Epigenetics and allergy: from basic mechanisms to clinical applications

Allergic diseases are on the rise in the Western world and well-known allergy-protecting and -driving factors such as microbial and dietary exposure, pollution and smoking mediate their influence through alterations of the epigenetic landscape. Here, we review key facts on the involvement of epigenetic modifications in allergic diseases and summarize and critically evaluate the lessons learned from epigenome-wide association studies. We show the potential of epigenetic changes for various clinical applications: as diagnostic tools, to assess tolerance following immunotherapy or possibly predict the success of therapy at an early time point. Furthermore, new technological advances such as epigenome editing and DNazymes will allow targeted alterations of the epigenome in the future and provide novel therapeutic tools.

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Allergic diseases display a broad spectrum of clinical manifestations and conditions. The major group of them is mediated by immunoglobulin E (IgE) and is commonly referred to as atopic disorders. These include allergic bronchial asthma (main manifestations in the lung and the lower airways), allergic rhinitis (AR; hay fever; the upper respiratory tract), allergic conjunctivitis (the eyes), (extrinsic) atopic dermatitis (AD; eczema; the skin) and food allergies (the upper and lower gastrointestinal tract) (Figure 1). Although each patient shows an individual spectrum of these conditions during the entire life span, certain patterns are well recognized. This includes the 'atopic (allergic) march', which describes the prototypical sequential manifestation throughout life, starting with atopic eczema or food allergies, shifting then later to respiratory conditions, during which compromised barrier function at one surface will lead to other dysfunctional epithelial barriers [1–3]. Furthermore, several

of the conditions may present simultaneously such as eczema together with food allergies or eczema together with bronchial asthma [4,5]. As this co-occurrence might arise even prior to the first actual ingestion of the food, the early postnatal period seems to be of crucial importance for the development of IgE-mediated allergies [6]. Although still under debate, IgE-mediated food allergies seem to be increasing at a high rate in the Western countries and concern now approximately 3–5% of children and about 1–3% of adults, with substantial geographical and ethnical differences [7–9]. Typical childhood associated food allergies, such as cow's milk allergy (CMA), also increasingly persist until later age. At early age, there is a strong sex bias in favor of males in the development of asthma and other allergic diseases including food allergy, which is inverted after puberty and throughout adulthood with a higher prevalence in women [10–14]. Allergic disorders not associated with IgE do also exist such as allergic

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contact dermatitis, non-IgE-mediated food allergy or intrinsic AD [15,16]. Furthermore, different subtypes of asthma can arise unrelated to IgE (non-atopic asthma) and although these might represent different disease entities, they share a number of similarities including the below described cytokine shift and resulting inflammation as well as mast cell activation [17,18]. As many publications studying epigenetic modifications in asthma do not provide extensive information on the type of asthma in the recruited individuals, it should be kept in mind that this review describes results from studies including probably a variety of asthma phenotypes, which might be a confounding factor for epigenetic changes.

Common to the atopic syndrome is the presence of chronic inflammation at body–environment interfaces [19,20]. This inflammation covers the mucosal membranes and the skin, and reflects an active, chronic misguided immune response directed against otherwise harmless environmental antigens. Such antigens are termed ‘allergens.’ This imbalanced immune response is characterized by allergic inflammation including the development of T-helper (Th)-2 (Th2) immune responses as the consequence of allergen presentation by innate immune cells termed antigen-presenting cells (e.g., dendritic cells, macrophages) to naive T cells. Following the exchange of a set of signaling events, these naive T cells develop into Th2 effector cells, which are defined and characterized by the production of a unique set of so-called ‘type-2’ (see section on ‘T cells’) cytokines including interleukin (IL)-4, IL-5, IL-13 and IL-9, the latter considered now to be synthesized predominantly by Th9 cells (Figure 1) [21–24]. These cytokines direct the effector phase of the allergic response [21–23]. IgE molecules bind to high- (FcεRI) and low-affinity (FcεRII, CD23) IgE receptors. FcεRI is expressed particularly on mast cells and basophils. Allergen binding to IgE molecules occupying FcεRI on the surface of those cells results in cross-linking of these receptors and subsequent cell degranulation and mediator release, leading to the development of allergic symptoms typical for type I (immediate) hypersensitivity reactions (Figure 1) [21,25]. Recently, it was shown that not only Th2 cells secrete type-2 cytokines but also innate lymphoid cells (ILCs) type 2 (ILC2) are important producers of these mediators. The orchestration of the inflammatory response by the above-mentioned cellular components together with the cytokine mediators is now termed ‘type-2 inflammation’ (Figure 1) [22,23].

The development of the atopic syndrome requires a genetic predisposition. Great efforts have been undertaken to unravel the genetic basis of allergic diseases and it is now accepted that allergic/atopic diseases

are characterized by a polygenetic signature. A great variety of genes encoding molecules regulating and/or participating in T cell activation, B cell development and allergen presentation, cytokines and cytokine receptors, chemokines, growth factors, proteins involved in remodeling, wound healing and repair, epithelial signatures, members of metabolic pathways and others have been identified as susceptibility loci for allergic diseases and related intermediate phenotypes or quantitative traits [21,26–29]. Despite these advances, the polygenetic signature cannot explain the dramatic increase in incidence and prevalence of atopic diseases throughout the past 70 years. It is now well accepted that environmental factors and (Western) lifestyle conditions play an important role in initiating and maintaining type-2 inflammation in predisposed individuals [19,22,23,30–32].

Based on this paradigm, allergic diseases are now considered prototypic examples of conditions determined by gene × environment interactions [33,34]. A few decades ago, research efforts focused on the identification and characterization of risk factors, which may favor the development and/or maintenance of allergic diseases. Key risk factors include smoking (active or passive), allergen exposure levels, ozone, diesel exhaust particles (DEPs), SO₂ and NO₂ [32,35]. Furthermore, viruses play an important role in disease exacerbation or maybe even development, especially in case of asthma [36–38]. Bacterial superinfections, particularly with endotoxin-positive *Staphylococcus aureus* strains and other microbes represent important contributors to the pathophysiology of AD and other allergic diseases [39–41].

Since not everybody with a genetic predisposition eventually develops an atopic disease, recent research focuses on potential protective factors. Particularly, the insufficient exposure to (infectious) microorganisms (the hygiene hypothesis) or dietary and bacterial metabolites, and frequent use of antibiotics and antipyretics have gained broad attention [30,42]. An important example for this concept is the traditional farming environment, particularly present in small Alpine villages, which confers a strong protection to allergy. This has been attributed to the direct exposure to farm animals, consumption of non-homogenized and non-pasteurized milk, a high prevalence of breastfeeding, intra-uterine exposure to airborne microbial factors and the inhalation of microbes and microbial compounds throughout early childhood [19,33–35,43]. This concept is now further expanding and offers great opportunities for the development of novel allergy and asthma protective strategies.

In order to advance in this field, it is critical to better understand the cellular and molecular mechanisms

Figure 1. Allergic (type 2) inflammation: basic mechanisms and pathophysiology, and selected clinical consequences. Th2 cytokines can be synthesized also by innate lymphoid cells type 2 (ILC2), which is not shown. Contribution of Th9 cells, major IL-9 producers, is also not presented. MHC-II denotes MHC class II molecules; TCR, T cell receptor. For remaining abbreviations, detailed description of the figure content and additional information, please, refer to the main text. This figure was inspired by several previously published images, especially by the one by Gandhi *et al.* [23].

through which environmental factors influence the initiation and/or maintenance of the disease. In this regard, epigenetic mechanisms may contribute significantly to the answer [19,31,32,44]. Epigenetics may explain the high degree of plasticity among immune responses observed throughout life, and the impact of external

environmental factors, microbial and non-microbial components on disease development. Epigenetic pathways could thus mediate the gene × environment interactions. This field is also gaining increasing attention due to the potential use of epigenetic signatures as biomarkers or interfering therapeutically with the epigen-

etic code. The knowledge gained over the last few years in this emerging field of research will be summarized in this article.

Basics of epigenetics

DNA methylation & histone modifications

Epigenetic modifications are biochemical changes of the chromatin, in other words, DNA or histones, that are functionally relevant, but do not affect the nucleotide sequence of the genome. Although they are thought to contribute to some other processes, for instance response to damage and DNA repair, epigenetic modifications are best known for their effects on the accessibility of certain genomic loci to transcription enzymes and thus their expression. DNA methylation, a covalent addition of a methyl group, occurs at the cytosine nucleotide belonging to CpG dinucleotide (called 'CpG site'), which is a DNA sequence where a cytosine nucleotide (C) is directly followed by a guanine nucleotide (G). CpG sites frequently cluster to form 'CpG islands', typically located in the elements of a gene regulatory element with impact on its transcription, for example, promoters or enhancers [45–47]. Low DNA methylation levels in a promoter region are often, but not necessarily, associated with a higher transcriptional activity (or at least the potential of the gene to become expressed), while high DNA methylation levels in the CpG island of a promoter are usually associated with lower gene expression up to full gene silencing. The reaction of DNA methylation is catalyzed by DNA methyltransferases (DNMTs), including DNMT1 (target: hemimethylated dsDNA; role: maintenance of DNA methylation patterns after DNA replication) and DNMT3A and DNMT3B (target: hemimethylated and unmethylated dsDNA; role: *de novo* DNA methylation) [48–51]. The best-characterized post-translational histone modifications include phosphorylation, ubiquitination, acetylation and methylation, the last two of which are the most extensively studied [52,53]. Histone acetylation occurs at the lysine residues and it is catalyzed by histone acetyltransferases, while the opposite reaction by histone deacetylases (HDACs). Higher histone acetylation levels lead to less tight wrapping of the proteins around the DNA increasing its accessibility to the transcriptional machinery, whereas decreased histone acetylation has the opposite effect. Histone acetylation independent of the position of the lysine amino acid generally correlates with potentially active genes or gene regulatory elements [54,55]. Histones can become methylated at lysine or arginine residues and this reaction is catalyzed by histone methyltransferases. How histone methylation influences the chromatin condensation and thus gene expression depends on the location of the amino acid residue and on the number of methyl

groups added [32,56–62]. DNA methylation and histone modifications mutually interact [63]. As a more detailed description is beyond the scope of this review, the interested reader is referred to more detailed review articles, for example, those referenced in this subchapter.

MicroRNA

In addition to the 'classical' epigenetic mechanisms including DNA methylation and histone modifications, also some post-transcriptional control elements such as microRNAs (miRNAs) have become widely recognized as important epigenetic regulators of gene expression [64,65]. These approximately 22-nt noncoding RNA molecules are highly abundant, with more than 2500 mature miRNA molecules characterized in humans. Canonical miRNAs are encoded in humans mostly (but not solely) by introns of both non-coding or coding transcripts. They are transcribed from dsDNA usually by RNA polymerase II. The transcripts are further processed by two RNase III-type enzymes, first in the nucleus (Drosha) and then in the cytoplasm (Dicer). To exert its function, mature miRNAs become incorporated into the RNA-induced silencing complex. The RNA-induced silencing complex is in turn guided by miRNAs to specifically target mRNAs. This leads to the cleavage or degradation of the bound mRNA molecule or suppression of its translation by reducing the speed of the ribosomal machinery. The magnitude of the silencing effect depends on the level of complementarity between the miRNA and the targeted mRNA [64–69]. It has been proposed that the post-transcriptional regulation of gene expression facilitated by miRNAs plays an important role in the fine-tuning of transcriptional programs in the context of bigger regulatory networks and in buffering fluctuations in gene expression resulting from random internal cellular modulation and/or environmental influences [70]. Considering their biological importance, miRNAs have been involved in multiple human pathologies [66]. These include also allergic diseases, in which the role of miRNAs has been rather extensively studied [71–76]. Not surprisingly, analyzing miRNAs might become an important diagnostic tool in allergies [77–79]. It is also worth mentioning that the mechanism of RNA-mediated silencing of gene expression has been utilized in biomedical research as a powerful laboratory tool [80] and in therapeutic applications as one of the possible antisense approaches [81]. However, due to space restrictions, we will in this review focus on the 'classical' epigenetic mechanisms, DNA methylation and histone modifications. For more information on miRNAs, readers are referred to other articles including those referenced in this short subsection. Connections between miRNAs and DNA methyla-

tion/histone modifications have also been described in detail elsewhere [64,66,68,69,82].

T cells: importance in allergy & epigenetic regulation of differentiation

Many types of immune cells contribute to the development of atopic predisposition and to the effector phase of allergic inflammation (Figure 1), with several subpopulations of Th cells differentiating from naive CD4⁺ T cells playing a pivotal role in those processes and their modulation (Figure 2). The direction of naive CD4⁺ T cell maturation determines their subsequent effects, including those on allergy. T cell differentiation is strictly regulated, with changes in epigenetic marks at main lineage-determining loci playing a pivotal role. In this review, only the most important humoral and selected epigenetic mechanisms modulating naive T cell fate along with the major allergy-related effects of mature Th subpopulations, such as Th1, Th2 (and Th9), regulatory T cells (Treg cells) and Th17, are shortly outlined (Figure 2).

Differentiation toward each of the above-mentioned T cell types is driven by a precisely defined cytokine milieu (one major cytokine or a set of most important cytokines) influencing expression of lineage-specific transcription factors (TFs), so-called master TFs or master regulators (Figure 2) [31,83–87]. Th1 cells develop from naive CD4⁺ T cells under the influence of interferon- γ (IFN- γ ; gene: *IFNG*) and IL-12. Stimulation with those cytokines leads to the expression of T-box 21 (TBX21, traditionally called also T-bet; gene: *TBX21*), a master TF of the Th1 lineage and secretion of IFN- γ , a major Th1-produced cytokine [88–90]. The most important allergy-related effect of Th1 cells is the inhibition of Th2 lineage differentiation and thus type-2 inflammation [91]. The maturation of naive CD4⁺ T cells toward Th2 cells occurs under the influence of IL-4, which stimulates the expression of GATA binding protein 3 (GATA3; gene: *GATA3*) [31,83,92], a Th2 master TF. Th2 cells produce IL-4 (gene: *IL4*), IL-13 (gene: *IL13*), IL-5 and IL-9 (gene: *IL9*), the latter considered recently to be synthesized predominantly by a separate T cells subpopulation, namely Th9 cells (see further) [22]. The importance of Th2 cytokines for allergic (type 2) inflammation is actually fundamental as they simply mediate its development [21–24,91]. In more detail, major effects of those cytokines include differentiation of further Th2 cells and B cell class switching toward IgE (IL-4), activation and survival of eosinophils (IL-5), airway hyper-responsiveness, hyperplasia of goblet cells and mucus production (IL-13) and proliferation/survival of mast and ILC2 cells and mucus production (IL-9) [21–24,91] (Figures 1 & 2). Th9 cells develop from

naive CD4⁺ T cells under the influence of IL-4 and transforming growth factor- β (TGF- β) but they can possibly differentiate also from TGF- β stimulated Th2 cells [83,93]. Although several TFs such as interferon regulatory factor 4 (IRF4; gene: *IRF4*), PU.1 (gene: *SP11*) and others are involved in the development of the Th9 phenotype, none of them can be considered a master TF here (in the same way as e.g., GATA3 for Th2 or TBX21 for Th1). On the other hand, some believe that PU.1 is the most important Th9 regulator since it is the only TF able to convert another Th lineage into Th9 (Figure 2) [24,93–95]. TGF- β and IL-6 together stimulate naive CD4⁺ T cells to express their master TF, RAR related orphan receptor C isoform 2 (RORC2, traditionally called also ROR γ T), which is one of the two isoforms expressed from the same encoding locus (gene: *RORC*), and thus to differentiate toward Th17 cells [85,96–98]. As indicated by the name, Th17 cells produce IL-17A (traditionally called also IL-17; gene: *IL17A*) and IL17F (gene: *IL17F*), but also IL-21 and -22. Allergy-related effects of those cells comprise airway hyper-responsiveness as well as promotion of neutrophils and therefore their participation in the forms of asthma, in which neutrophils predominate [91,96,98,99]. Stimulation of naive CD4⁺ T cells with only TGF- β results in the expression of the forkhead box protein 3 (FOXP3; gene: *FOXP3*) TF and their maturation toward Treg cells, which secrete IL-10 and TGF- β [84,98,100,101]. Major effects of these cytokines in allergy include suppression of inflammation (TGF- β and IL-10), and promotion of airway remodeling (TGF- β) (Figure 2) [91,98].

Differentiation and synthesis of specific cytokines by the mature T cells populations is strictly controlled, with a prominent role of epigenetics (Figure 2). In general, important regulatory regions of the genes encoding master (or very important) TFs and cytokines characteristic for a ‘target’ T cell lineage undergo changes of the epigenetic landscape favoring transcription. Simultaneously, regulatory elements in respective genes of TFs or cytokines specific for the other T cell types are subjected to modifications having silencing properties, especially if their expression would oppose the development of the ‘target’ phenotype [31,83,102–104]. DNA methylation of the genes specific for Th1 cells is a very good example here. In Th1 cells, loci characteristic for this lineage such as *TBX21* and *IFNG* are demethylated during the maturation, whereas those essential for Th2 (*IL4*, *IL13*) or Th17 (*RORC*, *IL17A*) cells remain methylated. DNA methylation of *IFNG* is in turn preserved in Th2 and Th17 cells. In addition, *IL4* and *IL13* loci are hypomethylated in Th2 cells [31,83,88,104–108]. *RORC* remains methylated and *FOXP3* undergoes demethylation in developing Treg

Figure 2. Major types of Th cells, their differentiation and its epigenetic regulation, and their crucial allergy-related functions.

DNA methylation (CpG) status of the loci pivotal for Th lineages is shown. Levels of epigenetic histone marks at loci specific for Th subpopulations is depicted (selection). For more details and abbreviations, please, refer to the main text. This figure was inspired by several previously published images, especially by the one by Suarez-Alvarez *et al.* [83].

cells, while the situation in Th17 cells is completely opposite, with DNA demethylation of *RORC* and sustained methylation of the *FOXP3* locus. DNA demethylation of *IL17A* and *TBX21* is also observed in Th17 cells (Figure 2) [31,83,106,108–112].

Our knowledge on the epigenetic regulation of Th9 cells differentiation is comparatively limited, which may result from the relative novelty of this only recently identified Th cell lineage. Not much is known about Th9-specific DNA methylation changes; at least our search failed to identify any relevant information

in the literature focusing on Th9 cells [24,94,95,103] as well as in the papers describing mechanisms of CD4⁺ T cell differentiation in a wider context [87,91,93,102,104]. However, other types of epigenetic modifications such as histone acetylation or methylation have been reported to contribute to Th9 lineage differentiation (Figure 2) [95,104]. The levels of total H3 (H3ac) and H4 (H4ac) as well as specific H3K9 [H3K9ac; K corresponds to a lysine residue which can be further modified with acetyl or methyl (here: acetyl) groups] and H3K18 (H3K18ac) histone acetylation, all having a

permissive character, have been found to be the highest, while trimethylation of H3K27 (H3K27me₃), a repressive histone modification [62], to be lowest at the *IL9* locus in naive T cells cultured under Th9 differentiating conditions when compared with Th cells cultured toward the Th1, Th2 or Th17 lineage [95,113]. In addition, the negative ratio of permissive to repressive histone modifications (H3ac/H3K27me₃ and H3K4me₃/H3K27me₃) at the *SPII* promoter characteristic for naive T cells has been reported to revert under Th9-polarizing conditions leading to the induction of PU.1 expression [104,114]. Histone modifications play an important role also in the development of the other Th lineages. For example, the *IFNG* locus in Th1 cells is characterized by high H4ac, high H3K4me₂ (di-methylation; a permissive mark), high H3K9me₂ (usually a repressive mark; hence its role in this context requires further investigation) and low H3K27me_{2/3} levels. At the same time, an opposite pattern has been identified in Th2 cells, with the exception of transient increases in H3K4me₂ and H3K9me₂ during the early phases of the differentiation [88,115]. Moreover, high levels of H3K4me₃ were observed at *IFNG* in Th1, *IL4* in Th2, *IL17A* and *IL17F* in Th17, *RORC* in Th17 and a subgroup of Treg cells, and *FOXP3* in Treg cells [116]. Simultaneously, *IFNG*, *IL17A*, *IL17F* and *RORC*, and to some extent also *FOXP3* and *IL4*, were characterized by high levels of H3K27me₃ in Th lineages listed in the previous sentence, except for those in whom H3K4me₃ levels have been high [116]. It has been also demonstrated that promoters of *IL17A* and *IL17F* are in Th17 cells characterized by high levels of not only H3K4me₃ but also H3ac [117]. In addition to H3K4me₃, increased levels of several other histone marks, especially H3K9/14ac (both permissive modifications), are present at the *FOXP3* locus in Treg cells (Figure 2) [118]. Inhibition of histone deacetylation, especially targeting HDAC9, increased the number and suppressive potential of Treg cells concomitant with a demethylation of the *FOXP3* regulatory regions and decreased inflammation in inflammatory bowel disease [119].

Naturally, we cannot address here the whole complexity of Th cell differentiation and its epigenetic regulation. As such, only major cell lineages and related cytokines are described. Gene expression mechanisms with more complex epigenetic aspects, for example the role of the Th2 locus control region or conserved noncoding sequence (CNS) elements present in several loci, for example in *FOXP3* (Treg-specific demethylated region – TSDR; also known as CNS2) [21,86,93,120–123], are not discussed due to space restrictions. We also do not distinguish between human and mice, and the simplified genetic nomenclature used always refers to

human. Finally, although Th cells play a crucial role in the development of allergy and in the mechanisms of allergic inflammation, many other cells whose maturation is also epigenetically regulated contribute as well, for instance B cells (Figure 1) [83].

Epigenetic modifications mediating the influence of environmental factors in allergic diseases

The genetic susceptibility to allergic disorders is known to have a polygenic character [21,26–29,43,124,125]. Since it had not been possible to explain the increase in incidence and prevalence of allergy and related disorders throughout several decades solely by changes in the genetic background, the contribution of modifying factors of environmental character became evident, which gained further support from epidemiological observations. For instance, although the genetic profile of the population remained unchanged, the incidence of allergies increased dramatically in the former German Democratic Republic (East Germany) in a period of only 20 years since the reunification of Germany [126–128]. Furthermore, epigenetic mechanisms, known to convey genomic adaptation to the external circumstances, became a natural candidate to explain these effects of the environment on the development of allergy [31,129].

The unfavorable change in the incidence of allergic disorders could be explained by an increased contribution of negative (risk or risk-increasing) environmental modifiers, decreased exposition to positive (protective or risk-reducing) environmental factors or both. Although this review does not aim to profoundly discuss the mechanisms of inheritance, it is worth mentioning that, theoretically, environmental influences could not only modify genetic predisposition, i.e., change it to a certain degree, but also completely eliminate it. For example, a person with genetic susceptibility to bee allergy living in an island not inhabited by *Hymenoptera* will never develop this type of atopic sensitization and thus related symptoms of allergic reaction.

According to the hygiene hypothesis, early-life microbial exposure correlating with the number of children in a family offers protection against the development of allergic conditions. Considering changes in the life-style, such as declined family size but also improvements in public health and hygiene, it should not be surprising that the incidence of allergic disorders has substantially increased over the last several decades [19,30,33–35,42,43,126]. However, exposure to microorganisms or their structural parts present in various indoor or outdoor environments can occur also in the absence of infection. Such microbial substances,

recognized by the innate immune system, can also stimulate chronic inflammation leading in turn to the development of the protection against inflammatory disorders [30,33,35,126,130–133]. This has been demonstrated also for atopic diseases, especially in the investigations on the effects of farming on allergic predisposition. Those studies have shown that children exposed early in life to microbiota present in farming/rural environments develop less allergic conditions (than their inner-city counterparts) [30,33,35,126,131,132,134].

Substantial efforts have been undertaken to clarify the mechanisms of this protective phenomenon, exemplified by the studies on *Acinetobacter lwoffii* (*A. lwoffii*) F78, a strain, which has been identified in the farming environment along with *Lactococcus lactis* G121, *Staphylococcus sciuri* W620 and a number of other bacteria not listed here [135,136]. All three bacterial isolates have been shown to reduce allergic reactions in mice and to induce Th1-polarizing program in human dendritic cells *in vitro* [135,137]. A subsequent study in mice demonstrated protective effects of maternal intranasal exposure to *A. lwoffii* against the development of atopic asthma-mimicking allergic airway inflammation in the progeny [138]. Finally, this transmaternal protection was shown to be IFN- γ -dependent, which was, at least partly, mediated by the *A. lwoffii*-induced preservation of histone H4 acetylation at the *IFNG* promoter of CD4⁺ T cells isolated from spleens of the mice offspring [139].

Transmaternal anti-allergic protection induced by *A. lwoffii* is in line with some more recent concepts. It used to be commonly accepted that the first contact of fetus/neonate with microorganisms occurs during the delivery, and results from the exposure to maternal vaginal and fecal bacterial microflora [30,126,131,140]. Indeed, children born by Caesarian section have been reported to be at higher risk of allergies when compared with those delivered by vaginal birth, although the data are conflicting [131,140–143]. However, it has become quite evident that microorganisms can stimulate the development of anti-allergic protection even earlier, in the prenatal period [144,145] either through a direct *in utero* contact with fetus, as suggested by the studies demonstrating the presence of bacterial DNA in placenta or meconium of preterm babies [146,147], or indirectly, with the mother influencing the neonatal immune system development in response to contact with microbiota. Interestingly, differential expression (mRNA level) of a number of immune-related genes in placenta in respect to lifestyle (anthroposophy and living on a farm) and parental allergen sensitization has been reported [148,149]. A follow-up study on one of those loci, namely *CD14*, revealed a relation between DNA methylation of the *CD14* promoter region with the level of *CD14* mRNA expression, suggesting epigenetic

intrauterine regulation of *CD14* to be involved in the protective effects of farming against allergy development [148]. Prenatal exposures not only to microorganisms but also to other environmental factors such as pollutants or nutrients (and resulting changes in the epigenetic status) are in general getting more and more recognition as important if not crucial players in the development of allergic predisposition [32,129,150–152] and are discussed in more detail in other sections of this review. It is also worth mentioning that another major factor involved in direct or transmaternal anti-allergic effects of farming is consumption of unprocessed cow's milk obtained directly from a farm, not necessarily due to its microbial contamination/content [153–155]. In any case, *FOXP3* demethylation and activation of Treg cells seem to be involved in protective effects of farm milk against allergies (see also later) [144,153]. However, longitudinal studies following children from *in utero* and during childhood with repeated biological samplings will be required to assess if the observed epigenetic changes precede the onset of allergic diseases.

In contrast, viruses and viral infections do not protect against atopy, but rather result in exacerbations or maybe even development of allergic disorders, with a remarkable example of airborne viruses and (atopic) asthma [36–38,156]. Among airborne viruses, human rhinoviruses (HRVs) and respiratory syncytial virus (RSV) seem especially important here. While RSV is frequently detected in infants with bronchiolitis and subsequent wheezing, HRV is the most common (viral) asthma exacerbation trigger at any age after [36,157]. Furthermore, it is thought that repeated early life viral infections, especially those involving the lower respiratory tract, can lead to the development of atopic asthma that can persist through childhood and into adulthood [36,37,157,158]. This effect is especially strong if repeated viral infections are combined with atopic predisposition, and mechanisms linking the contribution of innate antiviral and atopic immunoinflammatory pathways have also been proposed [37,159–161]. Fc ϵ RI is thought to play an important role in this mechanism [37,159,160], which seems to get some indirect support from genetic associations observed for single-nucleotide polymorphisms in *FCER1A* encoding the Fc ϵ RI α -subunit [162,163]. We are not aware of any data showing pro-allergic effects of airborne viruses to be epigenetically mediated but it seems the most reasonable possibility [164]. It is important to mention that the location of airway respiratory infection may be critical for its effect on allergic predisposition [158]. In addition, inverse associations between infections with some non-airborne viruses such as hepatitis A virus and allergies have been reported, although inconsistently between different populations/studies [156,165].

Pollen exposure (pollen season) is an obvious and well definable environmental factor influencing allergic disorders. An interesting study on seasonal AR (SAR) has been conducted, which combined the usage of DNA methylation and gene expression arrays. Methylation patterns observed in isolated and *in vitro* cultured CD4⁺ T cells (but not mRNA expression profiles) made it possible to clearly distinguish between samples obtained from SAR patients and controls [166]. An additional nice feature of the study was that the samples were collected from each participant within and outside the pollen season and thus also the environmental influence on the DNA methylation could be directly investigated. Moreover, the methylation profiles were found to be significantly associated with disease severity in patients during the season [166].

Furthermore, pollutants, especially air pollutants increase the risk of developing allergic disorders and these effects are at least partly epigenetically mediated and are shortly exemplified below. Greater average polycyclic aromatic hydrocarbons exposure has been shown to be associated with higher DNA methylation at several CpG sites within the *FOXP3* locus of peripheral blood mononuclear cells (PBMCs) derived Treg cells, with the effect being more pronounced in asthmatic than in non-asthmatic children [167]. In addition, maternal exposure to polycyclic aromatic hydrocarbons has been associated with DNA hypermethylation of *IFNG* promoter in cord white blood cells (from the offspring) [168]. Loss of DNA methylation at a single CpG site located in the promoter of the *TET1* gene has been demonstrated in a comparison of affected and non affected siblings to correlate in airway epithelial cells with asthma. At the same time, increased DNA methylation in response to traffic-related air pollution exposure at participants' current homes was also observed [169]. The family of TET enzymes is involved in the successive oxidation of methylated cytosines to 5-hydroxymethylcytosine and further to 5-formyl- and 5-carboxylcytosine [170,171] and preliminary data in a limited number of subjects have shown slightly altered hydroxymethylation levels in allergic individuals [172]. Coming back to *FOXP3*, an increase in DNA methylation levels in its 5' region has been shown in saliva samples to be associated with higher DEPs exposure. Moreover, children with higher *FOXP3* DNA methylation have been demonstrated to be at a higher risk of asthma, persistent wheezing or early transient wheezing [173]. Another study showed fine particle air pollutant exposure to correlate with buccal-brushing DNA methylation of several CpG sites in nitric oxide synthase genes (*NOS1*, *NOS2* and *NOS3*) in children; some current wheezing- or asthma medication-related differences in DNA methylation status of those genes

were observed as well [174]. It has been also found that intermediate or high levels of blood DNA methylation at a CpG island in the 5'-untranslated region of *ADRB2*, the gene whose polymorphic variants are known to affect the response to therapy with β 2-adrenoreceptor agonists [26,175], have been found to be associated with severe childhood asthma [176]. Moreover, a relationship between indoor exposure to NO₂ and severe asthma in children with high (but not low) *ADRB2* methylation levels has been observed [176].

Smoking, either as prenatal and/or postnatal tobacco smoke exposure or in its active form, is another extrinsic factor known to increase the risk of allergic disorders, especially asthma [177–181], and its effects on the epigenetic status have been clearly demonstrated [182–185]. For example, active smoking was shown to influence methylation of DNA isolated from peripheral blood [184], and *in utero* tobacco smoke exposure was found to affect methylation patterns of DNA obtained from buccal cells or whole blood (of the offspring), fetal lungs and placenta [182,183,185]. Moreover, an extensive study on the effects of prenatal smoking involving not only array but also whole genome sequencing DNA methylation data, additionally supplemented by the results of genome-wide histone modification and gene expression analyses, was recently published [186]. Whole blood samples obtained from mothers during gestation and from children and mothers up to several years after the birth of a child and cord blood (CB) specimens were analyzed. Maternal smoking-related differences in DNA methylation were found to be enriched in enhancer elements and to persist over years of life. Interestingly, differential DNA methylation in enhancers was found to be more often functionally relevant compared with other genomic regions, based on the combined analysis involving also histone modification and RNA expression data [186]. Finally, in a follow-up functional study including also animal experiments, epigenetic deregulation of the enhancer element targeting MAPK9 (gene: *MAPK9*), previously known as c-Jun N-terminal kinase 2 (JNK2), was linked to impaired lung function in early childhood [186]. Another study investigated a possible relationship between methylation of DNA isolated from cord white blood cells, prenatal smoking exposure ascertained by measuring CB cotinine levels and the development of AD in offspring (assessed at 2 years of age) [187]. It identified DNA methylation of thymic stromal lymphopoietin (TSLP; gene: *TSLP*) promoter to be associated with prenatal smoke exposure and AD. Additionally, the degree of *TSLP* methylation was shown to be inversely correlated with CB plasma TSLP protein levels [187]. Moreover, the differences in *TSLP* promoter DNA methylation and *TSLP* mRNA or TSLP protein

levels were observed between inflamed skin lesion samples obtained from children with AD and normal skin tissue sections [188]. Effects of exposure might not need even to be direct, as experiments in rodent models showed that exposure to nicotine altered the histone modifications profiles in the two subsequent offspring generations correlating with a reduced lung function [189]. Interestingly, modulatory effects of cigarette smoke on the epithelial innate immune response to HRV infection were observed *in vitro* [190,191], suggesting the possibility of the interaction also on the epidemiological level.

Very recently, epigenetic effects of smoking have been subjected to two large meta-analyses [192,193]. The first study focused on the influences of maternal smoking during pregnancy on newborn blood DNA methylation. Thirteen epigenome-wide association studies (EWAS 450K) cohorts with almost 6700 newborns were included [192]. More than 6000 CpGs demonstrated differential methylation in relation to maternal smoking at the genome-wide statistical significance level. Out of those, almost 3000 CpGs belonged to more than 2000 genes not previously related to smoking. For a number of differentially methylated CpGs, associations with gene expression were seen. Finally, a substantial persistence of the epigenetic signatures of prenatal maternal smoking observed in newborns into later childhood was observed [192]. Another large meta-analysis comprehensively determined the association between cigarette smoking and blood-derived DNA methylation. Sixteen EWAS (450K) cohorts comprising together over 15,900 individuals (including more than 2400 current, over 6500 former and approximately 7000 never smokers) were subjected to a meta-analysis [193]. Comparison between current and never smokers identified with genome-wide statistical significance more than 2600 differentially methylated CpGs annotated to over 1400 genes. Integration of transcriptome data revealed associations with gene expression at many differentially methylated CpGs. At a number of loci, the effects of smoking on blood-derived DNA levels were demonstrated to persist for many years as evidenced by a comparison between former and never smokers [193].

Besides all these environmental changes, the simple presence of allergic diseases is sufficient at least in rodent models to substantially increase the risk for the transmission of the allergy independent of the genetic background. Offspring of peanut-allergic mothers showed a Th2 dominated response at low levels of sensitization with increased IgE and IL-4 levels compared with control mice and the inflammatory response correlated with decreased DNA methylation at the *IL4* promoter [194].

Epigenetic modification as mediators of genetic susceptibility

Considering that the development of allergies is determined by gene $\times$ environment interactions [33,34], it is not surprising that genetic and epigenetic analyses are frequently conducted together. Although the assessment of epigenetic effects was frequently a major research target, potential influences of genetic variability were also taken into account. For example, both DNA methylation and genotyping data were used in the studies expanding our knowledge on the functionality of the 17q21 locus [195,196], the most widely replicated (childhood) asthma susceptibility locus ever identified by genome-wide association studies (GWAS) [26,125,197]. The association with asthma is stronger in boys compared with girls and sex-specific DNA methylation changes were found in the proximal promoter of the *ZBP2* gene with lower methylation levels in boys compared with girls that diminish with aging, suggesting that DNA methylation might mediate the sex- and age-specific associations [196]. DNA methylation analysis was also used in a study investigating the functional background of the association between total serum IgE levels and polymorphisms in *RAD50* [120], a gene encoding an important DNA repair molecule [21]. Further examples of the genes for which combined genetic–epigenetic analysis has been conducted in the context of allergy, specifically asthma and/or related phenotypes, include *NPSR1* [198], *ALOX12* [199] and *ADCYAP1R1* [200]. In addition, a very recent family-based study employing in parallel genome- (linkage scan) and epigenome-wide approaches identified a polymorphism located upstream of the *MTRN1A* locus to be associated with asthma and AR comorbidity and the effect was mediated by a differentially methylated intronic CpG site of *MTRN1A* [201]. A large GWAS in food allergy (milk, egg, peanut) analyzing children and their parents showed that the genetic variants at the major risk loci (*HLA-DBR1* and *HLA-DQB1*) correlated with differential DNA methylation and epigenetic changes might therefore mediate the risk of genetic factors to peanut allergy [202]. Given the ratio of reviews to original papers observed in the existing literature on the role genome $\times$ environment (epigenetic code) interactions in the development of allergic disorders, many further studies in the field are to be expected. These investigations are facilitated by the development of novel bioinformatics tools, such as the Gene, Environment and Methylation software package created to handle EWAS data, integrate genotype information and model interactions between genotype and the environment [203].

Table 1. Overview of epigenome-wide association studies performed in allergic diseases.

Study	Allergy-related trait	Asthma definition	Technology used	Sample size of primary screen (cases/controls)	Tissue used/ correction for cellular heterogeneity	Main findings	Validation	Comments	Ref.
Liang <i>et al.</i>	Serum IgE levels	Asthma with high and low IgE levels; physician-diagnosed asthma for replication	27K/450K	355 adults and children including 113 asthmatic children	PBMCs and eosinophils/yes	36 loci validated in all three cohorts with high confidence; a number of CpGs with correlation between methylation and IgE levels including <i>IL4</i> , <i>IL5RA</i> , <i>LPCAT2</i> , <i>ZNF22</i> , <i>SLC25A33</i>	Two cohorts: n = 149 (extreme IgE levels) and n = 160 asthmatic families)	Study shows a tenfold greater capacity of DNA methylation to explain variability in serum IgE levels compared with all so far performed GWAS	[210]
Hong <i>et al.</i>	CMA	-	450K	106/76 children	Whole blood/yes	575 DMPs (568 hypo/7 hyper) including CpGs in or close to <i>NDIP2</i> , <i>EVL</i> , <i>TRAPPC9</i> , <i>RPS6K2</i> (all > 5%), <i>ILIRL1</i> , <i>IL5RA</i> , <i>STAT4</i> , <i>IL4</i> , <i>CCL18</i>	Yes; 10 loci/2 cohorts: 5 CMA cases/20 controls whole blood, white children and 8 CMA cases/132 controls CB black children	Only 5 CpGs with diff. methylation >5%; reasonable replication rate in blood, not in CB; potential functional importance as in gene regulatory regions such as CTCF sites	[303]
Petrus <i>et al.</i>	CMA	-	450K	20/23 children and including 10 boys that outgrew CMA	Whole blood/no	Hypermethylation in CMA	Amplicon bisulfite sequencing of four genes (n = not specified)	No correction for cellular heterogeneity, small sample sizes	[207]
Martino <i>et al.</i>	Various IgE-mediated FA (mainly hen's egg)	-	450K	12 children with FA/12 controls; 2 time points (birth and 1 year)	CD4 ⁺ T cells from CBMCs and PBMCs/NA	179 CpGs associated with FA at 1 year, 136 CpGs at birth, 92 common to both time points	No	Longitudinal study with sample at birth and 1 year showing presence of epigenetic alterations predicting later food allergy; measurement of gene expression	[206]
Martino <i>et al.</i>	Multiple FA	-	450K	58 food-sensitized children (50% clinically reactive)/13 controls	PBMCs/yes	DNA methylation signature comprised of 96 CpGs predicted clinical outcome	Yes; 48 samples from an independent FA cohort (79% accuracy of the methylation signature)	DNA methylation signature outperformed IgE and skin prick testing for oral food challenge outcome → potential for novel diagnostic assays	[221]

Table 1 summarizes a selection of EWAS analyzing at least 500 genes simultaneously in allergic diseases in humans. Studies in animal models are not listed.

450K: Infinium Human Methylation450 Bead Array (485,000 CpGs covering all genes); 27K: Infinium HumanMethylation27 BeadChip (27,578 CpG covering >14,000 genes); 5 hmC: 5-Hydroxymethylcytosine; AEC: Airway epithelial cell; CMA: Cow's milk allergy; CB: Cord blood; CBMC: CB mononuclear cell; DDE: Dichlorodiphenylchloroethylene; Diff.: Differentially; DMP: Differentially methylated position; FA: Food allergy; FEV1: Forced expiratory volume in one second; GoldenGate: Illumina GoldenGate BeadArray (1505 CpG sites – 807 genes); PBMCs: Peripheral blood mononuclear cells; PUFA: Polyunsaturated fatty acid; SAR: Seasonal allergic rhinitis.

Table 1. Overview of epigenome-wide association studies performed in allergic diseases (cont.).

Study	Allergy-related trait	Asthma definition	Technology used	Sample size of primary screen (cases/controls)	Tissue used/correction for cellular heterogeneity	Main findings	Validation	Comments	Ref.
Nestor <i>et al.</i>	SAR	-	450K	8 cases/8 controls; sampled in and outside the pollen season	CD4 ⁺ T cells/NA	DNA methylation signatures separate patients and controls even outside the pollen (357 genes diff. methylated, including mainly CpGs in gene bodies of <i>HSPD1</i> , <i>TGFB2</i> , <i>CD134</i> (<i>TNFRSF4</i> , <i>OX40</i>), <i>CD45</i> (<i>PTPRC</i>), <i>SPN</i> (<i>CD43</i>), <i>IL2RA</i> , <i>IL13RA1</i>)	Technical validation of 3 CpGs in 4 SAR patients and 4 controls; similar affects seen by <i>in vitro</i> stimulation of PBMCs	Demonstrates the potential of DNA methylation based signatures; but very small sample size, and small differences per CpG site; expression profiles do not separate patients and controls; no single CpG differentiates patients and controls	[166]
Lockett <i>et al.</i>	Impact of season of birth on allergy development	-	450K	367 individuals (age 18)	Whole blood/yes	92 CpGs associated with season of birth, 20 CpGs nominally significant with allergy	207 children (age 8 years) replicates four of the CpGs	Methylation in newborns is not associated with season, suggesting a postnatal occurrence; longitudinal data available	[304]
Stefanowicz <i>et al.</i>	Atopy with/without asthma	IgE skin prick test, but combined analysis of asthmatics (independent of atopy status)	GoldenGate	25 children	PBMCs and AECs/No	No diff. methylated CpGs in PBMCs, eight in AECs (atopy vs asthma) including <i>STAT5A</i>	No	Identification of diff. methylated CpGs between PBMCs and AECs in each subject category (~10% of analyzed CpGs); expression analysis, but in a different data set	[209]
Morales <i>et al.</i>	Wheezing before age of 6 versus controls	-	GoldenGate	122 children	Whole blood/partly (eosinophil counts)	Hypomethylation of ALOX12 associated with persistent wheezing, partly related to DDE exposure	Independent cohort of 236 children	Epigenetic variation determined to a large part by genetic polymorphisms	[199]

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Study	Allergy-related trait	Asthma definition	Technology used	Sample size of primary screen (cases/controls)	Tissue used/ correction for cellular heterogeneity	Main findings	Validation	Comments	Ref.
Breton et al.	Asthmatics/ prenatal tobacco smoke exposure	Symptoms of asthma or use of asthma medication	27K	527 children (age 5–12 years)	Whole blood/ yes	19 CpGs altered in function of prenatal tobacco smoke exposure	2 CpGs replicated in independent cohorts (CB and adult whole blood)	Analyses show some loci to be replicated in different tissues; promoter-focused design limits the regions that can be analyzed	[183]
Joubert et al.	Prenatal tobacco smoke exposure	-	450K	1062 children	CB/yes	26 CpGs in 10 genes altered in function of prenatal tobacco smoke exposure	21/26 CpGs validated in a small cohort (n = 36)	Smoking assessed through maternal cotinine levels	[305]
Markunas et al.	Prenatal tobacco smoke exposure	-	450K	889 children	Whole blood shortly after delivery/yes	185 CpGs in 100 gene regions	<i>In silico</i> validation of ten gene regions using data from [305]	Smoking assessed through questionnaire; some overlap with previous EWAS	[306]
Richmond et al.	Prenatal tobacco smoke exposure	-	450K	800 children: three time points per child (birth, age 7 and 17 years)	CB/yes	15 CpGs sites associated with maternal smoking; dose – response relationship in function of smoking intensity and duration for some CpGs	No	Longitudinal design allows to identify CpGs that reverse methylation and where methylation persists	[307]
Joubert et al.	Prenatal tobacco smoke exposure	-	450K	6685 children (13 cohorts)	Various/yes	>6000 CpGs differentially methylated, including 2000 new ones	Replication in older children (n = 3187)	Large-scale EWAS identifying a huge number of new loci associated with maternal smoking supporting the need for large-scale analyses	[192]
Kim et al.	Atopic asthma	Differentiation of IgE-associated and non IgE-mediated asthma	27K	Atopic asthma (n = 7) versus nonatopic asthma (n = 7) versus controls (n = 10)	Bronchial mucosal tissue/ not precised	Differential methylation in 52 genes in atopic asthma versus asthma, most of them hypomethylated	No	Atopy to dust mite	[308]

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Table 1. Overview of epigenome-wide association studies performed in allergic diseases (cont.).

Study	Allergy-related trait	Asthma definition	Technology used	Sample size of primary screen (cases/controls)	Tissue used/ correction for cellular heterogeneity	Main findings	Validation	Comments	Ref.
Rosnerova <i>et al.</i>	Asthma/pollution	Not precised	27K	200 children (age 7–15 years)	Whole blood/ no	No methylation differences for asthma; 10K CpG sites diff. methylated between highly polluted and control regions	No	Very large number of diff. methylated CpGs might point to unknown confounders (e.g., blood cell composition)	[278]
Jiang <i>et al.</i>	Asthma/diesel exhaust exposure	Physician-diagnosed asthma	450K	16 asthmatics randomized to filtered air or diesel exhaust	PBMCs/yes	2827 CpG sites, mainly hypomethylated, enriched in protein kinase and NF- κ B signaling	No	In addition, methylation changes in repetitive elements were observed; however, small sample size, no validation, replication, no inclusion of healthy controls	[309]
Somineni <i>et al.</i>	Asthma/pollution	Physician-diagnosed	450K	12/12 African-American siblings	Nasal epithelial cells/no	Demethylation at a single CpG site in the <i>TEF1</i> promoter associated with asthma	35 additional siblings pairs with saliva, PBMCs, lung bronchial epithelial cells available; independent cohort n = 186	<i>TEF1</i> hypomethylation correlated with higher <i>TEF1</i> expression and higher global 5 hmC levels; increased methylation in function of traffic pollution; EWAS with robust multitissue validation	[169]
Clifford <i>et al.</i>	Allergen/diesel exhaust exposure	IgE-related diseases including asthma	450K	17 adult patients (47% asthmatics)	Bronchial epithelial cells/ not precised	Altered methylation at 7 CpG sites 48 hours after exposure; if allergen and diesel exhaust were separated by 1 month, 500 diff. methylated (mainly hypomethylated) CpGs were identified suggesting a priming mechanism	1 CpG by pyrosequencing in the same samples	Exposure to filtered air or diesel exhaust followed by bronchoscopy for allergen delivery; CpGs differ in function of the order of allergen/diesel exposure; small cohort size	[310]

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Table 1. Overview of epigenome-wide association studies performed in allergic diseases (cont.).

Study	Allergy-related trait	Asthma definition	Technology used	Sample size of primary screen (cases/controls)	Tissue used/correction for cellular heterogeneity	Main findings	Validation	Comments	Ref.
Yang <i>et al.</i>	Asthma	IgE-related asthma	450K	97 patients versus 97 controls	PBMCs/yes	81 DMRs between asthmatics and controls with hypomethylation of several immune related genes (<i>IL13</i> , <i>RUNX3</i> , <i>TIGIT</i>)	No	Few, but some DMRs related to IgE levels and percentage predicted FEV ₁ levels; integration of gene expression and DNA methylation data	[279]
Rastogi <i>et al.</i>	Asthma and obesity	Not precised	1.8–2 M CpG sites (sequencing based)	32 preadolescent children (8 per group asthmatics and controls with obesity or not)	PBMCs/no	Diff. methylated CpGs in immune related genes including <i>CCL5</i> , <i>IL2RA</i> , <i>TBX21</i> , <i>FCER2</i> , <i>TGFB1</i>	No	T cell signaling and macrophage activation pathways selectively hypomethylated in obese asthmatics	[311]
Amara-sekara <i>et al.</i>	Allergy protective effects of fish oil supplementation during pregnancy	-	450K	70 mother–infant pairs; fish oil supplementation (n = 36), control group (n = 34)	CD4 ⁺ T cells from CBMCs	No statistically significant CpGs at the genome-wide level in relation to treatment or PUFA levels	No	Some CpGs showed nonetheless a correlation to PUFA levels	[257]
Rodriguez <i>et al.</i>	Atopic dermatitis	-	27K	28 patients with atopic dermatitis, 29 controls	Whole, blood, T cells, B cells, skin epidermis	A large number of methylation differences between patients and controls are only found in the skin	No	Demonstrates the need for tissue-specific DNA methylation analyses; complementary expression analyses which show some overlap predominantly in genes implicated in epidermal differentiation and innate immune response	[208]

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Understanding allergic diseases through EWAS

Despite numerous large-scale studies, genetic polymorphisms confer only a low to moderate level of pre-disposition, which cannot explain the recent rise in prevalence of IgE-mediated allergic syndromes. Most studies have so far focused on the analysis of one (or several) candidate gene(s). Technological advances in sequencing and microarray technology do now allow for the genome-wide analysis of DNA methylation patterns with specific phenotypes and a number of large cohorts have been profiled for their DNA methylation patterns [204]. Although not without a number of potential pitfalls requiring a careful design and analysis of the data [205], the analyses have identified some CpGs associated with disease phenotype and early-life exposure in different diseases ranging from metabolic to psychiatric diseases, from cancer to cardiovascular complications as well as immune-related disorders. Few EWAS have so far been performed in allergic diseases with the exception of asthma and most have been analyzing a limited number of individuals and findings have often not been replicated (Table 1). To draw general conclusions on the studies involving asthmatic subjects is further complicated by the fact that studies report rarely the specific sub- or endotypes and rely on a phenotypic diagnosis (Table 1), thereby introducing probably some molecular heterogeneity in the studied population, which renders the detection of asthma-type specific changes very difficult even at the broad level of atopic versus nonatopic asthma.

EWAS in allergic diseases have notably shown that DNA methylation signatures can separate allergic patients from normal controls and show increased discriminatory power compared with gene expression based signatures [166]. However, due to small differences and significant interindividual variability of DNA methylation patterns, no single CpG showed sufficient discriminatory power to distinguish between the two groups. Signatures of differentially methylated positions are in some cases already present at birth and predict future disease onset of – in this case – food allergy [206]. EWAS have also shown that allergy-related autosomal DNA methylation changes differ substantially between boys and girls when compared with sex-matched controls [207]. Although the results were obtained in a cohort of limited size, they provide further evidence that DNA methylation alterations might underlie (or at least correlate with) the observed age- and sex-specific differences in the prevalence of allergic diseases [11–13]. As also discussed in more detail below, the optimal tissue is yet an unsolved issue and the choice of the tissue will have a major impact on the identified differentially methylated positions, as

for example observed in a study on AD, where only skin samples showed differential methylation between patients and controls, but neither purified blood cell populations nor PBMCs [208]. Similarly, although airway epithelial cells and PBMCs shared most of their methylation patterns, asthma specific methylation differences were only detectable in the lung epithelial cells [209]. While in a recent large and well-controlled EWAS no DNA methylation changes were found to be associated with asthma, this study found 36 loci (34 genes) at which DNA methylation levels associated with serum IgE levels and most of these genes were particularly important for gene regulation in eosinophils [210]. This study also demonstrated the power of well-conducted EWAS with a tenfold greater capacity of the genome-wide DNA methylation patterns to explain the observed variability in IgE concentrations compared with genetic variation.

While there is definitely room for improvement in sample size, replication efforts, but also phenotypic characterization of the analyzed samples, the available studies provide first encouraging steps toward deciphering the role of epigenetics in allergic diseases and have clearly demonstrated the power of integrating epigenetic analyses in the endeavor for understanding allergic diseases.

New generations of DNA methylation microarrays interrogating an ever increasing number of CpG sites such as the EPIC BeadChip analyzing DNA methylation levels at more than 850,000 CpGs and which increasingly focus on gene-regulatory elements outside genes and promoters have recently been devised [211,212], together with the decreasing cost of whole-genome bisulfite sequencing [213], will allow a more comprehensive assessment of the DNA methylation landscape and provide novel insights in the development and course of allergic diseases.

Clinical applications of epigenetics in allergies: current status & future perspectives

Epigenetic modifications as biomarkers for allergy or response to treatment

Current molecular biomarkers used in clinical practice for the management of allergic patients are mainly based on the measurement of circulating antigen-specific IgE levels as well as basophil activation tests, but lack sufficient sensitivity to predict onset of atopic diseases or early efficacy during therapeutic interventions [214]. While detection and quantification of disease-specific cell subsets using multilevel fluorescent activated cell sorting (FACS) or mass spectrometry (MS) do provide alternatives, it might be difficult to implement them in a routine clinical setting. Epigenetic modifications and

in particular DNA methylation represent a 'molecular memory' and might mediate the above described gene × environment interactions. DNA methylation signatures have in other complex diseases such as cancer and autoimmune disorders shown their value as biomarkers of diagnosis and/or therapeutic response [215]. As a first application, DNA methylation changes might thus be used as molecular biomarkers to quantify the different allergy enhancing or protective exposures.

Altered methylation patterns at genes implicated in the balance of Th1/Th2 populations such as *IL4*, *IL5*, *IL10* and *IFNG* have frequently been observed with generally higher methylation levels at Th1 key genes and lower methylation levels at Th2 genes in PBMCs or purified CD4⁺ T cells after exposure to potentially allergenic substances such as DEPs [216] or dust mite allergens [217]. Changes in the DNA methylation patterns have also been associated with acquisition of tolerance or better-termed sustained unresponsiveness in children with CMA [218] and with *in utero* exposition to the allergy-protective farm environment [219]. Epicutaneous immunotherapy against milk proteins followed by adoptive Treg cells transfer prevented sensitization to a new allergen (peanuts or house dust mite) through a mechanism including increased methylation of the promoter of the key TF *GATA3* in splenic cells of the recipient mice and persisted until at least 2 months after the end of the sensitization [220]. It remains nonetheless to be shown if the observed effects are due to changes in the cell composition of the analyzed blood or tissue samples or due to altered epigenetic profiles of a specific cell-type as, for example, *IFNG* DNA methylation levels have been shown to vary between cell types. The increasing number of EWAS will yield additional and perhaps more specific and sensitive diagnostic markers for allergic diseases, which will allow to identify allergic subjects at an early stage, but also allergy-prone individuals prior to the onset of disease. Examples include specific DNA methylation profiles in CD4⁺ T cells of patients with SAR both in but also outside the pollen season compared with healthy controls [166]. As mentioned above, the simultaneously performed genome-wide expression analyses did not allow to differentiate between the two groups showing the superior potential of DNA methylation as a diagnostic biomarker. In addition, differential DNA methylation in genes of the MAPK pathway were detected in children developing food allergies later on [206], which provides new opportunities for prevention or early therapeutic intervention if signatures are sufficiently validated. Furthermore, diagnostic DNA methylation signatures composed of 96 CpGs have been devised predicting oral food challenge outcome with an accuracy of 79%, which outperformed conventional biomark-

ers such as the measurement of allergen-specific IgE levels or skin-prick tests and have thus the potential to replace the food challenge, a procedure potentially associated with the risk of serious complications such as anaphylaxis [221].

Few studies have so far addressed the potential of epigenetic biomarkers as a surrogate for the efficacy of the treatment of allergic diseases, with the exception of methylation of *FOXP3*. *FOXP3*, expressed only by Treg cells, plays a crucial role in allergic diseases, with smaller numbers of Treg cells in allergic children. Several presumably allergy-protective stimuli such as consumption of raw milk [153] have been shown to increase the number of Treg cells correlating well with the demethylation of the *FOXP3* TSDR. Demethylation of the TSDR was observed in children with reduced atopic sensitization and asthma [153], as well as in children outgrowing IgE-mediated CMA after dietary intervention [222], children receiving oral immunotherapy (OIT) to peanut allergy [223], and patients receiving sublingual immunotherapy to timothy grass and house dust mite [224]. Of note, in children who remained unresponsive 12 weeks after the end of OIT to peanuts the *FOXP3* methylation level remained low, while in those who showed loss of tolerance the *FOXP3* methylation increased to baseline raising the possibility to potentially use methylation levels of the *FOXP3* TSDR as a biomarker for sustained unresponsiveness [223]. Similarly, the above-described DNA methylation changes correlating with serum IgE concentration [210] might ultimately be useful to select patients that are most likely to profit from treatment with anti-IgE antibodies such as omalizumab, an expensive medication that is currently only applicable to and effective in a subset of patients [225,226].

It remains unknown for the moment if these and other observed DNA methylation changes are simply reflecting the cellular defects or if the epigenetic changes precede the disease symptoms and set the stage for disease development. Furthermore, signatures composed on a number of epigenetic markers and/or combined with genetic or biochemical markers will be necessary to improve the sensitivity and specificity of clinically useful biomarkers.

Recently, a number of studies have shown the potential of the analysis of DNA methylation patterns in circulating cell-free (ccf) DNA (ccfDNA), the so-called liquid biopsy, for the detection and monitoring of complex diseases other than cancer including autoimmune or metabolic diseases [227–231]. In all examples, the tissue of origin had a high rate of apoptosis facilitating the detection of the DNA molecules from relevant cells. Due to the tissue-specificity of DNA methylation patterns, analysis of ccfDNA allows to identify the tissue

or even cell type of origin [232,233]. Blood cell type-specific DNA methylation markers are also used to deconvolute the confounding effects of cellular composition in EWAS [234] or can be used to accurately quantify the number of immune cell types and subtypes [235]. Potential applications in allergic diseases are numerous including monitoring changes in T cell balance or eosinophilic infiltration in less accessible tissues, such as lung or epithelial tissue in the gastrointestinal tract, or accurately and rapidly quantify low abundant cellular subtypes.

Treatments influencing directly or indirectly epigenetic marks in allergy

DNA methylation inhibitors such as 5-azacytidine have been little investigated in allergic diseases and data are currently conflicting with some studies finding beneficial actions through notably an induction of Treg cells [236] or demethylation of *IFNG* [237], while in other studies the use led to a worsening of the allergic conditions [188]. As most studies investigated only the effect on a single target gene, it is difficult to draw any conclusions on the potential use of these genome-wide epigenetic modifiers, but caution is warranted. Similarly the use of HDAC inhibitors has yielded conflicting result, with some studies showing anti-inflammatory effects and others pointing to enhanced inflammation requiring thus further investigation of the use of this treatment [73,238]. Little data are currently available on treatments targeting other histone modifications such as histone methylation, but overall the use of epigenetic drugs altering the genome-wide epigenetic landscape might be associated in allergic diseases with a number of drawbacks.

Recently, major technological advances using the CRISPR/dCas9 system allow the targeted engineering of the epigenome [239–241]. These approaches allow for the first time to functionally investigate and validate the importance of epigenetic modifications at any locus in the genome and might provide novel alternatives to modulate the epigenome in allergic diseases. The combination of CRISPR/dCas9, which does not introduce double strand breaks in the genome through the use of a nuclease-deficient Cas9 enzyme, allows to guide epigenetic enzymes to specific loci in the genome where they can specifically (de)methylate DNA or (de)methylate/(de)acetylate histones [242,243]. While only recently devised, this technology bears in the future great promise for the treatment of diseases without clearly-defined underlying mutations. As epigenetic modifications are in general reversible, the identification of key molecular changes induced by environmental exposure might provide new treatment alternatives in which these changes might be reversed prior to the

occurrence of allergic conditions. More importantly, as described in this review, the altered T cell polarization is largely driven by epigenetic modifications at key TFs, and cytokines as well as CNS. Epigenetic editing provides potentially the tools to, for example, alter the balance between different T cell populations by making use of two vectors where the expression of the key components of the editing complex is driven by two cell-type-specific promoters to achieve expression of the editing complex for example only in T cells [244].

Epidemiological evidence from asthma/allergy protective environments points to the prenatal and the early postnatal periods as critical windows for establishing an allergy susceptible or protective phenotype. This time window coincides with the establishment of the gut microbiota, maturation of the immune system and epicutaneous allergen sensitization, which are all important factors for the development of future allergies. The hope is that intervention in these processes might be sufficient to avoid or delay allergic reactions in many cases.

Treatment with probiotics such as the farm-derived *Acinetobacter lwoffii* F78 during pregnancy has been shown to confer protective effects in a mouse model of ovalbumin-induced sensitization through epigenetic modulation of Th1/Th2 balance genes (see also earlier) [139] and addition of *Lactobacillus rhamnosus* in a small nutritional intervention study including children with CMA, facilitated outgrowth of the allergy as evidenced again through epigenetic modification at Th1/Th2 regulatory regions [222]. Overall, modification of the gut microbiota and increasing the abundance of bacteria such as *Clostridia* or *Roseburia*, which produce epigenetically active short chain fatty acids including butyrate, seems to be beneficial for decreasing inflammation and re-establishing barrier function notably through induction of Treg cells and/or activation of ILCs and IL-22 production [245–248]. However, evidence is still controversial with several large-scale studies do not providing solid evidence for the prevention of allergies through postnatal administration of probiotics [249,250]. A few studies have shown that already at birth epigenetic changes associated with an asthma-protective status or later development of allergies were present [206,219] suggesting a very early altered (epigenetic) programming of future immune responses and raising the question on the utility of *in utero* interventions. Supplementation with methyl donors has been shown in mouse models to actually exacerbate asthmatic conditions in the offspring [251]. However, dietary intervention programs during pregnancy with polyunsaturated fatty acids present, for example, in high concentrations in fish oil have been shown to reduce the risk of developing

allergies of the offspring [252] through modulation of the neonatal immune response [253,254], which is at least partly mediated by changes in the DNA methylation patterns [255]. In addition, the above-described allergy-protective effects of unprocessed farm milk are at least partially attributable to its increased content of polyunsaturated fatty acids [154]. While first candidate studies analyzing the DNA methylation patterns of genes in T-lineage differentiation in CB of neonates were only showing evidence for altered DNA methylation patterns in children with mothers continuing to smoke during pregnancy [256], a recent genome-wide study in CB CD4⁺ T cells identified some CpGs showing a dose-response effect in function of the polyunsaturated fatty acid levels as well as differentially methylated CpGs [257]. However, the observed changes were small raising questions on the biological significance and did not reach statistical significance after correction for multiple testing requiring thus confirmatory analyses in larger cohorts or other cell types. Other possible protective treatments could include the ingestion of small amounts of an allergen during pregnancy and lactation. This strategy has been applied to peanut sensitivity and shown in a mouse model to reduce severe allergic reactions in the offspring when exposed to peanuts [258] and is supported by epidemiological evidence [259]. There are, however, no data on epigenetic modifications following this approach available.

Strategies modifying the epigenome in allergy

Although no US FDA approved specific therapy for allergy exists and the long-term benefits of different immunotherapies require further investigation, preliminary data suggest that immunotherapy allows desensitizing patients to an allergen and leads to at least some degree of sustained unresponsiveness [260–263]. Immunotherapy might provide life-changing alternatives to subjects suffering from food allergy, where the standard care is strict food avoidance and accidental ingestion leads to a high number of emergency room visits [260,261]. Immunotherapies including mainly oral (OIT), sublingual (SLIT), subcutaneous or epicutaneous (EPIT) administration of the allergen are thought to correct the shift from a largely Th2- to a more Th1-dominated response, as well as inducing immunosuppressive Treg cells, and these changes in the polarization of T cells are considered critical for the efficacy of immunotherapy [264,265]. Since epigenetic modifications are critically involved in the T cell differentiation as outlined above, these therapies will necessarily act on the epigenome.

There are currently no epigenetic biomarkers and few biomarkers at all predicting the success of the different immunotherapies. As immunotherapies require

year and maybe lifelong application periods it will be essential to define molecular signatures that will predict the success of an immunotherapy as early as possible enabling a switch to alternative therapeutic approaches (if available) without further delay. Similarly, when a state of unresponsiveness is achieved, signatures based on or including epigenetic markers might help to distinguish between patients that will show sustained unresponsiveness or even tolerance without requiring further therapy and those who will be in need of continued administration of therapy. Preliminary evidence in peanut allergic subjects shows that about 30% of the initial patient cohort remained unresponsive after a 4-week interruption following 5 years of OIT [266] and response rate might be increased through concomitant administration of the above-described probiotics [267] suggesting the contribution of epigenetic mechanisms in achieving the state of unresponsiveness. As described above, methylation of *FOXP3* has been shown to be lower in patients with increased unresponsiveness including those with peanut allergy at both the end of an OIT administration and 12 weeks after the completion of the treatment [223] as well as in patients receiving dual sublingual immunotherapy against timothy grass and house dust mites [224].

Another option to modulate the epigenome toward a lower risk of atopic diseases or at least toward the development of less pronounced allergic phenotypes would be a direct influence on the differentiation of the cells playing the major regulatory in the immune system, in other words, T cells, for example by reducing the expression of the TFs promoting Th2 responses such as *GATA3*/*GATA3* (see also earlier). This targeted knock-out or at least knock-down could possibly be achieved using antisense strategies. In principle, through specific binding and subsequent degradation of mRNAs, antisense molecules prevent translation of those mRNAs to proteins contributing to the pathogenesis of a certain disease [81]. Such pathogenic proteins could be either normal (wild-type) translation products that are only too abundantly expressed (e.g., *GATA3* in atopic asthma, $\alpha 4$ -integrin in relapsing-remitting multiple sclerosis or coagulation factor XI in prevention of venous thromboembolism) [268–270] or they can be synthesized based on the incorrect (mutant) genetic background (e.g., superoxide dismutase-1 in familial amyotrophic lateral sclerosis or type II keratin-6A, in pachyonychia congenita) [271,272] or otherwise inappropriately spliced mRNA (e.g., acetylcholinesterase read-through transcript in myasthenia gravis) [273].

The possibility that antisense approaches could be used to edit the genome of developing T cells, is sup-

Abbreviations	
<i>A. Iwoffii</i>	<i>Acinetobacter Iwoffii</i>
AD	Atopic dermatitis
AECs	Airway epithelial cells
AHR	Airway hyper-responsiveness
APCs	Antigen-presenting cells
AR	Allergic rhinitis
CB	Cord blood
CBMCs	CB mononuclear cells
ccfDNA	Circulating cell-free DNA
CMA	Cow's milk allergy
CNS	Conserved non-coding sequence (e.g., CNS2)
CpG (site)	DNA sequence where a cytosine nucleotide (C) is directly followed by a guanine nucleotide (G)
DDE	Dichlorodiphenyldichloroethylene
DEPs	Diesel exhaust particles
Diff.	Differentially
DMPs	Diff. methylated positions
DNMTs	DNA methyltransferases (e.g. DNMT1, DNMT3A)
EPIT	Epicutaneous immunotherapy
EWAS	Epigenome-wide association study
FA	Food allergy
FACS	Fluorescent activated cell sorting
Fc RI	High-affinity IgE receptor
Fc RII (CD23)	Low-affinity IgE receptor
FOXP3	Forkhead box protein 3
GATA3	GATA binding protein 3
GEM (software)	Gene, Environment and Methylation (software)
GoldenGate	Illumina GoldenGate BeadArray (1,505 CpG sites - 807 genes)
GWAS	Genome-wide association study
H3/H4ac	Histone H3 or H4 acetylation (e.g. H3K9ac, H3K18ac)
H3K(X)me(x)	Histone H3K (X = e.g. K4, K27) di- (x = 2) or tri- (x = 3) methylation (e.g. H3K4me2, H3K27me3)
HATs	Histone acetyltransferases
HDACs	Histone deacetylases (e.g. HDAC9)
HDACi	HDAC inhibitors
HMTs	Histone methyltransferases
HRVs	Human rhinoviruses
IFN-	Interferon-
IgE	Immunoglobulin E
IL	Interleukin (e.g., IL-4, IL-5)
ILCs	Innate lymphoid cells (e.g., ILC2)
IRF4	Interferon regulatory factor 4
JNK2	c-Jun N-terminal kinase 2

ported by recent studies on hgd40/SB010, a DNAzyme-type antisense molecule against the above-mentioned *GATA3*. DNAzymes are antisense molecules possessing inbuilt, internal catalytic activity, making it possible for them to deactivate targeted mRNAs upon specific binding without a need of accessory molecules possessing enzymatic activity, which are additionally characterized by good stability and low toxicity [81]. First proofs for preventive and therapeutic effectiveness of DNAzymes targeting *GATA3* were obtained in mouse models of allergic airway inflammation mimicking human atopic asthma [268]. After successful preclinical testing [274], hgd40/SB010 entered Phase I clinical trials which demonstrated its safety and tolerability in humans [275]. Finally, a randomized, double-blinded, placebo-controlled, multicenter Phase II clinical trial showed hgd40/SB010 to significantly attenuate late and early clinical asthmatic responses after allergen provocation in patients with atopic asthma. In addition, the analysis of biomarkers showed also a weakening of Th2-regulated inflammatory responses in patients receiving hgd40/SB010 [276].

Unsolved issues: are easily accessible cells suitable for the study of allergic diseases?

Although there has been increasing interest in the analysis of epigenetic modifications in allergy, a few issues have been raised among which are interrogations of the biological significance of the commonly observed relatively small changes in DNA methylation patterns as well as the relevance of the biological material used for epigenetic analyses, especially as cell lines display highly divergent DNA methylation patterns compared with the tissue or cell-type it was derived from [277]. Epigenetic modifications are in human studies analyzed in multiple types of biomaterials, among which blood is probably most common. Whole peripheral blood [183,199,278] or its cellular isolates, such as PBMCs [169,196,222,279] or subpopulations of cells further sorted from PBMCs [167,280] are frequently used. Comparative analyses of the results of DNA methylation studies obtained from different types of blood specimens (sorted cells, PBMCs or whole blood) should be conducted with great caution. In addition, interpreting the epigenetic data obtained from whole blood or PBMCs may sometimes be difficult, as these samples comprise varying proportions of diverse cell populations with different biological characteristics. The comparative analysis of DNA methylation profiles from whole blood, PBMCs, granulocytes and seven selected purified cell lineages collected from healthy male donors showed clear differences in DNA methylation profiles between myeloid cells and

lymphocytes as well as the distinct methylation pattern of B cells [281]. Comparison between PMBCs and granulocytes revealed that at least one probe was differentially methylated for 85% of the analyzed genes. On the individual gene level, in spite of apparently similar general methylation profiles present in main types of the blood cells, patterns observed for some individual CpGs might be opposite (hypo- vs hyper-methylation) [281]. To avoid potential confounding by cellular heterogeneity in the analyzed samples in studies which are not performed using sorted blood cell populations, it is important to estimate blood cell populations in the analyzed samples [282]. If no cell count data are available, a number of bioinformatic tools have been developed that can estimate and correct for cell type heterogeneity in the analyzed samples based on either reference datasets or reference-free statistical algorithms [283–288]. While reference based approaches are commonly used for blood and CB-based studies, reference-free methods can be applied to any biological material for which no reference datasets are presently available including saliva (see also later, [289]) and studies in young children, for which current reference data sets are not adequate [290]. The choice of the reference dataset can have a major impact on the detection of differentially methylated positions and therefore only an appropriate reference dataset should be used for deconvolution [289]. Otherwise reference-free methods should be used.

Other types of relatively easily accessible biomaterials include saliva [169,173,291] or buccal brushings [173], conventional sources of DNA for genetic testing that have also been used in studies on DNA methylation. A study comparatively analyzing epigenome-wide DNA methylation profiles in saliva and PBMC samples from the same subjects (either patients with respiratory allergies or healthy controls) showed that, indeed, saliva can be successfully used for this type of analysis [289,292]. Although samples such as blood or saliva do not represent local tissues directly affected by allergic inflammation such as lungs, gut or skin, epigenetic analyses conducted in them, especially in peripheral blood cells, are very informative since a huge part of allergic disease-related pathophysiological processes take place not (only) in the end-organs but also systemically, in circulation, and their presence there frequently precedes the establishment of a clinical diagnosis [37,159,293–295]. Hence, being easily accessible, peripheral blood samples can possibly provide the results and might possibly be sufficiently good proxies of epigenetic changes in end-organs, although as detailed above some results not really supporting this statement have also been published [208,209]. Thus, epigenetic testing of local tis-

Abbreviations	
LCR	Locus control region
MAPK9	Mitogen-activated protein kinase 9
MEDALL (consortium)	Mechanisms of the Development of Allergy (consortium)
MHC-II	Major histocompatibility complex class II
miRNA	MicroRNA
NOS	Nitric oxide synthase (e.g. NOS1, NOS2)
OIT	Oral immunotherapy
PACE (consortium)	Pregnancy And Childhood Epigenetics (consortium)
PAHs	Polycyclic aromatic hydrocarbons
PBMCs	Peripheral blood mononuclear cells
PUFAs	Polyunsaturated fatty acids
RISC	RNA-induced silencing complex
RORC2	RAR related orphan receptor C isoform 2 (traditionally called also ROR T)
RSV	Respiratory syncytial virus
SAR	Seasonal allergic rhinitis
SNPs	Single-nucleotide polymorphisms
SLIT	Sublingual immunotherapy
TBX21	T-box 21 (traditionally called also T-bet)
TCR	T-cell receptor
TET1	Tet methylcytosine dioxygenase 1
TFs	Transcription factors
TGF-	Transforming growth factor-
Th (cells)	T-helper (cells; e.g. Th2, Th9)
Treg cells	Regulatory T-cells
TSDR	Treg-specific demethylated region
TSLP	Thymic stromal lymphopoietin
27K	Illumina HumanMethylation27 BeadChip (27,578 CpG covering >14,000 genes)
450K	Illumina HumanMethylation450 BeadArray (485,000 CpGs covering all genes)
5hmC	5-hydroxymethylcytosine

sues directly affected by allergic inflammation, such as skin [188,296], nasal epithelium [169,297] or bronchial/lung epithelium [169,209,298], seems very important to verify if the results obtained in them correspond to those deriving from the analyses of easily accessible tissues such as blood. Taken together, all these might be of huge importance not only for research but also for diagnostic purposes, especially in case of bronchi/lungs or the gastrointestinal tract, locations not as easily accessible as skin or nose (see also earlier). Although as mentioned above it is at the moment difficult to assess the (potential) usefulness of liquid biopsy in allergic diseases, one might speculate that

methylation analysis of these DNA samples could provide valuable information on the epigenetic status of end-organ tissues directly affected by allergic inflammation [227,299,300].

Finally, considering that epigenetic modifications in the prenatal period seem to play a very important, if not crucial, role in the development of allergic disorders (see also earlier) [43,150,151], CB [120,199,219], CB mononuclear cells [301] and further cell subpopulations isolated from CB mononuclear cells [302] as well as placental tissues [148,182] may represent valuable biological materials for epigenetic testing.

Future perspective

Epigenetic mechanisms play a key role in immune regulation and are influenced by or might mediate a variety of environmental exposures leading to persistent molecular alteration of the gene regulatory landscape. Although the field of epigenetics in allergic diseases has only recently gained momentum and studies on the epigenetic components of allergy have so far been limited, analyzing often only a small number of individuals and little attempts have been made to confirm the findings in replication studies or assess the functional relevance of the observed changes, the importance of epigenetic mechanisms in the development of allergic diseases has now clearly been demonstrated. Large-scale projects with a focus on epigenetic changes such as the Pregnancy And Childhood Epigenetics (PACE) in the US and the EU-funded Mechanisms of the Development of Allergy (MEDALL) consortia are currently underway and are combining data from multiple large cohorts to investigate the impact of environmental factors on the epigenome and their role in the initiation of allergic diseases during childhood. Moreover, further studies are required to better characterize the mechanisms underlying different forms of (allergic) asthma and their correlation with clinical characteristics. With a number of studies demonstrating a transgenerational inheritance of disease-associated phenotypic traits, which are recurrently accompanied by alterations in DNA methylation patterns in the different generations, the study of epigenetic modifications might also provide some clues on the transgenerational effects of environmental exposure. Technological advances allow the detailed analysis of DNA methylation changes in a truly genome-wide manner using highly purified disease-relevant cells or even single cells, particularly in distant gene-regulatory features such as enhancers where subtle changes might lead to a major reorganization of the chromatin landscape. First studies support the potential of DNA methylation changes as biomarkers for the diagnosis or assessment of treat-

ment efficacy for allergic diseases. Following extensive validation and refinement, these signatures might in the future complement, and perhaps even replace, current diagnostic tests and might predict the success of different treatment protocols using various forms of immunotherapy that are currently developed for the treatment of allergic diseases. Finally, new tools allow now in a cell-type-specific manner to interfere with the epigenetic code enabling the assessment of the functional relevance of the epigenetic alterations in experimental systems. These tools might also in the future provide new therapeutic modalities to correct the epigenetically defined imbalance in Th cell subpopulations or activate/repress regions that are aberrantly altered in allergic diseases. Epigenetic changes in allergic diseases will be a major subject of research in the next few years and the information gained will probably have a major impact on clinical practices in the near future.

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Executive summary

- The prevalence and persistence of allergic diseases including asthma and food allergies has recently experienced a major increase in the Western World.
- Epigenetic modifications play a key role in the differentiation of T cell lineages and influence the balance between distinct Th cell populations.
- Epigenetic modifications mediate the effect of (or are influenced by) various environmental exposures, both allergy-protecting and conferring susceptibility to allergic diseases.
- DNA methylation-based signatures in the relevant tissue differentiate patients with allergic diseases from controls.
- Changes in the DNA methylation patterns might predict susceptibility to disease prior to its onset and correlate with sustained unresponsiveness to allergens.
- Novel technological approaches such as epigenetic editing and DNazymes provide new avenues for the treatment of allergic diseases.

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