic disease via DNA-M. One example is folate, an essential dietary B vitamin that provides the substrate for methylation of a wide variety of molecules, including DNA, and whose absence affects the DNA-M enzymatic machinery [88]. In a landmark study, Hollingsworth and colleagues used a mouse model to demonstrate that prenatal methyl donor supplementation not only increases the severity of allergic inflammatory responses, but also alters DNA-M at 82 loci including *Runx3*, a known negative regulator of allergic airway disease [89]. Because folate provides the substrate for DNA-M, DNA-M is a strong candidate mechanism for the effect of folate on allergic disease. Indeed, maternal folate supplementation increases DNA-M at *axin fused* [90] and alters DNA-M at *Pepck* [91], and deprivation reduces DNA-M at *Slc394a* [92] in mice.

Other risk factors

There are numerous other exposures that also affect allergic disease outcomes, and may well do so via DNA-M, but the involvement of DNA-M awaits investigation. Examples include exposure to house dust mites and dampness [93,94], older siblings and number of siblings [95,96], pollen [97], paracetamol, both prenatally and in adulthood [98,99], maternal acid-suppressive medication during pregnancy [100] and birth by cesarean section [101,102]. The evidence linking vitamin D levels to risk of allergic disease is conflicting [103,104], but if a correlation does exist it could potentially be epigenetically mediated.

Confounding factors—Exposures are frequently confounded with one another [95,105], making it difficult to isolate the effects attributable to each. For instance, consumption of food and nutrients are correlated with other factors such as social class [106]. Social class itself has been associated with adult DNA-M [107], highlighting the potential for confounding evidence, inherent to epidemiological studies. To truly isolate the effects of specific factors on DNA-M, experimental studies, such as intervention trials in humans and laboratory experiments in animals, are required. In addition, novel approaches measuring DNA-M provide a huge number of markers that may be used to identify unknown confounders such as confounding by differential cell counts (see section ‘Cell-type correction’). If there are exposure-specific DNA-M patterns and these patterns are stable over time, we may be able to identify the presence of unknown confounders/exposures and adjust for those covariates. Hence, a major future task is to determine whether specific exposures leave specific fingerprints or unspecific footprints in the pattern of DNA-M.

DNA-M in disease: cause or consequence?

The vast majority of ongoing research in the area of epigenetic modifications and the development of allergic diseases does not consider the chronological order of events [108]. For instance, DNA-M is measured among individuals who already have disease or disease-related symptoms and a comparison is made with DNA-M profiles of those who are disease-

free. This approach cannot distinguish whether alterations in DNA-M are causes or consequences of the disease. To overcome these limitations, researchers can collect or retrieve previously stored blood samples and consider DNA-M in these samples as the baseline. Such an approach will allow us to disentangle the time order of DNA-M and disease outcome. Curtin *et al.* used a candidate-gene approach to show that elevated DNA-M of the *IL-2* promoter, measured in stored cord blood samples, is associated with increased risk of severe asthma exacerbations and hospital admissions for asthma/wheeze at age 2–8 years [18]. This finding supports the possible role of DNA-M in the development of subsequent allergic diseases.

However, before any cause/effect inferences can be made, it is important to consider the possible influence of the DNA sequence (e.g., genetic variants) on epigenetic modifications. For example, cancer cells contain many epigenetic aberrations, as well as many DNA mutations, suggesting two possible directions: DNA mutations are responsible for epigenetic modifications or epigenetic alterations 'cause' genetic instability [109]. A complex and ongoing interplay between genetic and epigenetic factors could also prove to be indispensable. To this end, epidemiological studies that consider the time order of events, and, possibly the prenatal and transgenerational effects, are needed to understand the role of epigenetic modifications in disease development and prognosis.

Approaches to the epigenomic analyses of allergic disease

In the past, for many diseases, etiologic research has generally progressed in two separate streams of investigation: studies of environmental or psychosocial exposures, and studies of genetic and cellular pathways. Since epigenetic changes can result from multiple exposures and may lead to molecular changes that then predict disease, epigenetic research has combined these dual streams and established new links among multiple disciplines. However, new challenges have emerged [19]. For instance, an altered disease-related status such as allergic sensitization may lead to immune cell differentiation, which can result in epigenetic changes [110] that are consequences of the original disease. It is therefore essential to establish that epigenetic changes result from exposures (possibly conditional on the DNA sequence, such as methylation quantitative trait loci [methQTLs] [111–114]), but are not consequences of the disease being studied. Since the intermediate role of the epigenome between the environment and disease can be confused, epigenetic studies require considerations of appropriate study designs. Study design scenarios become even more complex as epigenetic effects can be inherited. For instance, Li *et al.* demonstrated that grandmaternal smoking shows an association with the grandchildren's asthma, even when adjusted for maternal smoking [59]. Hence, multiple generations may be needed to establish that disease-associated DNA-M results from responses to exposure and is not simply inherited from grandmother to mother with maternal smoking propensity, and that the related epigenetic changes are genuinely a response to grandmaternal smoking.

Study design considerations

The advantages and disadvantages of basic study designs are summarized in Table 1. Case–control studies cannot distinguish whether differences in epigenetic markers are due to exposures or diseases. However, if blood spots were collected at birth and stored [115], it is possible to determine, in both cases or controls, whether differential DNA-M is a consequence of disease. On the other hand, regarding parental exposures, DNA-M from blood spots at birth will not help to distinguish whether differential methylation is due to past exposures experienced by the child (*in utero*) or due to inheritance of intergenerational DNA-M. Cross-sectional studies and baseline investigations of follow-up studies in disease-free populations (e.g., birth cohorts) can help to identify epigenetic marks related to exposures. Methylation profiling of early-life blood spots and repeated DNA-M later in life

may establish the necessary time order. DNA-M is likely to result from exposure if a specific DNA-M mark was not detected early in life, but occurred after the onset of exposures and before the onset of the disease. In contrast to case–control or cross-sectional designs, follow-up studies allow one to establish whether a disease is due to a prior epigenetic status. The most advanced design is an experimental study during gestation with additional information on early maternal epigenetic markers (e.g., blood spots from the mother at her own birth). In intervention trials during gestation, we can establish whether a change in maternal exposure results in differentially methylated DNA, which then, in turn, can be linked to later health outcomes in the offspring. Furthermore, if early-life blood spots of the mother and information on grand-maternal exposures are available, it is possible to differentiate between epigenetic inheritance and epigenetic responses due to intervention in exposures. In addition, the effects of maternal epigenetics on the susceptibility to interventional changes during gestation can be quantified. Such a design would conclusively show whether a change in exposure during the developmental period of gestation has the potential to change offspring DNA-M, independently of grandmaternal exposure and maternal epigenetics.

While ‘causal’ associations can be established in experimental studies, these are difficult to establish in epidemiological studies. Mendelian randomization (MR) is the concept that genotypes are random within a population due to random assortment of alleles during meiosis and, therefore, should be randomized across exposure [116], similar to the intention-to-treat effect of experimental studies. MR designs are considered to support causal associations and are suggested to substitute experimental trials [117]. MR assumes that genotypes are randomly distributed and that their effect is not confounded by external variables; however, because epigenetic marks may modify the penetrance of genes [118], the epigenetic status will confound the genetic effect. In this case, it has been demonstrated that the MR assumption does not hold [119]. Although genes may be randomly distributed, their effect is modified by nonrandomly distributed DNA-M related to nonrandomly distributed exposures and so become confounded. This dilemma is worsened when we consider that effect modification or confounding may have occurred in previous generations and these effects may be sustained (e.g., smoking of the grandmother leads to smoking of the mother, leads to smoking of the grandchild).

Phenotyping

As in genetic epidemiology, for epigenetic studies, the definition of the disease phenotype is critical in determining the outcome of any analysis. Allergic diseases occur throughout the life course, from neonates to geriatrics. A key question is, what is the starting time point and what is the earliest identifiable marker or phenotype? In fact, latency periods (i.e., time period between exposure and onset of the disease) of allergic diseases are quite short: cord serum IgE indicates differences in susceptibility immediately following gestational exposures; eczema typically develops in the first year of life; wheezing can be detected in infancy and the certainty of an asthma diagnosis increases with age; and allergic rhinitis typically begins around adolescence. Immune responses to allergens may be initiated during fetal development [120] and allergic sensitization can occur as early as 22 weeks of gestation [121].

Diagnosing allergic diseases in infancy and preschool children can be challenging [122], for example, for asthma, clinical presentation continuously evolves with the developing lung and immune system and is difficult to differentiate from other conditions with similar symptoms such as viral infection-induced wheeze. The majority of children with early-life wheeze do not have persistent symptoms in later life, but those with persistent wheeze are highly likely to develop asthma [123]. Sensitization marks the underlying physiological background of allergic diseases, but, can be asymptomatic [124]. These three examples

highlight the problem of using symptoms or markers at a single point in time. To counteract this, the collection of repeated information starting in early childhood to track allergic development can be used to reduce disease misclassification. Observations of symptoms at different ages are applied to improve definitions of wheezing/asthma, eczema and rhinitis. Lung function tests are feasible starting at school age; repeated lung function measurements provide critical information to characterize individual lung growth. Repeated tests of allergic sensitization, ascertained by a simple skin prick test or by measuring specific IgE in serum, will help to determine the stability of these assessments.

Tissue source of DNA to be analyzed

Unlike in genetic epidemiology, for epigenetic studies, the cellular source of DNA samples is an essential consideration given the extent of tissue-specific methylation. Peripheral blood leukocytes are often used for DNA-M analysis since blood samples are less expensive and invasive than other tissue sampling methods, such as bronchial biopsy. While this may be appropriate for immune-mediated disorders, it will not allow the identification of disease-associated DNA-M in other tissues, for example, lungs in the case of asthma. Given the difficulty of procuring bronchial tissue versus the relative ease of measuring methylation levels in blood, a question of great practical importance is to assess the extent to which blood tissue is a reasonable surrogate for lung tissue (i.e., airway epithelium and smooth muscle cells). Since peripheral blood is composed of many distinct cell populations in varying proportions and DNA-M is cell type-specific, this may affect the interpretation of DNA-M changes. Evidence of tissue-specific variance in methylation has been reported [125], yet few DNA-M studies examine correlation across different tissue types [125,126]. A recent study showed that smoking has a direct effect on the epigenome in lung tissue, as well as in peripheral blood [127]. In a small sample (n = 25), Stefanowicz *et al.* analyzed the effects of disease status (healthy vs asthmatic and atopic vs nonatopic) and tissue type (blood cells vs airway epithelial cells) on DNA-M [128]. The authors reported tissue-specific DNA-M differences at 7.8% of 1023 CpG sites. A factorial analysis, disentangling the effect of tissue and disease, was not presented.

Cell-type correction

Since whole blood is composed of several cell types, the interpretation of DNA-M differences is challenging. The cell populations in whole blood include lymphocytes (T cells [$CD4^+$ and $CD8^+$], NK cells [$CD56^+$] and B cells [$CD19^+$]), monocytes ($CD14^+$), neutrophils, basophils, granulocytes and eosinophils. The proportion of eosinophils in the circulation is relatively low, but this type of cell is closely involved with allergic disease. In methylation studies, the frequency of differentially methylated CpGs in eosinophils compared with the whole blood is concealed by the partial overlap with the granulocyte compartment [129]. The use of flow cytometry to quantify the overall lymphocyte population composition in peripheral blood is exhaustive since it requires larger amounts of fresh blood samples and extensive work on antibody tagging. Houseman *et al.* proposed a method to correct for differences in cell mixture in DNA-M studies without differential cell counts [130], which utilizes the concept of differentially methylated regions of DNA as markers of cell type. The authors used flow cytometry to establish cell type-specific DNA-M profiles. They then estimated the cellular composition of each sample based on DNA-M signatures of each blood cell type. They demonstrated that sample DNA-M information can be used to correct for the (unknown) differential cell counts.

Genome-wide analysis of DNA-M

The invention of DNA-M arrays that simultaneously interrogate DNA-M levels of thousands of CpGs across the genome has revolutionized the field of epigenetic medicine. Arrays are

powerful tools that have allowed the use of hypothesis-independent approaches, and dramatically increased the throughput and speed with which DNA-M can be measured. Two widely used DNA-M arrays are the Infinium® HumanMethylation27 and HumanMethylation450 beadchips (Illumina). The latter interrogates >484,000 CpG sites associated with approximately 24,000 genes and covers 99% of RefSeq genes with multiple sites in the annotated promoter, 5′ untranslated regions, first exon, gene body and 3′ untranslated regions. Accompanying increasing awareness of the importance of non-CpG island DNA-M, the 450K array includes an increased proportion of non-CpG island CpGs, including intragenic CpGs [131]. Alternatively, there are nonarray-based approaches to measuring DNA-M: capture-based selection of methylated sequences may miss important, but isolated methylated CpGs (e.g., intragenic CpGs), but is useful for studying CpG islands and diseases such as cancer that dysregulate CpG island methylation. Sequencing-based approaches can also be used to measure genome-wide DNA-M, but currently are not as cost effective as arrays.

Another method used to map DNA-M on a genomic scale is the immune-precipitation and sequencing-based method, MeDIP-seq. A recent study demonstrated that 450K arrays and MeDIP-seq are comparable on a genome-wide scale and can be used to determine differentially-methylated loci in RefSeq genes, CpG islands, shores and shelves [132]. The latter method allows a wider interrogation of methylated regions of the human genome, including thousands of non-RefSeq genes and repetitive elements. These CpGs are difficult to assay by 450K array due to the problems of cross-hybridization. However, 450K arraying is a high-resolution technique that can detect methylation down to a single nucleotide resolution, whereas MeDIP-seq only detects methylated regions of approximately 150–200 bp [132].

Before raw DNA-M data can be analyzed by using commercial statistical platforms, quality control and validation analyses must be performed. GenomeStudio software (Illumina) is widely used to assess quality control. After quality control of DNA preparations and chip performance, raw DNA-M data are further analyzed using bioinformatical tools. Computational packages such as Illumina's Methylation Analyzer and MethyLUMI in R, have been designed to automate the pipeline for exploratory analysis and summarization of site-level and region-level methylation changes in epigenetic studies [133,201]. Analysis of the 450K array is considered challenging owing to the inclusion of two different probe chemistries, Infinium® I and II (Illumina) [134]. Infinium II assays have larger variance and are less able to detect extreme methylation values. Various normalization algorithms have been proposed to rescale the Infinium II data to the Infinium I. These include the 'peak-based correction', 'subset quantile within array normalization' and 'β-Mixture Quantile (BMIQ) dilation' methods. BMIQ is preferable as it incorporates a preprocessing step for the correction of probe-design bias, while simultaneously reducing technical variability [135].

Several new methodologies have been developed to correct for technical artifacts such as background noise, the positional effects on a single beadchip, probe-type bias and batch effects. Normalization of the data is used to avoid any enrichment toward any probe type, and to avoid within-batch effects or technical variations [134]. In addition, appropriate intra- and inter-plate controls could be used for assessing and controlling for batch effects. Marabita *et al.* recently evaluated analysis pipelines for DNA-M profiling [134] and demonstrated first, the importance of correcting for probe design type and batch effect and, second, that quantile normalization and BMIQ [135] are the most effective methods for normalization.

Another effective computational package used to preprocess raw DNA-M data is Illumina's Methylation Analyzer [136]. This multifaceted program includes options for filtering out loci with missing β values, from the X chromosome or with median detection p-values > 0.05, and loci whose methylation array probe sequences contain SNPs in or near the target CpG. Other options include normalization and correction for background noise, batch effects, color channel imbalance and multiple testing-correction algorithms. Additional factors to consider in cleaning data are the probes on sex chromosomes – most DNA-M studies exclude probes on sex chromosomes.

Recently, a study by Chen *et al.* discovered that 6% of the Illumina 450K array probes are cross-reactive, cohybridizing to nontarget sequences highly homologous to the intended target, and that some probes target polymorphic CpGs that overlap SNPs [137]. The crossreactivity is due to repetitive genomic sequences, pseudogenes and homologous genes. The authors provided a list of all crossreactive probes and polymorphic CpGs.

Pathway analysis

Once a list of CpGs of interest – whose methylation levels correlate with the variable of interest – has been generated, what next? A popular method to infer biological meaning from such lists is genetic pathway analysis. Many of the CpGs in DNA-M arrays are located within or near known genes or their regulatory regions. Using these annotations, one can establish a list of genes colocated with the CpGs of interest, then genetic pathway analysis can be used to determine whether any particular genetic pathway is overrepresented in this gene list, relative to a background list. Such genetic pathways are important in understanding the role of DNA-M differences in cellular function and disease. Popular genetic pathway tools (e.g., DAVID; National Institute of Allergy and Infectious Diseases, MD, USA [138] and Ingenuity®; Ingenuity Systems, Inc., CA, USA) use computationally determined gene ontology classifications; however, more advanced tools (e.g., MetaCore) use manually curated databases of genetic pathways. A recent example of genetic pathway analysis in relation to allergic disease comes from Reinius *et al.*, who used DAVID to compare differentially methylated genes between $CD4^+$, $CD8^+$, $CD14^+$, $CD19^+$ and $CD56^+$ T cells and eosinophils [129]. They showed differential enrichment of a range of immune-related genetic pathways between these cell types. In addition, hypermethylation in the promoter of a single key upstream regulator could shut down an entire biological pathway without this being detected by pathway analysis. Harper *et al.* demonstrated that pathway analysis can yield spurious associations when genes are differentially represented on an array [139]. Critically, a recent study by Geeleher and colleagues suggests that the varying number of CpGs associated with different genes on DNA-M arrays can severely bias the results of pathway analysis, although this can be corrected by modeling the relationship between the number of probes associated with a gene and its probability of appearing in the gene list [140].

MethQTLs & modifiable genetic variants: a two-stage model

Traditional genomic research investigates simple associations of either DNA-M or genetic variants with disease outcomes [141,142]. However, some studies have explored the combined effects of DNA-M and genetic variants on disease outcome [11,143,144]. Specific SNPs, named methQTLs, can influence the level of DNA-M at specific CpGs [136]. In addition, the role of methQTLs may depend on exposures such as gestational smoking and environmental pollutants (conditional methQTLs) [63,145]. Once a CpG site's methylation level has been changed by methQTLs or conditional methQTLs, this may influence the penetrance of other SNPs (e.g., modifiable genetic variants [modGVs]). Recently, Karmaus and colleagues conceptualized the idea of a two-stage model incorporating both methQTLs and modGVs [11]. We reported that CpG sites and genetic variants interact, dramatically

increasing the relative risk of disease, with risk ratios between 4 and 40 [11,143,144]. The effects of modGVs have been addressed in a recent review, emphasizing how DNA-M may alter the penetrance of genetic variants [119]. Information on possible cellular level mechanisms that may explain changes in gene regulation in response to specific exposures is scarce. We consider the two-stage model to be one of multiple potential explanations of the interplay of DNA-M, environmental exposures and genetic variants in producing disease. For instance, for allergic diseases, several genetic variants within the 17q12-21 chromosomal region have been repeatedly associated with asthma and asthma severity [5], and genotype-dependent DNA-M patterns have been observed at this locus [146]. Previously, we have shown that genetic variants and adjacent DNA-M within *filaggrin* had an interactive effect on the risk of eczema [143]. Similarly, we demonstrated that DNA-M modulates the risk of asthma related to genetic variants in *IL-4R* [144].

DNA-M & the potential for preventative treatments

If the burden of evidence for the association between DNA-M and allergic diseases continues to increase, then it will be desirable to alter DNA-M and so mitigate, cure or prevent allergic disease development. Since epigenetic marks can be inherited transgenerationally, it may be possible to reduce risk in this and future generations.

There are currently very few drugs available to alter DNA-M; these are cytidine analogs that competitively bind to and inhibit DNA methyltransferase enzymes, thereby lowering levels of DNA-M across the genome. 5-azacytidine (Vidaza™) and 5-aza-2′-deoxycytidine (decitabine; Dacogen™) are US FDA-approved to treat certain types of cancer, and trials have found that they can be used successfully at lower doses [147]. Zebularine is a recent synthetic cytidine analog with much higher stability [148], which has been used successfully in animal models [149], but is not approved for human use. Despite identical mechanisms, 5-azacytidine, decitabine and zebularine have surprisingly different effects on gene expression [150].

A major issue with treating allergic disease with DNMT inhibitors is that these drugs affect genome-wide DNA-M; they are not sequence-specific. Considering the sheer number of other processes in which DNA-M is involved, from behavioral memory to disease predisposition, it would not be desirable to administer these drugs to treat allergic disease. However, recent research has yielded two approaches for targeting changes to DNA-M, which could be tailored to produce interventions to preventatively vaccinate against or cure allergic disease via DNA-M.

Mahfouz showed that siRNAs can be used to direct DNA methylating complexes to target sequences in the genome of plants [151]. The chromatin structure was also altered, which could be a mechanism for maintaining DNA-M. Rivenbark and colleagues showed that site-specific methylation can be achieved in cancer cells *in vitro* by fusing the catalytic domain of DNMT3a to a sequence-specific array of zinc fingers [152]. The authors showed that this successfully altered DNA-M at the target promoters and that this effect was stable through cell division. The difference in the extent of DNA-M changes associated with cancer compared with allergic diseases could be an issue in the implementation of this technique. Cancer is typically associated with dramatic changes to DNA-M concomitantly at many CpGs within CpG-dense regulatory sequences (CpG islands), whereas the DNA-M patterns associated with allergic disease are frequently more subtle changes and associated with isolated CpGs [19,63].

Regarding environmental effects on DNA-M, while it is ostensibly tractable to modify exposure levels to prevent disease, this too can present difficulties. For example, high levels of folate, which provides the methyl substrate for DNA-M, may pose a risk for allergic

disease as discussed above. But dietary folate supplementation is common in the western world to reduce the incidence of neural tube defects, so it is not practical to reduce levels of folate to mitigate allergic disease. Nevertheless, there is a great need to develop novel intervention strategies to alter DNA-M profiles for lower susceptibility to allergic disorders.

Conclusion

DNA-M has been extensively linked to allergic disease development: a long list of environmental exposures contributes to allergic disease, many of which have also been shown to modify DNA-M, suggesting that their effects on disease susceptibility may occur via epigenetic mechanisms. Determining the direction of causality between epigenetic changes and allergic disease is an important issue faced by the growing field of epidemiological epigenetics, and one which is complicated further by transgenerational epigenetic inheritance. Experimental study design needs to fit the question being asked and also to include considerations to mitigate issues such as transgenerational inheritance, tissue heterogeneity and variation in disease phenotypes. Genome-wide methylation data normalization is essential in such studies and cutting edge normalization methods are numerous and are evolving rapidly. Methylation array data can be given biological meaning with pathway analysis, and an increasing number of studies are highlighting the importance of methQTLs and modGVs in interpreting epigenetic profiles. With our broadening understanding of the role of the epigenome in allergic disease, we may one day be able to modify the epigenome to prevent or mitigate allergic disease.

Future perspective

Several characteristics unique to allergic disease will define new avenues of future epigenetic research over the next decade. First, allergic diseases have short latency periods compared with other ailments, allowing the effects of exposure to be detected with minimal delay. We predict that this short latency will allow great advances in the coming years towards establishing the time order of exposure, DNA-M and allergic disease – an important question for the subsequent minimization of disease risks and development of treatments. The issue of causality is perhaps the biggest obstacle to further understanding the role of the epigenome in allergic disease. Future research needs to focus on linking environmental exposures in specific time windows with conditional DNA-M upon genetic variants (methQTLs); and investigating the modifying role of DNA-M on the penetrance of genetic risks (modGVs).

Second, in the framework of short latency periods and shorter follow-up periods until disease onset, past exposure can be measured with great detail. This will allow us to establish whether specific exposures leave specific finger-prints or unspecific footprints in the pattern of DNA-M across the genome. If, in addition, these patterns are stable over time, then we can use those fingerprints in future studies to adjust for unknown past exposures.

Third, given the key role of gestation and early life in establishing allergic disease, there is a need to establish time windows during pregnancy or in early life at which critical alterations of DNA-M occur that then may result in manifestations of different allergic diseases. Once such time windows have been identified, then future research can develop intervention strategies to maintain a low-risk environment in the appropriate time period.

Finally, allergic diseases affect various tissues (e.g., airway epithelium, skin and blood), which offers an opportunity, as well as a challenge. We predict that similarities in DNA-M between different tissues will be utilized by research to further extend the relevance of peripheral tissue (e.g., blood) DNA-M patterns to those in the disease-affected tissue, ultimately reducing the need for invasive sampling. This increasing ability to use peripheral

tissue DNA-M may allow data sharing of DNA-M profiles between entirely different diseases, greatly increasing the available sample sizes.

Together, we predict that these factors will allow us to greater define the causality of allergic disease-associated DNA-M in the coming decade. We may be able to identify whether past exposure establishes specific fingerprints or unspecific footprints that then can be used to correct for past exposures and unknown confounders. We stand to gain not only knowledge, but also the potential to develop novel treatments to lighten the burden of allergic disease on the individual and society in this and future generations.

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Executive summary

DNA methylation & allergic disease

- There is evidence supporting the association between allergic diseases and DNA methylation.
- A wide range of environmental exposures affect allergic disease.
- DNA methylation is proposed to mediate effects of the environment on allergic disease.
- It remains unknown whether allergic disease alters DNA methylation, or whether altered DNA methylation influences allergic disease, or a combination of both.
- Determining the causality of this relationship is further complicated by transgenerational inheritance of DNA methylation.

Approaches to the epigenomic analysis of allergic disease

- The appropriate study design must be selected, depending on what type of epigenetic response is being investigated.
- Allergic disease phenotype definitions and choice of tissue are important experimental considerations.
- Genome-wide methylation array data must be normalized and corrected for a range of factors, including cell type. New tools for 450K array data normalization are being developed constantly.
- Interpretation of array results can be performed using pathway analysis, and must be considered in relation to altered methylation changes depending on the DNA sequence (methylation quantitative trait loci), and methylation which influences the extent of the DNA sequence effect on phenotype (modifiable genetic variants).

DNA methylation & the potential for preventative treatments

- In the future, it will be possible to prevent or mitigate allergic disease via DNA methylation, although the major obstacle in this will be targeting these treatments to specific DNA sequences.

Table 1

Basic study designs for epigenetic research vary in their ability to detect different types of epigenetic responses.

Study design	Ability of study design to detect types of epigenetic differences		
	Epigenetic differences related to exposure	**Epigenetic differences related to exposure in past generations**	**Epigenetic differences not a consequence of the disease of interest**
Case–control studies	Cannot be determined by this study design	Can be established if exposures in past generations are known and DNA samples collected at birth can be assessed†	Cannot be determined by this study design
Cross-sectional studies	Can be established only if healthy individuals are analyzed or sampling is conducted before the disease developed (e.g., at birth)	Can be established if exposures in past generations are known and DNA samples collected at birth can be assessed†	Due to disease misclassification using one point in time, it is uncertain whether epigenetic differences are also responses to the disease
Follow-up studies	Can be established only if healthy individuals are analyzed or sampling is conducted before the disease developed (e.g., at birth)	Exploit exposure information and samples from more than one generation	Can be established if healthy subjects are followed-up and the disease developed after onset of epigenetic changes (time order)
Randomized intervention trials (experimental study) with follow-up	An experimental change of exposure (e.g., smoking cessation) can demonstrate whether the intervention results in an epigenetic response	Can differentiate between epigenetic inheritance and epigenetic responses due to intervention in exposures	Can differentiate whether a disease is a response to epigenetic changes or due to epigenetic inheritance

†Some states in the USA (e.g., CA, MI, NY) and some countries store blood spots collected at birth (Guthrie cards) that can be used for epigenetic studies.