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Epigenetic Regulation: The Interface Between Prenatal and Early-Life Exposure and Asthma Susceptibility

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Abstract

Asthma is a complex disease with genetic and environmental influences and emerging evidence suggests that epigenetic regulation is also a major contributor. Here, we focus on the developing paradigm that epigenetic dysregulation in asthma and allergy may start as early as *in utero* following several environmental exposures. We summarize the pathways important to the allergic immune response that are epigenetically regulated, the key environmental exposures associated with epigenetic changes in asthma genes, and newly identified epigenetic bio-markers that have been linked to clinical asthma. We conclude with a brief discussion about the potential to apply newly developing technologies in epigenetics to the diagnosis and treatment of asthma and allergy. The inherent plasticity of epigenetic regulation following environmental exposures offers opportunities for prevention using environmental remediation, measuring novel biomarkers for early identification of those at risk, and applying advances in pharmaco-epigenetics to tailor medical therapies that maximize efficacy of treatment. '*Precision Medicine*' in asthma and allergy is arriving. As the field advances this may involve an individually tailored approach to the prevention, early detection, and treatment of disease based on the knowledge of an individual's epigenetic profile.

Keywords

epigenetic regulation; asthma; environment; precision medicine; prenatal exposures

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AUTHOR CONTRIBUTIONS

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INTRODUCTION

Asthma is a heterogeneous chronic inflammatory disorder of the airway resulting from complex interactions between genetic predispositions and environmental exposures. The environmental component is critical, as supported by many studies documenting shifting asthma rates by geographical region and level of urbanization (Asher, 2011; Masoli et al., 2004), and others documenting differences in disease incidence between monozygotic twins (MZTs) (Thomsen et al., 2010). Despite this well-established premise, the underlying biological basis for this heterogeneity still needs to be elucidated.

It is becoming increasingly evident that epigenetic regulation following environmental exposures may underlie the interface between prenatal and early life environmental exposure and asthma susceptibility. The term 'epigenetics' refers to all meiotically and mitotically heritable changes in the phenotype. Epigenetic changes result in altered gene activity and expression, while the DNA sequence remains unchanged (Cheung and Lau, 2005; Lee and Workman, 2007; Li et al., 2007). These changes include DNA methylation at cytosine-guanine dinucleotides (CpG) residues, posttranslational modifications of histones proteins and noncoding RNA-mediated gene silencing (Koppelman and Nawijn, 2011). These mechanisms regulate gene expression and translational output by blocking the ability of transcription factors to bind to the recognition sites on CpG nucleotides, allowing binding of transcription-inhibiting proteins, inducing chromatin remodeling through methylation, acetylation, phosphorylation or ubiquitylation of histone tails, or by binding of the microRNA (miRNA) to the 3′-untranslated regions of mRNA and inducing degradation of target mRNA (Yang and Schwartz, 2012).

Emerging evidence suggests that crosstalk among epigenetic pathways and epigenetic marks exist. High levels of DNA methylation in gene promoters, for example, can be associated with the induction of repressive deacetylation of histones and the presence of relatively transcriptionally inactive chromatin regions, known as heterochromatin (Fuks, 2005; Vaissière et al., 2008). Alternately, methylation and histone acetylation may work in conjunction to affect gene transcription, as found in the case of expression of interleukin (IL)-13 in proallergic T helper (Th) 2 cells; it is induced by both DNA methylation and permissive histone acetylation, as described in more detail later in this review (Webster et al., 2007). There is communication between small RNAs and DNA methylation as well. Knockout mice with disruption of the RNAase III family nuclease Dicer, critical in the generation of small RNAs and RNA interference, exhibited defects in global DNA methylation (Benetti et al., 2008). Greater DNA methylation at the suppressor of cytokine signaling (SOCS) 3 gene promoter and subsequent decreased gene expression of SOCS3 has been shown to impair microRNA-122 (miR122) function and enhance interferon-stimulated response element (ISRE) activity (Yoshikawa et al., 2012). New advances in our understanding of rheumatoid arthritis suggest that cross talk between DNA methylation and miRNAs may be important in this disease as well (Miao et al., 2013).

Changes in DNA methylation can occur throughout life, but much of the epigenome is established during embryogenesis and early development of the fetus (Reik, 2007). In support of this premise is growing evidence that several prenatal environmental exposures

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have been associated with altered risk for asthma development. Cigarette smoking during pregnancy, for one, has been shown to modify fetal lung development (Carmines et al., 2003; Hylkema and Blacquiere, 2009) and fetal immune function (Noakes et al., 2003; Singh et al., 2011) important to the later development of asthma and other respiratory diseases. Other intrauterine exposures, including the maternal diet (Chatzi et al., 2008; Dunstan et al., 2003) and microbial exposures (Heederik and von Mutius, 2012), also are known to modify the risk of allergic disease in the offspring.

The maternal phenotype more often than the paternal phenotype has been shown to predict asthma and allergy in the child, indicative of a 'parent-of-origin' effect. For example, in children under 5 years of age, the risk of transmission of asthma from an affected mother was approximately four times higher than the risk associated with paternal asthma (Litonjua et al., 1998; Moffatt and Cookson, 1998). Childhood atopy also was linked strongly with maternal asthma in a New Zealand cohort (Sears et al., 1996). Cookson provided the first evidence of specific maternal linkage to atopy and asthma in the child at the 11q13 marker for the β subunit of the high affinity IgE receptor, FC∈RI-β (Cookson et al., 1992). Although previously attributed to differences related to the metabolism of environmental toxins in the intrauterine environment, these studies also support the possibility that specific changes in the epigenome of the fetus and genomic imprinting following prenatal environmental exposures may be contributing.

However, some studies suggest that the apparent 'parent-of-origin' effect can be relatively complex. As an example, in the Isle of Wight Birth Cohort (n = 1,456), maternal asthma was associated with asthma in girls (ages 4, 10, and 18 years) (prevalence ratio [PR], 1.91; 95% CI, 1.34–2.72), but not in boys; paternal asthma was associated with asthma in boys (age 4, 10 and 18 years) (PR, 1.99; 95% CI, 1.42–2.79), but not in girls. Maternal eczema was associated with increased risk of eczema in girls only (ages 2, 4, 10, and 18 years) (PR, 1.92; 95% CI, 1.37–2.68), whereas paternal eczema did the same for boys (1, 2, 4, and 10 years) (PR, 2.07; 95% CI, 1.32–3.25) (Arshad et al., 2012). Known differences in the prevalence of asthma by sex likely would not explain these results based on the several epidemiological studies that have shown that asthma generally is more common among boys than girls. Following puberty that sex effect may switch (De Marco et al., 2004; Tantisira et al., 2008). Instead, these data suggest that the mother's versus father's epigenome is important, and its expression is potentially mediated by sex of child, in contrast to a simple 'parent-of-origin effect' or 'sex effect.'

In this review, we will address the role of epigenetic regulation and the influence of the environment on the development and pathogenesis of asthma, with special attention on exposures during the prenatal and early post-natal period. We will start by presenting a description of the key pathways important to the allergic immune response that are epigenetically regulated followed by reviewing evidence that environmental exposures implicated in asthma induce epigenetic alterations. We will discuss the development of new epigenetic biomarkers and the evidence supporting a relationship between these and clinical asthma. We will conclude with a brief discussion about novel tools and applications in asthma epigenetic research.

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EPIGENETIC REGULATION OF KEY PATHWAYS IN THE ALLERGIC IMMUNE RESPONSE

Just as the clinical presentation of asthma and the response to associated environmental exposures is heterogeneous, so are the underlying immune pathways. Although there are some reports that epigenetic regulation may play a role in various asthma phenotypes including obesity-associated asthma (Rastogi et al., 2013), the bulk of the scientific literature in this field focuses on the role in allergic immune pathways leading to asthma. For the key allergic immune pathways, epigenetic regulation already has been widely reported, and the field is growing, as reviewed below.

Antigen Presentation/Dendritic Cell Differentiation

Differentiation of antigen presenting dendritic cells is critical to the differentiation of naïve T cells into effector T cells (i.e., Th1, Th2, and Th17 cells) and T regulatory (Treg) cells, and is linked to the development of allergic asthma (Kuipers and Lambrecht, 2004). In a murine study designed to evaluate the effects of maternal allergen exposure on offspring, pups of mice that were sensitized with ovalbumin (OVA) in an experimental model of allergic asthma were found by genome-wide DNA methylation studies to have different DNA methylation profiles in splenic CD11c(+) dendritic cells compared to pups of nonallergic female mice. Using this genome-wide approach the authors identified 40 differentially methylated gene loci CpG sites that demonstrated about ninefold or greater (ranging from 8.9- to 716.7-fold) differences in methylation between the pups born to asthmatic mothers and the controls. Furthermore, the overall methylation was higher in the dendritic cells of mice born to allergic *versus* nonallergic mothers (Fedulov and Kobzik, 2011). This difference in dendritic cell DNA methylation profiles as it related to allergic asthma suggested there was a functional capacity of this cell type to mediate asthma susceptibility through epigenetic mechanisms.

T Helper (Th) Cell Pathway

Naïve helper T cells are capable of differentiation into Th1 cells of which interferon (IFN)-γ and interleukin-12 are predominating cytokines or Th2 cells that are primarily responsible for the production of proallergic IL-4, IL-5, and IL-13. Some evidence suggests allergic asthma represents a skew toward a Th2 profile and suppression of regulatory Th1 cytokines (Calderon et al., 2009). The production of Th2 cytokines also is thought to be critical in the development of airway hyperresponsiveness (Kuperman et al., 2002; Venkayya et al., 2002). Early exposure to allergens may be responsible for this imbalance. For example, Prescott et al. demonstrated increased production of Th2 cytokines in cord blood and peripheral blood mononuclear cells (PBMCs) of infants from birth to 2 years when samples were cultured with house dust-mite allergen (HDM) (Prescott et al., 1999). Emerging evidence suggests Th1 cytokines also play a significant role and may be more important in severe compared with mild asthma (Abdulamir et al., 2008). As described below, numerous studies to date have demonstrated that this imbalance between the Th1 versus Th2 phenotypes may be regulated through epigenetic mechanisms.

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For example, chromatin remodeling, whereby hyperacetylation of histones is responsible for the open chromatin structure that allows for binding of transcription factors necessary for gene transcription, is critical in helper Th cell differentiation. Avni et al. demonstrated that conversion of H3 and H4 histone hypoacetylation in naïve cells to a hyperacetylated profile upon stimulation under Th1 and Th2 conditions induced the differentiation of CD4+ T cells. The differentiated Th1 and Th2 cells exhibited reduced acetylation in IFN-γ and IL-4 gene loci that was considered responsible for the associated silencing of gene transcription in these differentiated cells (Avni et al., 2002). Further studies demonstrated that GATA-3, a proallergic Th2-specific transcription factor, induced chromatin remodeling at the IL-4/ IL-13 intergenic regulatory region of developing Th cells allowing for accessibility and thus differentiation to Th2 cells (Takemoto et al., 2000).

DNA methylation also plays an important role in the control of Th2 cytokine expression and stabilization during Th cell development. When naïve T cells are activated under Th2-polarizing conditions, demethylation occurs at the IL-4 genetic promoter (Janson et al., 2009; Lee et al., 2002). As mentioned above, Webster et al. examined CD4+ T cells from human cord blood samples and found that CpG methylation levels in the proximal promoter of the IL-13 gene was reduced significantly in Th2 when compared with Th1 cells. This finding corresponded with higher histone H4 acetylation levels in the IL-13 proximal promoter, but not in the IFN-γ promoter (Webster et al., 2007).

DNA methylation of the counter-regulatory Th1 cytokine IFN-γ also has been the subject of much investigation. Several studies have demonstrated the association between Th1 differentiation and hypomethylation in the promoter region of the IFN-γ gene (Dong et al., 2013; Webster et al., 2007). Interestingly, White et al. also found a reduction in IFN-γ methylation levels of CD8+ T cells in atopic versus nonatopic children. In addition, hypermethylation of the IFN-γ promoter region was associated with decreased IFN-γ gene expression in splenic CD4+ T cells (Brand et al., 2012; Jones and Chen 2006).

Kumar et al. transfected primary cultured T cells with let-7 family miRNAs (a highly conserved family of miR-NAs known to be involved in Toll-like receptor 4 signaling) and found reduced IL-13 levels. Intranasal administration of let-7 also reduced the allergic phenotype (airway inflammation, airway hyperresponsiveness, mucus metaplasia, and subepithelial fibrosis) in sensitized mice (Kumar et al., 2011). Let-7 miRNAs were tested further in a murine model of asthma by Polikepahad et al. Short RNAs and miRNAs were highly enriched in both naïve and allergen-sensitized and challenged mouse lungs, with the most abundant ones belonging to the let-7 family. However, targeted inhibition of let-7 miRNA suppressed Th2 cytokine production, eosinophil recruitment to the lung, and airway hyperresponsiveness, suggesting that in this *in vivo* model let-7 miRNAs exerted a proinflammatory role (Polikepahad et al., 2010). The authors attributed these unexpected findings to the large (>800) repertoire of targets of let-7 miRNAs and possible secondary effects.

T Regulatory (Treg) Cell Pathway

Treg cells suppress Th1/Th2 activity and may be impaired in patients with allergic disease including asthma (Larche, 2007). Activation of the forkhead box transcription factor 3

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(FOXP3) is responsible for the differentiation of Treg cells and thus is a target for investigating this pathway. Hinz et al. used a novel method of determining Treg number by measuring the percent of demethylation in the Treg-specific demethylated region (TSDR) of FOXP3. Using this method they found that low Treg numbers in cord blood DNA was associated with increased risk of atopic dermatitis at age 1 year. They also demonstrated that environmental exposures, such as prenatal maternal smoke exposure, were associated with a decrease in cord blood Treg numbers (Hinz et al., 2012). Several studies also have shown the importance of histone modifications in making the FOXP3 locus available for Treg gene transcription and expression. Using chromatin immunoprecipitation (ChIP), Floess et al. (2007) found the FOXP3 gene locus in an open euchromatin structure in Treg cells as compared to the closed structure in a conventional T cell, and this finding was associated with differences in histones H3 and H4 acetylation and trimethylation profiles. Furthermore, inhibition of histone deacetylases (HDAC) promoted acetylation in the FOXP3 gene locus, which resulted in both an increase in FOXP3 gene expression and Treg function.

Th17 Pathway

Th17 is a distinct lineage of Th cells that has been newly linked to allergic diseases including asthma. Epigenetic mechanisms are thought to be involved in Th17 differentiation. Th17 lineage, whose cells are responsible for the production of several cytokines, including IL-17a, IL-17f, IL-21, and IL-22 may be regulated through histone modifications. In one study, Koenen et al. demonstrated that human Treg cells can differentiate into Th17 cells, likely through HDAC activity (Koenen et al. 2008). In another publication, Mukasa et al. examined the chromatin structure of the IL-17a, IL-17f, and IFN-γ gene loci in Th17 cells and also found differences in cytokine expression associated with changes in histone methylation (primarily in histone H3) in the presence of extrinsic cell manipulation with various cytokines including TGFβ and IL-12 (Mukasa et al., 2010).

Arginase-Nitric Oxide Synthase Pathway

The nitric oxide synthase (NOS) pathway recently has emerged as an important mechanism in the development of asthma, and the enzyme arginase may play a key role in limiting the production of nitric oxide (NO). L-arginine can be metabolized to either urea or L-ornithine via arginase activity, or nitric oxide, and L-citrulline via NOS activity. Increased arginase activity results in decreased NO production by competitive inhibition for the substrate L-arginine. Conversely, increased NOS activity results in increased NO production which has been associated with asthma (Benson et al., 2011). Several studies have related elevated fractional exhaled nitric oxide (FeNO), a bio-marker of airway inflammation, to early environmental exposures, and allergic airway inflammation, respiratory symptoms, and asthma (Cornell et al., 2012; Dweik et al., 2010; Fitzpatrick et al., 2006; Kalliola et al., 2013; Strunk et al., 2003).

In a recent publication by Breton et al., the conventional understanding of this pathway was somewhat challenged with the novel finding that DNA methylation of the arginase gene promoter (thus silencing of gene transcription), but not the iNOS gene, was associated with decreased FeNO production in children both with and without asthma (Breton et al., 2011). This pathway is complex, as Kuriakose et al. illustrated in their response to the publication

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where they reported pilot data of an inverse association between iNOS methylation and proximal but not distal FeNO components (Kuriakose et al., 2012). This later finding supports the theory that decreased methylation and increased expression on iNOS results in increased NO production. Although Breton's findings appear to contradict our current understanding of this pathway, it was the first paper to identify an association between DNA methylation changes and an airway inflammatory marker of asthma.

ASTHMA-RELATED ENVIRONMENTAL EXPOSURES AND EPIGENETIC DYSREGULATION

Exposure to a number of environmental elements has been associated with the development of asthma and allergies in children and altered epigenetic regulation (Table I). These relatively ubiquitous exposures include allergens, ambient air pollution, environmental chemical compounds, folate, and other prenatal supplements, as discussed below.

Allergens

Exposure to allergens is critical to the process of allergic sensitization and the development of atopic diseases, such as asthma. Emerging literature supports the importance of the prenatal time period of allergen exposure to the development of atopic disease in childhood, or in the case of household pets, to its protection (Lodrup Carlsen et al., 2012; Ownby et al., 2002; Perzanowski et al., 2013). The literature supporting allergen exposure-induced epigenetic changes is beginning to accumulate. Brand et al. documented that allergic sensitization in mouse models increased DNA methylation in the IFN-γ promoter and silenced IFN-γ gene transcription. The effect was reversed with administration of DNA methyl-transferase (DNMT) inhibitor *in vitro* and *in vivo* (Brand et al., 2012). In a subsequent study by our group, Niedzwiecki found that the grand-offspring of mice sensitized with *A. fumigatus* during pregnancy had lower DNA methylation at IL-4 promoter CpG-408 and CpG-393 as compared to mice exposed after birth or those without exposure ($P < 0.005$ across all doses). The prenatally exposed grand-offspring paradoxically had lower allergic IgE levels (Niedzwiecki et al., 2012). Shang et al. also, in an experimental allergic sensitization model involving treatment of mice with HDM extracts, found that treated mice exhibited changes in global methylation compared to controls. Using methylation sensitive restriction finger-printing, the group identified several asthma candidate genes in cAMP signaling, AKT signaling, ion transport, and fatty acid metabolism that exhibited altered methylation (Shang et al., 2013).

Collision et al. characterized the miRNAs that are expressed in the airway wall after allergen provocation of mice sensitized to HDM. Interestingly, the investigators measured elevated levels of miR-145, miR-21, and let-7b in the HDM-induced allergic airways as compared to non-allergic mice. Notably, miR-145, miR-21, and let-7b have been implicated in airway smooth muscle function, allergic inflammation, and airway epithelial cell function, respectively, suggesting that these miRNAs may regulate aspects of the host response to HDM. Selective inhibition of miR-145 significantly reduced IL-5 and IL-13 production by Th2 cells, eosinophils recruitment to airways, mucus hypersecretion, and airway hyperresponsiveness to a level similar to that observed following treatment with

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dexamethasone (Collison et al., 2011). Furthermore, in one human cell study, *ex vivo* exposure of CD4+ T lymphocytes to dust mite antigens resulted in hypomethylation of several CpG sites in the IL-4 promoter region (Kwon et al., 2008).

Dietary Supplementation with Methyl Donors

Despite the publication of several studies suggesting that alterations in diet during pregnancy may protect against the risk of asthma or allergies in the child (Chatzi et al., 2008; Dunstan et al., 2003), only prenatal supplementation with folate has been linked to epigenetic changes and an asthma phenotype. This was first demonstrated in a mouse model of experimental asthma where pregnant and weaning mice received a diet supplemented with methyl-donors including folic acid. Offspring of these mice showed enhanced features of experimental asthma following OVA sensitization, including more severe airway hyperresponsiveness and airway eosinophilia. This allergic phenotype also was associated with altered DNA methylation and protein suppression of several genes including runt-related transcription factor 3 (RUNX3) (Hollingsworth et al., 2008), a gene known to regulate CD4+/CD8+ T lymphocyte development by silencing CD4+ expression during T cell lineage decisions (Ehlers et al., 2003). This study also found that a diet rich in methyl donors during lactation or adulthood did not induce airway disease in this experimental model, suggesting that the timing of methyl donor supplementation may be key in epigenetic regulation (Hollingsworth et al., 2008).

The association of prenatal folate supplementation with child asthma risk has since been tested in human cohorts. In an Australian prospective birth cohort, the intake of supplemental folic acid late in pregnancy was associated with increased risk of asthma in the child at 3.5 and 5.5 years (Whitrow et al., 2009). Håberg et al. (2009) found that the use of folic acid supplements in pregnancy during the first trimester was associated with an increase in the risk of early respiratory infections and wheeze. A later study from the same group measured concentrations of blood plasma folate during the second trimester of pregnancy and found an increased risk of asthma at age 3 years for children with high maternal plasma folate levels (Håberg et al., 2011). In contrast, Magdelijns et al. (2011) found that folic acid use during pregnancy was not associated with a greater risk of wheeze, asthma, or eczema. At age 2 years, serum folate levels were associated inversely with total IgE levels, atopy and wheeze (Matsui and Matsui, 2009). These latter human studies further support the premise that the current evidence is insufficient to recommend any change from the current practice of peri-conceptional folic acid supplementation to protect children from neural tube defects.

Particulate Matter (PM)

Several studies have associated exposure to traffic-related air pollution, in particular diesel soot and fine particulate matter ($PM_{2.5}$), with asthma exacerbations and hospitalizations among children (Bell et al., 2009; McConnell et al., 2006; Patel et al., 2009; Spira-Cohen et al., 2011). In the Columbia Center for Children Environmental Health birth cohort of children from New York City (NYC), our group found a positive association between measured residential indoor levels of $PM_{2.5}$ and the development of new wheeze that was reported between the ages of 5 and 7 years, a symptom characteristic of asthma at young

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ages (Jung et al., 2012). Furthermore, two studies to date in adults have demonstrated that exposure to $PM_{2.5}$ (Salam et al., 2012) and PM_{10} (Tarantini et al., 2009) is associated with decreased methylation of the inducible nitric oxide synthase (iNOS) promoter, an important enzyme in NO production and airway inflammation (Benson et al., 2011). Interestingly, the association between iNOS DNA methylation and PM_{10} exposure was only evident in short term (after 3 consecutive days of work in a steel production plant) and not long-term exposure (after 2 consecutive days off from work, reflecting baseline levels of exposure) (Tarantini et al., 2009).

Polycyclic Aromatic Hydrocarbons (PAHS)/Diesel Exhaust Particles (DEP)

Exposure to airborne polycyclic aromatic hydrocarbons (PAH), traffic-related combustion products, has been linked to epigenetic changes associated with childhood asthma and allergy. Our group first reported that high levels of ambient PAH measured during pregnancy was associated with DNA methylation of the acyl-coenzyme A synthetase long-chain family member 3 (ACSL3) promoter in cord white blood cell DNA and with increased asthma risk in children (Perera et al., 2009). Moreover, levels of ambient PAH measured during pregnancy were associated with hypermethylation at multiple CpG sites in the IFN-γ promoter from the same cord blood DNA (Tang et al., 2012), offering a mechanism whereby the counter-regulatory cytokine is suppressed or silenced in asthma.

Exposure to PAH also has been shown to alter DNA methylation of genes important in Treg function. Nadeau et al. demonstrated that FOXP3 was hypermethylated in children with higher measures of ambient PAH exposure (Nadeau et al., 2010). In further mechanistic experiments in cell systems by the same group, high dose administration of the PAH phenanthrene increased DNA methylation and reduced gene expression of FOXP3. Most interestingly, these changes were associated with conversion of the Treg to a more Th2 phenotype (Liu et al., 2013), suggesting immunemodulation of key pathways in allergic sensitization, as reviewed below.

Exposure to diesel exhaust particles (DEP), another environmental air pollutant, also has been associated with epigenetic regulation of asthma genes. Our laboratory (Liu et al., 2008) exposed mice almost daily to 3 weeks of inhaled DEP while undergoing intranasal sensitization to *A. fumigatus* to study whether DEP would induce methylation changes of the asthma genes IL-4 and IFN-γ. Inhaled DEP exposure and intranasal *A. fumigatus* induced hypermethylation at CpG-45, CpG-53, CpG-205 sites of the IFN-γ promoter and hypomethylation at CpG-408 of the IL-4 promoter. Altered methylation of both genes correlated significantly with changes in IgE levels. This was the first study to demonstrate that inhaled environmental exposures influenced methylation of Th genes important in IgE regulation *in vivo*. In human cohort work, measures of DEP exposure, estimated by land use regression analysis, was associated with altered expression of FOXP3 by increased DNA methylation in saliva DNA in children (n =92) selected from the Cincinnati Childhood Allergy and Air Pollution Study. This group reported a 4% (95% CI, 1.83–6.18%) increase in FOXP3 methylation per interquartile range increase in estimated DEP exposure (Brunst et al., 2013).

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Environmental Tobacco Smoke (ETS)

Prenatal and early childhood exposure to tobacco smoke represents a major risk factor for the development of childhood asthma (Thomson, 2007). Substantial literature suggests that some of these effects may be epigenetically regulated. To demonstrate epigenetic effects on lung tissue in smokers, one study examined lung biopsy specimens and alveolar macrophages obtained from bronchoalveolar lavage in a group of otherwise healthy tobacco smokers and age-matched, healthy, nonsmoking adults. They found a reduction in the expression and activity of HDAC-2 as well as enhanced gene expression of the inflammatory mediator, IL-1β-induced tumor necrosis factor (TNF)-α that was correlated with HDAC activity (Ito et al., 2001). Reported maternal smoking during pregnancy was associated with higher methylation levels of leukocyte DNA as well (Terry et al., 2008). In contrast, Breton et al. examined global and gene-specific methylation patterns in buccal cells from children in the Children's Health Study in relation to history of prenatal ETS exposure. Although there was no strong clustering of gene methylation patterns by prenatal smoke exposures, they found that methylation of the DNA repetitive short interspersed nucleotide element AluYb8 was decreased in association with maternal smoking during pregnancy. Methylation of the DNA repetitive long interspersed nucleotide element (LINE-1) was decreased only among children with the common GSTM1 null genotype (Breton et al., 2009), a genotype previously associated with early onset asthma following prenatal ETS exposure (Gilliland et al., 2002). Even more recently, using plasma cotinine levels measured during pregnancy to document *in utero* exposure to cigarette smoke in the Norwegian Mother and Child Cohort Study researchers discovered differential DNA methylation for 26 CpGs mapped to 10 genes, including aryl-hydrocarbon receptor repressor (AHRR), cytochrome P450 family 1 subfamily A (CYP1A1), and growth factor independent 1 transcription repressor (GFI1) (Joubert et al., 2012).

Volatile Organic Compounds (VOCs)

Childhood exposure to benzene, toluene, xylene and other VOCs has been shown to be associated with a greater prevalence of asthma and nonspecific respiratory symptoms, although adverse respiratory effects of prenatal exposures to VOC are less apparent. While effects of VOC exposure on asthma gene methylation or other epigenetic alterations have not yet been demonstrated, *in vitro* experiments have shown that benzene exposure induces hypermethylation of poly (ADP-ribose) polymerase-1 (PARP-1), a gene involved in DNA repair (Gao et al., 2010). Airborne measures of benzene were associated with demethylation of LINE-1 and Alu, repetitive elements that are indicators of global methylation (−2.33% for a tenfold increase in airborne benzene levels; $P = 0.009$; −1.00%; $P = 0.027$, respectively). Hypermethylation in p15 promoter (+0.35%; $P = 0.018$) (a gene whose hypermethylation has been associated with acute myeloid leukemia) and hypomethylation in melanoma-associated antigen (MAGE)−1 (−0.49%; $P = 0.049$) (a gene known to be hypomethylated in malignancy) were associated with increasing airborne benzene levels (Bollati et al., 2007). Given this and the link between benzene exposure and asthma respiratory symptoms, further study into epigenetic mechanisms is this area may be informative.

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Microbes

Several studies support the hypothesis that the risk of atopic sensitization and allergic disease is reduced by higher microbial exposure prenatally or during early life (Lampi et al., 2011; Leynaert et al., 2001; Remes et al., 2003; Riedler et al., 2001; Von Mutius 2007). This protective effect may persist into adult life (Leynaert et al., 2001). First, there is mounting evidence that microbes can alter asthma gene IFN-γ regulation. For example, infection with several viruses like human immunodeficiency virus, lymphocytic choriomeningitis virus, and Epstein Barr virus have been shown to induce either hypermethylation or demethylation of IFN-γ in CD8+ T cells (Vuillermin et al., 2009), although effects on allergy were not determined in these studies. Second, in one clinical trial, prenatal exposure to probiotics intended through supplementation with *Lactobacillus rhamnosus* GG during pregnancy and lactation suppressed the development of allergic disease in children through age of 4 years (Blümer et al., 2007). Third, in a birth cohort study, the protection against the development of allergy following prenatal microbial exposure via exposure to farming during pregnancy was associated with enhanced neonatal Treg function in cord blood cells, as well as greater FOXP3 expression and DNA demethylation (Schaub et al., 2009). Finally, in the PASTURE (protection against allergy: study in rural environments) birth cohort, cord blood DNA methylation levels in regions in ORM1-like protein (ORMDL)1, and STAT6 were hypomethylated in DNA from farmers' as compared to nonfarmers' children. In comparison, regions in RAD50 and IL-13 were hypermethylated. Changes in methylation between birth and age 4.5 years occurred in 15 gene regions and these differences clustered in the genes highly associated with asthma (ORMDL family) and IgE regulation (RAD50, IL-13, andIL-4), but not in the Treg genes (FOXP3, RUNX3) (Michel et al., 2013).

EPIGENETIC BIOMARKERS AND CLINICAL ASTHMA

Despite the growing literature relating epigenetic changes in asthma genes following environmental exposures, the literature supporting that such changes are associated with clinical asthma is just beginning to emerge. For example, in the Cincinnati Childhood Allergy and Air Pollution Study described above, a twofold increase in the mean level of FOXP3 methylation was observed among persistent wheezers compared with nonwheezers. Early transient wheezers and asthmatic children had higher mean levels of FOXP3 methylation when compared with nonwheezers and nonasthmatic children (Brunst et al., 2013). Isidoro-Garcia et al. compared CpG methylation levels of the D-prostanoid receptor (PTGDR) gene, a mediator of the production of prostaglanding D_2 (PDG2), in a cohort of allergic asthmatic patients and controls. PTGDR methylation levels were decreased in multiple CpG sites in asthmatic patients compared with controls (Isidoro-García et al., 2011). Further, bronchial biopsy specimens from adult asthmatic patients exhibited lower histone deacetylase (i.e., HDAC1 and HDAC2) protein expression and activity, when compared with those derived from healthy controls, indicative of impaired transcriptional repression via altered removal of acetyl groups in asthma (Ito et al., 2002). Decreased HDAC activity and increased histone acetyltransferase (HAT) activity (i.e., increased transcriptional activity) also was observed in alveolar macrophages collected via bronchoalveolar lavage in adult asthmatics when compared to controls (Cosío et al., 2004). Royce et al. demonstrated that administration of an HDAC inhibitor (valproic acid) in a

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murine model of allergic airways disease suppressed airway hyperresponsiveness and inhibited the development of airway epithelial thickening and fibrosis (Royce et al., 2011), highlighting the potential importance of HDAC activity to asthma and suggesting a novel epigenetic approach to the treatment of airway remodeling.

Moreover, when bronchial epithelial miRNA expression was studied by microarray analysis among steroid-naive adults with asthma versus controls, those with asthma had evidence of 217 miRNAs that were differentially expressed. Another 200 were identified in steroid-using subjects with asthma (false discovery rate, 0.05). Treatment with inhaled corticosteroids induced changes in nine of the miRNAs identified in steroid naïve asthmatics (Solberg et al., 2012). Using microarray expression profiling of mRNA and noncoding RNA (ncRNA) to examine RNA patterns in peripheral CD4+ and CD8+ T cells, large differences among severe as compared to nonsevere asthmatics were detected in the CD8+ T cell samples, particularly in natural antisense, pseudogenes, intronic, and intergenic long noncoding RNAs (lincRNAs), consistent with an activated CD8+ T cell pattern (Tsitsiou et al., 2012).

Also, in nasal cells collected from asthmatic children aged 8–11 years, lower promoter methylation of both IL-6 (+29.0%; P = 0.004) and iNOS (+41.0%; P = 0.002) were associated with higher FeNO level (Baccarelli et al., 2012). In the Infancia y Medio Ambiente (INMA) Project: Menorca and Sabadell cohorts, lower whole blood DNA methylation at age 4 years in the arachidonate 12-lipoxygenase (ALOX12) gene was associated with persistent wheezing in children at age 6 years (Morales et al., 2012). Finally, in the Isle of Wight Cohort, IL-4 receptor (IL4R) gene methylation levels, identified using the Illumina Infinium Human Methylation 450 Bead Chip, showed an association with asthma at age 18 years. Testing for an interaction between eight different single nucleotide polymorphisms (SNPs) and IL4R methylation level on the risk for asthma revealed a significant interaction between SNP rs3024685 and IL4R methylation levels (P =0.002; after adjusting for false discovery rate) (Soto-Ramírez et al., 2013).

However, much more discovery is needed to gain a better understanding of the contribution of epigenetic bio-markers to clinical asthma. Several studies have demonstrated that variability in DNA methylation levels is cell-and tissue-specific (Nadeau et al., 2010; Talens et al., 2010). This was well-demonstrated by Jacoby et al., who assessed DNA methylation levels of 58 CpG sites from eight immune response genes and identified very different patterns of interindividual variability across neighboring CpG positions depending on cell type (i.e., highest for CD56+, CD8+, unsorted cord blood mononuclear cells or PBMCs; lowest for CD4+ cells). This particular pattern also exhibited an age effect and tended to be greater in adult versus newborn cord blood specimens in all cell types, and differed depending on the immune response gene (Jacoby et al., 2012). Stefanowics et al. documented substantial differences when comparing the DNA methylation signatures, determined by Illumina GoldenGate Methylation Cancer Panel I, of airway epithelial cells (AECs) versus PBMCs among asthmatic, atopic, and healthy children undergoing elective surgery for nonrespiratory conditions. They found 57 CpG sites across 47 genes that were differentially methylated in AECs as compared to PBMCS. Moreover, they identified 8 methylated sites including CpGs in STAT5A and CRIP1 genes that were differentially

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methylated in asthmatic versus atopic alone-derived AECs. Such differences were not evident in PBMCs, quesitoning the suitability of using methylation levels in DNA derived from PBMCs as bio-markers of methylation patterns in asthma (Stefanowicz et al., 2012).

Pharmaco-Epigenetic Studies

Epigenetic regulation also has emerged as a potential mechanism for the action of asthma-related pharmacologic therapies and differences in therapeutic drug responses across individuals. For example corticosteroids, which suppress some of the hyperactive immune responses in the airways, are believed to exert some of their anti-inflammatory action by inducing histone acetylation of anti-inflammatory genes and by recruiting HDAC2 to activated pro-inflammatory genes (e.g., glucocorticoreceptor) (Barnes, 2009). Theophylline, although used less frequently in the management of asthma, also has been shown to reverse the effects of corticosteroid resistance by restoring HDAC2 activity and inducing deacetylation of proinflammatory genes (e.g., IL-8, NF-kβ) (Barnes 2009). Wu et al. tested the DNA methyltransferase inhibitor 5-azacytidine in asthma animal models and found that treated mice exhibited reduced airway hyperreactivity, pulmonary eosinophilia, and other allergy related biomarkers while the number of FOXP3+ cells was increased (Wu et al., 2012).

NOVEL TOOLS AND NOVEL APPLICATIONS IN ASTHMA EPIGENETIC RESEARCH

In contrast to observational studies of singletons, twin studies can provide several advantages for studying epigenetic regulation. Monozygote twin (MZT) pairs are matched on age, genome, and intrauterine and usually early childhood environment and cultural milieu. Yet they are often discordant for diseases including asthma (Runyon et al., 2012; Strachan et al., 2001). For example, Fraga et al. reported that with age, MZTs showed large differences in global DNA CpG island methylation, gene expression profiles on microarray analysis and levels of histone acetylation (Fraga et al., 2005). Wong et al. measured the levels of DNA methylation of three neuropsychiatric genes (dopamine receptor 4 [DRD4], serotonin transporter [SERT], and X-linked monoamine oxidase A [MAOA] in MZT and dizygotic twin [DZT]) pairs at 5 and 10 years old, and found significant differences in methylation patterns of both DZT and MZT pairs, as well as differences over time (Runyon et al., 2012; Wong et al., 2010). MZT studies in which there is discordance in environmental exposures and asthma are certainly a powerful method for studying epigenetics as they can eliminate potential confounders such as genetic predisposition and *in utero* exposures.

Epigenome-wide association scan (EWAS) is another tool that has the potential to discover risk factors and molecular and disease consequences. This fairly novel application to epigenetic lung research allows for assessment of methylation pattern variations across numerous candidate gene loci. For example, such arrays from peripheral blood DNA have been useful in identifying genes alternatively regulated by epigenetics mechanisms following current and past exposure to cigarette smoke (Breitling et al., 2011). Moreover, Zeilinger et al. conducted EWAS comparing the association of tobacco smoking on DNA methylation with the illumina 450 K BeadChip using DNA obtained from whole blood

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(Zeilinger et al., 2013). They identified 972 CpG sites with differential methylation levels, depending of the smoking status. This new technique may prove useful in determining epigenetic marks in complex diseases such as asthma, recognizing however that whole blood may still not be the optimal tissue for studying epigenetic regulation in this disease.

CONCLUSION

The inherent plasticity of epigenetic regulation following environmental exposures offers opportunities for prevention using environmental remediation, measuring novel biomarkers for early identification of those at risk, and applying advances in pharmaco-epigenetics to tailor medical therapies that maximize efficacy of medical treatment. Future studies that focus on the contribution of interacting environmental exposures, cell, and tissue-specific effects, and possible multigenerational effects, are needed. 'Precision Medicine' in asthma and allergy is arriving. This may involve an individually tailored approach to the detection, prevention, and treatment of disease based on the knowledge of an individual's epigenetic profile as this field advances. A greater understanding of epigenetic regulation in asthma and allergy is at its core.

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TABLE I

Environmental Exposures and Their Epigenetically Regulated Asthma Genes

Environmental exposure	**Gene(s) affected**	**Epigenetic modifications induced by environmental factor**	**References**
Allergens, including	IL-4, IFN-γ	DNA methylation	Liu et al. (2008) Niedzwiecki et al. (2012)
A. fumigatus, dust mite	IL-4	DNA methylation	Kwon et al. (2008)
	PDE4D, AKT1s1, TM6SF ACLS3, POM121/2	DNA methylation	Shang et al. (2013)
	IFN-γ	DNA methylation	Brand et al. (2012)
Dietary supplement with methyl donors, including folate	RUNX3	DNA methylation	Hollingsworth et al. (2008)
Particulate matter (PM)	iNOS	DNA methylation	Salam et al. (2012), Tarantini et al. (2009)
Polycyclic aromatic hydrocarbons (PAHs)/Diesel exhaust particles (DEP)	ACSL3	DNA methylation	Perera et al. (2009)
	IFN-γ	DNA methylation	Liu et al. (2008)
	FOXP3	DNA methylation	Liu et al. (2013), Brunst et al. (2013), Nadeau et al. (2010)
Cigarette smoke	GSTM1/GSTP, IL-8 and IL-1β-induced TNF-α	Histone deacetylation	Ito et al. (2001)
	AHRR, CYP1A1, GFI1	DNA methylation	Joubert et al. (2012)
VOCs	LINE-1, Alu1	DNA methylation	Bollati et al. (2007)
Microbes	FOXP3	DNA methylation	Schaub et al. (2009)
	RAD50, IL-13, IL-4	DNA methylation	Michel et al. (2013)