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# Methylation risk scores for childhood aeroallergen sensitization: Results from the LISA birth cohort

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Author contribution statement

A.H., M.S. and A.K. conceived the idea for the presented study. A.K. conducted the analyses and wrote the initial paper draft. A.H. conceptualized the methodology and supervised the analyses. R.W. and N.G. helped with data processing of DNAm data. E.T and T.E. provided input on the statistical analysis. M.W. answered questions regarding laboratory sample processing. J.C. wrote the initial code for calculation of MRS and H.Z. J.C.C and E.B. provided further summary statistics on the used EWAS results. A.H., M.S. and A.P. jointly supervised the present project. All authors revised and commented on the final manuscript version.

Conflicts of Interest

Dr. Celedón has received research materials from Pharmavite, GSK, and Merck in order to provide medications free of cost to participants in NIH-funded studies, unrelated to the current work. All other authors have no further conflicts of interest to declare.

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## Abstract

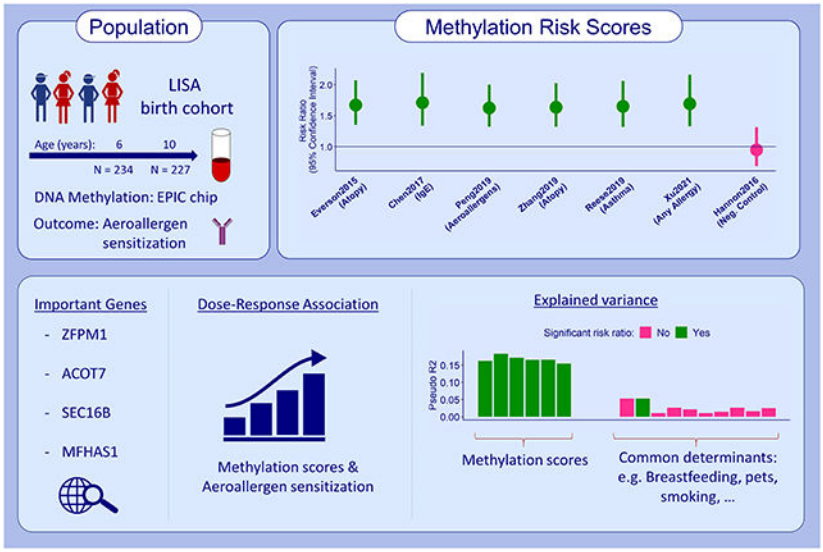
**Background—**Epigenomic (e.g., DNA methylation [DNAm]) changes have been hypothesized as intermediate step linking environmental exposures with allergic disease. Associations between individual DNAm at CpGs and allergic diseases have been reported, but their joint predictive capability is unknown.

**Methods—**Data were obtained from 240 children of the German LISA cohort. DNAm was measured in blood clots at six (N=234) and ten years (N=227) using the Illumina EPIC chip. Presence of aeroallergen sensitization, was measured in blood at six, ten and 15 years. We calculated six methylation risk scores (MRS) for allergy-related phenotypes, like total and specific IgE, asthma or any allergies, based on available publications and assessed their performances both cross-sectionally (biomarker) and prospectively (predictor of the disease). Dose-response associations between aeroallergen sensitization and MRS were evaluated.

**Results—**All six allergy-related MRS were highly correlated (r>0.86) and seven CpGs were included in more than one MRS. Cross-sectionally, we observed an 81% increased risk for aeroallergen sensitization at six years with an increased MRS by one standard deviation (best-performing MRS, 95% confidence interval=[43%; 227%]). Significant associations were also seen cross-sectionally at ten years and prospectively, though the effect of the latter was attenuated when restricted to participants not sensitized at baseline. A clear dose-response relationship with levels of aeroallergen sensitization could be established cross-sectionally, but not prospectively.

**Conclusion—**We found good classification and prediction capabilities of calculated allergy-related MRS cross-sectionally, underlining the relevance of altered gene-regulation in allergic diseases and providing insights into potential DNAm biomarkers of aeroallergen sensitization.

## Graphical Abstract



**Keywords**

Methylation risk scores; DNA methylation; Allergic diseases; Epidemiology; Polygenic risk scores

## Introduction

The link between genetic variation and allergic diseases is already well established by several genome-wide association studies (GWAS)[1]. However, non-genetic and environmental determinants, like birth order[2,3] or pet ownership[4], have also been discussed, and might explain further variance in allergic diseases (e.g., asthma and allergic rhino-conjunctivitis) through epigenetic mechanisms such as DNA methylation (DNAm)[5].

Over the past years, epigenome-wide association studies (EWAS) have identified differential DNAm at several CpG (addition of a methyl-group to a cytosine in the context of CpG dinucleotides) sites to be associated with allergic phenotypes including atopy, defined as allergic reaction in skin prick test, high total (>=200kU/L) or specific immunoglobulin E (IgE) (>=0.35kU/L)[6-9], childhood asthma[10] or any allergic disease plus sensitization[11].

Compared to large-scale GWAS, current EWAS often have limited sample size[12] with the maximum sample size in allergic phenotypes being 3,493[10]. Age-, tissue-, and cell type-specific differences in DNAm patterns further limit the generalizability of results[5]. Additionally, identified CpGs usually have small effect sizes, similar to single genetic variants for common diseases[13]. Given the unknown generalizability and replicability of recent EWAS of allergy-related phenotypes, mainly due to small sample sizes, a proof of the applicability of their results in a predictive context is of great interest for methylation studies.

Following the same methodology as previously employed for polygenic risk scores (PRS)[14], methylation risk scores (MRS) could be used to evaluate the reproducibility of published atopy-related EWAS and their prediction accuracy cross-sectionally (as biomarkers of aeroallergen sensitization) and prospectively (as predictors of future aeroallergen sensitization). MRS have been reported as biomarkers for exposures like smoking[15], as predictors of poor survival outcomes in hepatocellular carcinoma[16] and disease indicators for prostate cancer, even outperforming other known risk factors[17].

MRS are calculated by using external evidence from published EWAS and weighting the CpGs in the target cohort with the respective effect sizes from external EWAS on the same phenotype to calculate a weighted average. Thereby, small effects of single CpG sites are accumulated, which increases the statistical power and prediction accuracy[14]. The objective of this study is to calculate MRS that are derived from published EWAS, in order to classify cross-sectional, and predict prospective, childhood aeroallergen sensitization in the prospective German LISA birth cohort. For this, we evaluated (1) the predictive accuracy of six different MRS in both cross-sectional and prospective models, (2) their overlap and correlations and (3) compared their associations and prediction accuracy to other known determinants of allergic sensitization and individual CpG sites.

# Methods

## Study population

We used data from the prospective German birth cohort on the Influence of Life-style factors on Development of the Immune System and Allergies in East and West Germany (LISA), which recruited 3,097 full-term healthy newborns of European ancestry between 1997 and 1999 in four study centers (Munich, Wesel, Leipzig and Bad Honnef). The study was approved by the local ethics committees (Bavarian General Medical Council, Medical Council for North-Rhine-Westphalia and the University of Leipzig) and informed parental consent was given. More information can be found elsewhere[18].

Allergen specific serum immunoglobulin E (IgE) concentrations were assayed by the CAP-RAST FEIA system (Pharmacia Diagnostics, Freiburg, Germany) according to the manufacturer's instructions and in line with global recommendations[19]. An overall screening test was used to test allergic sensitization against aeroallergens at six, ten and 15 years. Our outcome was defined by a specific IgE threshold of >0.35 kU/L (Radio-Allergo-Sorbent-Test (RAST) class 1) to the screening test of common aeroallergens (Dermatophagoides pteronyssinus, cat, dog, rye, timothy grass, Cladosporium herbarum, birch and mugwort). Further RAST classes were defined according to common cut-offs[20], where RAST 0 implies no allergic sensitization and RAST 5 or 6 (combined into one category) is the highest possible value. Questionnaire-based information on symptoms of rhino-conjunctivitis (concurrent running nose and itching eyes) and wheezing in the previous twelve months was collected at the same time-points.

We assessed potential determinants of allergic diseases, which have been shown to be associated with different allergic diseases or lung function, such as parental education[21], breastfeeding[21,22], birth order[2], pet holding[21], maternal smoking during pregnancy[23], environmental tobacco smoke exposure[23] (ETS) or bronchitis infections[23] in early childhood, as well as polygenic risk scores calculated as weighted scores from genome-wide significant GWAS hits for any allergic disease[1,24], asthma[25], dermatitis[26], allergic rhinitis[27] and total IgE[28]. Additional information on the study design and on the definition of determinants of allergic diseases can be found in the online supporting information (Table S1, Methods 1).

## DNA methylation (DNAm) data

Samples using genomic DNA (gDNA) from blood-clots at six and ten years were analyzed using the MethylationEPIC BeadChip (Illumina, Inc., San Diego, CA). Paired samples were placed on the same chip to avoid batch effects among pairs. CpGs on sex chromosomes and those having missing values low intensities were excluded. We used functional normalization[29] to normalize the data and ComBat[30] to adjust for technical variation. After outlier removal, the final dataset includes information on 774,330 CpG probes for 461 DNAm samples, 234 at six and 227 at ten years, with an overlap of 221 participants with DNAm data at both time points. Cell type proportions were estimated both with the Houseman method[31] using a new reference panel[32] and with the EpiDISH[33] package, which

additionally includes eosinophil estimates. Further information on processing and quality control can be found in the online supporting information (Methods 2 & Fig. S1).

## Calculation of MRS

We calculated MRS based on the effect estimates or other summary statistics for CpG sites that have previously been associated with allergic diseases[6,8,10,11] or additionally provided summary statistics[7,9] for associations with up to a raw p-value of 0.1 for each EWAS. A weighted sum of DNAm beta values, defined as estimated methylation level, was then transformed to z-scores and MRS were produced for each respective EWAS and differing p-value thresholds. A literature review identified EWAS of phenotypes related to atopy or high IgE. Further publications for any kind of allergic disease were included, if they were conducted in a larger consortium framework (asthma[10] and any allergic disease[11]). Seven MRS were calculated, one for high IgE[7], one for aeroallergen sensitization[8], two for atopy, defined as high total IgE or positive skin-prick-test and sensitization, respectively[6,9], one for asthma[10] and one for any allergic disease[11] as well as one MRS for schizophrenia[34] as negative control. In all seven EWAS, DNAm was measured in whole blood. Varying p-value thresholds from $1\times10^{-1}$ to the lowest reported p-value per EWAS were considered, resulting in several scores per EWAS with a decreasing number of CpGs for smaller p-values, similar to what is known as “thresholding” for PRS[14]. To correct for correlations between included CpG sites, co-methylated regions were calculated using the CoMeBack method[35], which identifies co-methylated regions based on correlation and proximity of CpGs. In accordance to the original publication, we did this based on residuals corrected for Houseman cell type proportions of the LISA study. Only one CpG per co-methylated region was included in the final MRS, a procedure similar to “clumping” in PRS approaches[14]. All MRSs were calculated as z-scores following a standard normal distribution. A more detailed description is further provided in the supplementary information (Methods 3).

## Statistical analysis

Associations between each MRS and aeroallergen sensitization were estimated using logistic and Poisson regression with robust standard errors. Poisson regression was used to assess risk ratios (RR), as aeroallergen sensitization was not a rare outcome in our sample and thus odds ratios would not resemble RR. All models were adjusted for sex, age, whether the blood was taken in the allergy season (March to August), as current pollen exposure might influence DNAm[36] as well as circulating IgE levels, and estimated cell type proportions using EpiDISH. We applied the following criteria to evaluate and compare the performance of different MRS: 1) RR and corresponding 95%-confidence intervals (95% CI) were used to evaluate the strength and accuracy of the association with aeroallergen sensitization; 2) C-statistic, the area-under-the-curve and explained variance (Pseudo $R^2$) were used to evaluate the prediction accuracy for aeroallergen sensitization. The different MRS were compared and evaluated under four different scenarios: Two cross-sectional models assessing the association at six and ten years and two prospective models, assessing the association between the MRS and subsequent aeroallergen sensitization (MRS at six and ten years as predictor of aeroallergen sensitization at ten and 15 years, respectively). As a sensitivity analysis the prospective models were calculated in the non-sensitized population only, excluding all participants with sensitization at the time of DNA methylation measurement,

thereby analyzing only those who could develop new sensitization between the two time points. We furthermore calculated the receiver operating characteristic (ROC) for the cross-sectional analyses to assess the diagnostic ability of our MRS.

The best MRS per EWAS were selected based on the highest c-statistic in the cross-sectional model at six years. Correlations between the seven “best MRS” (one per EWAS) and the corresponding CpGs were evaluated. All CpGs reported in the available EWAS were tested for replication in the LISA study, both with the Houseman (as done in the original EWAS[6-10]) and EpiDISH cell type proportions, with successful replication being defined as a p-value below 0.05 after adjusting for the total number of tested CpGs from all EWAS using the Benjamini-Hochberg correction[37].

Associations between the MRS and the six RAST classes were investigated using boxplots to evaluate a potential dose-response relationship with increasing levels of aeroallergen sensitization as well as ordinal logistic regression analyses.

To compare the strength of association and prediction accuracy of the MRS to those of other common determinants of allergic diseases (including allergy-related PRS, Table S1) and the most common single CpGs, we calculated the explained variance and strength of association (RR and 95% CI) with aeroallergen sensitization and compared it to the performance of the MRS.

We further assessed the association of all MRS with allergic disease symptoms, namely rhino-conjunctivitis and wheezing, using the same approach as described above. In addition, we calculated correlations between the MRS and the different estimated cell type proportions to assess if a specific cell type was overrepresented in the MRS.In a sensitivity analysis, we tested the impact of co-methylated regions on the robustness of MRS: Namely, we calculated MRS with and without application of the CoMeBack method and used a reference population instead of the LISA study to determine the co-methylated regions (see Gatev et al., 2020[35]for details)

All statistical analyses were run in R[38] V4.1.2.

## Results

### Description of Study Participants

We included 461 samples, collected from 240 participants of the LISA birth cohort, in our analysis, both from six (N=234) and ten (N=227) years of age (Table 1), of which 221 were paired with DNAm data available at both time points (Fig. S1). The sample included slightly more males than females (58% vs. 42%) and the prevalence of rhino-conjunctivitis symptoms increased, while that of wheezing symptoms decreased, between six and ten years. Relevant outcome measures used in the six-year sample are aeroallergen sensitization at six years (prevalence: 32.6%, 74 cases) and at ten years (44.9%, 105 cases). In the ten-year sample, aeroallergen sensitization at ten years (44.5%, 101 cases) and at 15 years (37%, 84 cases) were analyzed in the main analysis. Differences seen between the two time-points are due to sample removal, as originally all samples were paired and are presenting the

same baseline characteristics. Baseline characteristics from our analysis sample (N=240) are similar to the total study population of the LISA Munich cohort (N=1464, Table S2).

## Methylation Risk Scores

Table 2 shows information on the seven EWAS, phenotype, age group and sample size from which MRS were calculated. The EWAS reported between 13 and 395 significant signals and varied by age, from four to 18 years, and ethnicity, covering not only European but also Hispanic and multi-ethnic populations. The best MRS per EWAS were selected based on the highest c-statistic in the cross-sectional model at six years across all p-value thresholds that were tested (Figure 1 and Table S3). The best performing MRS included two (Everson2015[6], atopy) to 24 (Zhang2019[9], atopy) CpGs for p-value thresholds ranging from $1\times10^{-4}$ (Zhang2019[9], atopy) to $1\times10^{-13}$ (Peng2019[8], aeroallergens). CpG sites and the corresponding weights for the best MRS are listed in Table S4

The six allergy-related MRS were highly correlated with each other but not with the negative control (age six years: Figure S3, age 10 years: Table S5). A total of seven CpGs are included in more than one allergy-related MRS, with cg11699125 being the most common one included in all but one allergy-related MRS. All of these seven CpGs could be successfully replicated in LISA (Table 3) and were annotated to the genes *ZFPM1*, *ACOT7, MFHAS1* and *SEC16B* using UCSC reference genes from the Illumina annotation file. Replication of reported EWAS signals (1501 in total) in LISA at six (N=234) and ten (N=227) years yielded 554 and 288 replicated hits correcting for Houseman and 111 and one replicated hits correcting for EpiDISH cell type estimates, respectively. Thus, we observe highly reduced replication rates when including eosinophils as a cell type confounder. Of note, of the published EWAS[6-11], only one (including the cohorts in PR-GOAL and GALA-II)[7] controlled for eosinophils in their analyses. Complete results can be found in Table S6.

## MRS as cross-sectional biomarkers

Figure 1 and Table S3 present results from evaluating MRS that were calculated based on different p-value thresholds and EWAS for the cross-sectional (age 6 and 10 years) as well as prospective analyses (6-10 years and 10-15 years). To improve clarity, Figure 1A presents the mean MRS over all p-value thresholds per EWAS. All allergy-related MRS were significantly associated with aeroallergen sensitization in LISA (Fig. 1A). Effect sizes were very similar between different MRS ranging from RR=1.47 [95% CI: 1.19; 1.84] to RR=1.81 [1.44; 2.27] in the cross-sectional model at six years and from RR=1.12 [0.87; 1.44] to RR=1.40 [1.19; 1.64] at ten years (Table S3). Classification accuracy (Fig. 1B, c-statistic), was about 0.7 for all allergy-related MRS and the best scores explain more than 15% of variance in aeroallergen sensitization, quantified with pseudo $R^2$, at six years and more than 12% at ten years (Fig. 1C). The negative control (MRS for schizophrenia) was not associated with aeroallergen sensitization in LISA. The ROC curves display similar patterns for allergy-related MRS and the negative control performs worst (Figure S2).

### MRS as prospective predictors

In the prospective models, all allergy-related MRS are significantly associated with aeroallergen sensitization, even though the effect estimates are smaller than in the cross-sectional models (Fig. 1, D&A). The prediction accuracy and the explained variance of the prospective models was smaller than in the cross-sectional models. For example, the pseudo $R^2$ decreased from explaining roughly between 12% and 15% of the variance in the cross-sectional models to only 8% to 12% in the prospective ones (Fig. 1, F). The c-statistic was also slightly lower with ~0.65 in the prospective models instead of ~0.7 in the cross-sectional models. In a sensitivity analysis, we analyzed whether prospective associations are observed because of participants that were already sensitized at the time of DNAm measurement. For this we ran the prospective analyses restricted to the non-sensitized population only. Looking only at the non-sensitized population (N = 160 from six to ten, N = 99 from ten to 15 years), the effect of MRS on prospective aeroallergen sensitization was further attenuated and no significant association was observed (Table S7). This might imply that DNAm is a consequence or biomarker of aeroallergen sensitization rather than a predictor of sensitization development.

### Dose-response relationship

Figure 2 shows a clear and significant positive trend between higher MRS and higher RAST classes in the two cross-sectional analyses, except for the negative control (Fig. 2, A&B). This trend can be seen for all allergy-related MRS, independent of age group, ethnicity or specific phenotype in the original EWAS. The trend was weaker in the prospective models (Fig. 2, C&D). The prospective trend from six to ten years was significant for all allergy-related MRS, but with lower odds ratios than in the cross-sectional models. The prospective trend from ten to 15 years was only significant for two of the six allergy-related MRS (Fig. 2D & Table S8).

### Prediction accuracy of MRS in comparison to known determinants

As seen in Figures 1 and 3, the explained variance of allergy-related MRS is about 15% in the cross-sectional model at six years. Explained variance by other common determinants was lower, with family history of allergic diseases explaining around 5% and all others less than 3%, including PRS (Fig. 3). Significant associations with aeroallergen sensitization were only present for the MRS and having two parents with a history of allergic diseases. Of the seven CpGs, present in more than one MRS, all were significantly associated with a reduced risk for sensitization and the pseudo $R^2$ was similar to the MRS, especially for cg17971251 and cg11988722.

### Prediction accuracy of atopy-related MRS for other allergic symptoms

Associations between calculated MRS and allergic symptoms, such as rhino-conjunctivitis and wheezing, were weaker than associations with aeroallergen sensitization and those models explained less variance (pseudo $R^2$ <0.09 for rhino-conjunctivitis and <0.14 for wheeze) (Fig. S4, A&C). The prediction accuracy for rhino-conjunctivitis is similar to the accuracy for aeroallergen sensitization (c-statistic ~0.7). In contrast to this, the association between MRS and wheezing was stronger in terms of effect estimates and prediction

accuracy. However, the higher RRs for wheezing and their wide CIs can also be attributed to the lower case numbers for allergic symptoms (Rhino-conjunctivitis: n=20, Wheezing: n=25 at six years) and these results should be interpreted cautiously (Fig. S4 and Table S9). Results of best performing MRS at ten years and their association with symptoms of wheezing and allergic rhinitis can be found in Table S10.

### Correlations with cell type proportions

During bulk DNAm analysis, several different blood cell types with differing methylation profiles are analyzed. To assess if a specific cell type is overrepresented in the MRS, we calculated correlations between the MRS and the different estimated cell type proportions. There was little correlation ($r <= 0.3$) between the allergy-related MRS and estimated cell types apart from eosinophils ($r = [0.53; 0.59]$), indicating that the MRS represent differential DNA methylation-related to aeroallergen sensitization independent of most immune cell types, apart from the known association with eosinophils[39] (Fig. S5).

### Robustness of MRS to determination of co-methylated regions

In our main analysis, co-methylated regions were determined using the LISA cohort. Using a reference population instead of our own LISA cohort for the determination of co-methylation region (as described in[35]) or no filtering based on CoMeBack at all, did not have an impact on our main results or the number of included CpGs (Tables S11 and S12). Individual CpGs included in the final MRS were correlated, despite application of the CoMeBack method, which only removes correlated CpG sites that are in close proximity to each other (Figure S6).

## Discussion

In the present study we calculated different MRS from available EWAS of atopy, high IgE, asthma or any allergic disease and assessed their prediction accuracy for childhood aeroallergen sensitization using cross-sectional and prospective data on DNAm and sensitization from the German LISA study. We showed the superior performance of allergy-related MRS compared to well-known determinants of allergic diseases, like birth order, as well as their high correlation with each other. Seven CpGs were overlapping between the MRS, all located in previously reported genes associated with allergic diseases, and were successfully replicated in the LISA study. The best performing MRS show a clear dose-response relationship with RAST classes of aeroallergen sensitization and explain more variance in aeroallergen sensitization than common determinants or PRS. However, we noticed differences between cross-sectional and prospective analyses, with the latter showing smaller effect sizes, lower prediction accuracy and less explained variance.

Our results fit with the accumulating evidence of improved disease definition using DNAm patterns and more specifically MRS as biomarkers for exposures[15] or predictor of diseases[16]

Looking at other determinants, MRS outperform them in explained variance, with about 15% of explained variance versus less than 7% for the next best determinant. Similar values are achieved by the seven most commonly represented CpGs in the MRS. This highlights the role of DNAm as important allergy specific factor. Even though other

determinants of allergic disease have been widely established and are also included in clinical recommendations[40], we could only observe significantly increased risk for the epigenetic factors and if both parents had a history of allergic diseases. Lack of associations with the other determinants could be explained by the relatively small sample size in this sub-sample of the LISA cohort Furthermore, the low predictive capabilities of a PRS for asthma in childhood was published previously[41] and might underline our results of larger epigenetic associations as these lie on a level of omics closer to the phenotype[42].

We found that the seven most important CpGs included in more than one MRS mapped to the genes *ZFPM1*, *ACOT7, MFHAS1* and *SEC16B,* all of which have been reported in relation to allergic diseases[43-46]. The first three genes affect inflammatory responses through mast cell differentiation and development of cysteinyl leukotrienes. *ACOT7* has also been discussed as an important “cross-tissue allergy-associated methylation site” by one of the discovering EWAS[11]. The functional pathways of *SEC16B* have yet to be elucidated.

The MRS calculated for the six-year data showed a stronger effect with aeroallergen sensitization at six years (cross-sectional analysis) than with aeroallergen sensitization at ten years (prospective analysis; RR~1.7 vs ~1.4). The RR were further attenuated and not significant anymore when analyzing only the non-sensitized population in both prospective models. This might indicate that the MRS are in fact not predictive of sensitization at a later time point but coincide with or follow aeroallergen sensitization and the prospective models only capture the effect of already sensitized participants at baseline . However, the prospective analysis of the non-sensitized population is limited by a small sample size and thus limited statistical power. The positive trend between MRS and RAST classes seen in the cross-sectional models could not be seen in the prospective models, hence underlining the cross-sectional but not predictive nature of the association. A prospective prediction capability could have helped with early detection of allergic disease development and future studies might evaluate the prospective capabilities of combining IgE and DNAm measurements to improve prediction of allergic disease development. Development of an enhanced predictive tool is of great interest in the context of personalized medicine and might include genetic and epigenetic aspects, as well as IgE as already available biomarker. Nevertheless, the observed cross-sectional classification capability of MRS underline the relevance of altered gene-regulation in allergic diseases, aligning with previous publications noting that DNAm changes are more often seen as a consequence rather than the cause of a disease and that especially SNP-CpG associations are not necessarily causal[47].

We observed reduced replication rates of reported CpGs when adjusting for EpiDISH cell type estimates compared to the often used Houseman estimates (7.4% vs. 36.9% at six years). This might indicate that a high portion of previously seen associations may be attributable to eosinophils, which are not estimated in Houseman proportions. Notably, our MRS results remain significant even when adjusting for eosinophils, whereas EWAS replication is highly diminished.

In our study, we did not observe differences with ethnicity for our MRS in an population of European ancestry, as the MRS calculated from an EWAS of Hispanics with multiple racial backgrounds[7] performed just as well and sometimes even better than European-ancestry

derived MRS. This aligns with the meta-analysis results from the EWAS on asthma conducted in the pregnancy and childhood epigenetics consortium[48], which did not see any influence of ethnicity on detected CpG hits. We could, however, not evaluate the applicability of our MRS for non-European populations.

Taking into account, the relatively small sample sizes used in the applied EWAS ranging from only 367 samples in discovery[6] to 3,493 used for meta-analysis[10], the portion of replicated signals in the LISA study (38.8% at six years) indicates a good replicability of allergy-related EWAS results. Further, our MRS performed well over all included EWAS, independent of variation in ethnicity or age, ranging from childhood to young adulthood. EWAS results of allergy seem to be rather similar across childhood, indicated by replication of signals at six years, although only one EWAS obtained results in participants younger than six years[11], while the others were mostly older (Table 2) and similar patterns in both cross-sectional models.

Robustness of our findings was also confirmed across the different phenotypes used in the published allergy-related EWAS. Although main phenotypes were similar, as our outcome is a direct categorization of aeroallergen sensitization measurements, even broader ones like total IgE[7] or any allergic disease[11] result in the same patterns. Especially the similar findings across EWAS of different phenotypes, e.g. sensitization versus asthma, might hint in the direction of a general allergy phenotype[49], also in terms of DNAm patterns. MRS were also associated with symptoms of allergic diseases in the LISA cohort, though associations with rhino-conjunctivitis and wheezing were weaker than for aeroallergen sensitization, likely due to the lower prevalence of these symptoms.

We recognize additional study limitations. We could not test the accuracy of MRS across different tissues (e.g. nasal epithelium), as there are, to the best of our knowledge, no respective large-scale EWAS available for calculating further MRS. However, previous publications could replicate their signals from whole blood in other allergy-relevant tissues[10,50]. Additionally, we extracted gDNA from blood clots, whereas other studies used whole blood, so predictive accuracy of proposed MRS might be even higher using identical sample processing methodology. Our MRS approach uses CoMeBack to remove correlated CpGs located in co-methylated regions from the MRS. Future studies should evaluate if the prediction accuracy of MRS can be further improved by considering all correlations between CpGs instead of only those located in close proximity to each other and does not account for trans chromosomal correlations. Absence of significant associations between aeroallergen sensitization and established predictors of allergic diseases in our sub-cohort indicate a limited statistical power due to our relatively small sample size for this analysis. Therefore, future studies with larger sample sizes are needed to replicate our findings. However, the strong associations and prediction accuracy that we found for the MRS despite our relatively small study sample also demonstrates the applicability of this approach for small study populations and the robustness of previously reported EWAS results.

Strengths of this study include the objective assessment of aeroallergen sensitization in blood. This makes all of our main associations robust as neither aeroallergen sensitization diagnosis nor DNAm, estimated cell type proportions or sex are subject to recall bias.

Moreover, the prospective design of the LISA study enabled us to compare repeated measures at two time-points of DNAm with three time-points of measured aeroallergen sensitization.

In summary, we established well-working MRS for aeroallergen sensitization, which outperform commonly known determinants in identifying the disease. The presented results confirm the association of DNAm at some CpGs with allergic diseases and underline the relevance of altered gene-regulation in allergic diseases. The results support replication and applicability of available EWAS results and pave the way for future analyses investigating the specific functions between methylation patterns as biomarkers of disease manifestation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Data availability statement

Due to data protection reasons, the datasets generated and/or analyzed during the current study cannot be made publicly available. The datasets are available to interested researchers from the corresponding author on reasonable request, provided the release is consistent with the consent given by the LISA study participants. Ethical approval might be obtained for the

release and a data transfer agreement from the legal department of the Helmholtz Zentrum Muünchen must be accepted.

## Abbreviations

| | |
|---|---|
| **95% CI** | 95% Confidence interval |
| **CpG** | Cytosine and Guanine only separated by their phosphate backbone |
| **DNAm** | DNA methylation |
| **ETS** | Environmental Tobacco Smoke exposure |
| **EWAS** | Epigenome-wide association studies |
| **GWAS** | Genome-wide association studies |
| **gDNA** | genomic DNA |
| **IgE** | Immunoglobulin E |
| **LISA** | Influence of Life-style factors on Development of the Immune System and Allergies in East and West Germany study |
| **MRS** | Methylation risk score |
| **PRS** | Polygenic risk score |
| **RAST** | Radio-Allergo-Sorbent-Test |
| **ROC** | Receiver operating characteristic |
| **RR** | Risk ratio |

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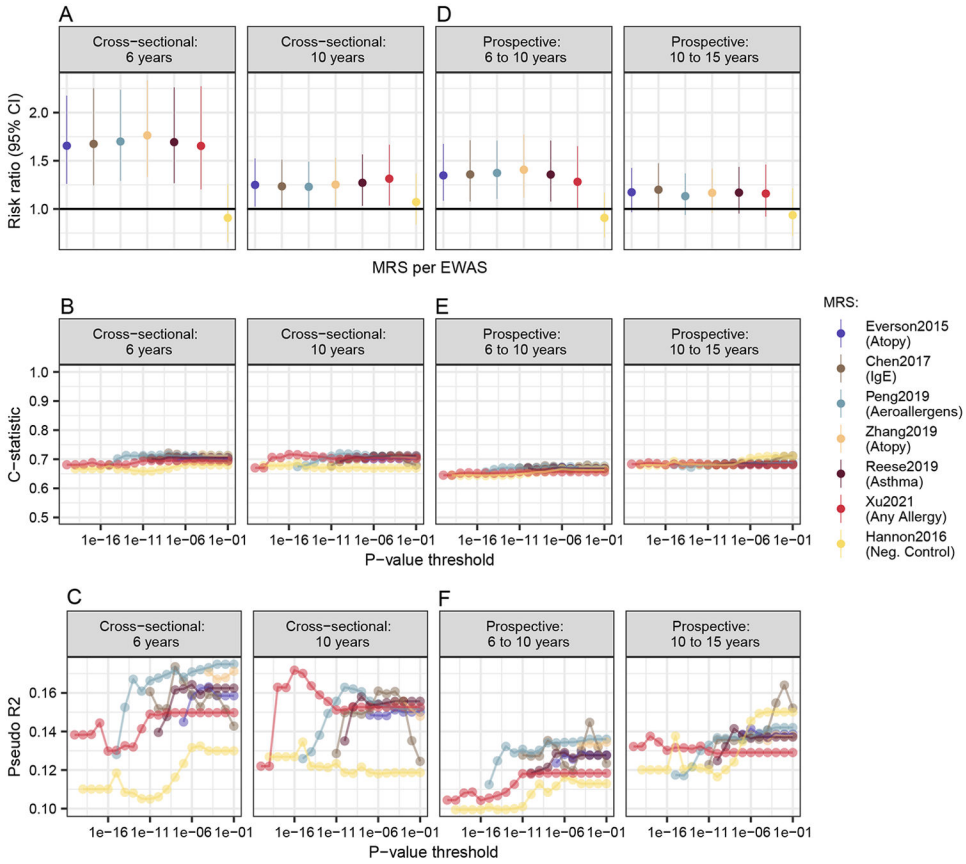


**Figure 1:**

Predictive capabilities of MRS on aeroallergen sensitization. Four different models and criterions are displayed, assessing the cross-sectional and prospective impact of MRS as well as their (A) mean effect size per publication over all p-value thresholds, (B) performance and (C) explained variance for the different p-values thresholds (determining how many CpG sites were included in the MRS). All models are adjusted for sex, age, whether the blood was taken within the allergy season and cell type proportions. RR (A) were derived from Poisson regressions, whereas the other criterions (B&C) were calculated using logistic regression. Sample sizes for the four models were n = 234, n = 227, n = 234 and n = 167, respectively.

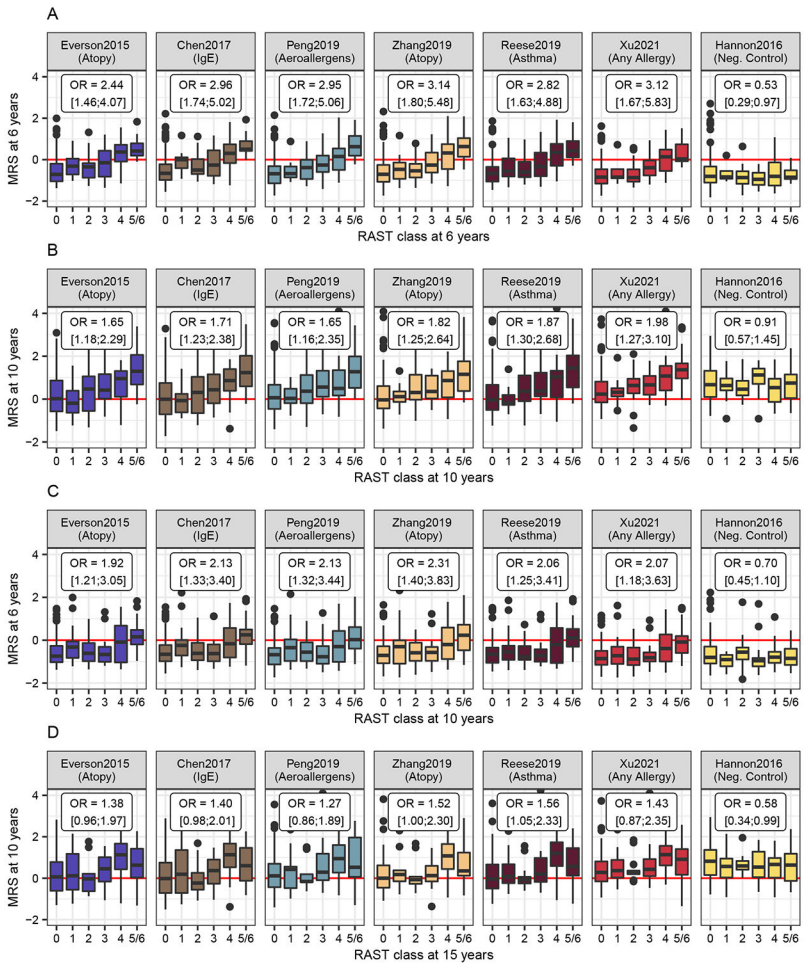


**Figure 2:**

Dose-response relationship of MRS z-scores and RAST classes of aeroallergen sensitization cross-sectionally at (A) six and (B) ten years and prospectively from (C) six to ten and (D) ten to 15 years. The fifth and sixth RAST classes are combined due to the low sample size in the highest class. Odds ratios (OR) and 95%-confidence intervals from ordinal logistic regression analysis of the association between RAST classes and MRS are displayed in their respective panels.

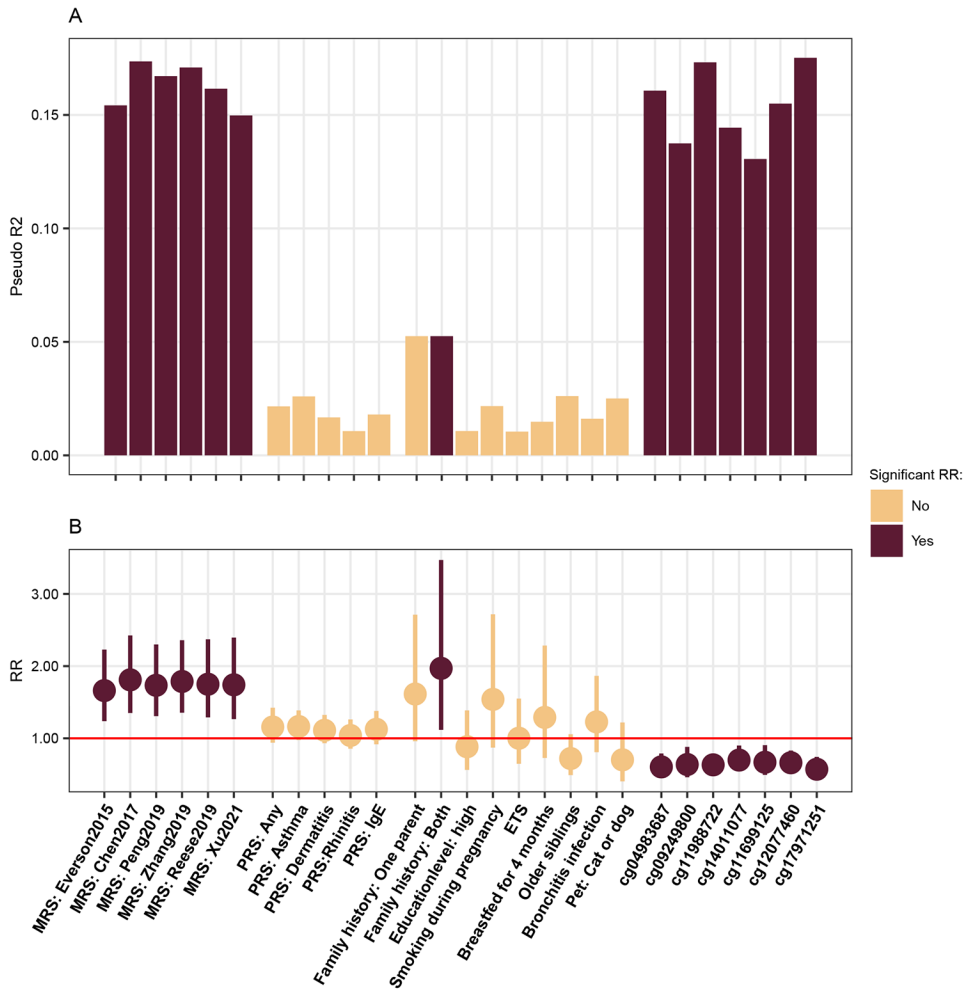


**Figure 3:**

Prediction accuracy of MRS in comparison to other known risk factors. (A) Explained variance was assessed using logistic regression and (B) RR and 95%-CI using Poisson regression with robust standard errors. For continuous variables (MRS, PRS and CpGs z-scores were used, hence the RR estimate can be understood per one standard error increase. All models were adjusted for sex, age and those with MRS and single CpGs additionally for cell type proportions and whether the blood was taken within the allergy season. Significance was determined in the Poisson model with a threshold of 0.05.

**Table 1:**

Sample information and variable distribution in the final analysis sets at six and ten years of age in the LISA birth cohort.

| | Analysis sample (N = 240) | |
|---|---|---|
| | **DNAm measured at 6 years (N = 234)** | **DNAm measured at 10 years (N = 227)** |
| Male sex, % (n) [n missing] | 57.69% (135) [0] | 57.71% (131) [0] |
| Age at DNA methylation measurement [years], mean (sd) | 6.072 (0.15) | 10.155 (0.14) |
| High parental education[1], % (n) [n missing] | 79.49% (186) [2] | 79.74% (181) [2] |
| Aeroallergen sensitization[2], % (n) [n missing] | | |
| At 6 years | 31.62% (74) [0] | 29.96% (68) [0] |
| At 10 years | 44.87% (105) [0] | 44.49% (101) [0] |
| At 15 years | 37.18% (87) [63] | 37.00% (84) [60] |
| Allergic symptoms in last 12 months, % (n) [n missing] | | |
| Rhino-conjunctivitis | 8.55% (20) [1] | 14.98% (34) [4] |
| Wheezing | 10.68% (25) [2] | 7.93% (18) [2] |
| Blood taken in allergy season (Mar - Aug), % (n) [n missing] | | |
| At 6 years | 67.52% (158) [0] | 66.96% (152) [0] |
| At 10 years | 52.99% (124) [0] | 51.10% (116) [0] |
| At 15 years | 41.45% (97) [55] | 39.65% (90) [52] |
| Polygenic Risk Scores[3], mean (sd) | | |
| Any allergic disease | 0.187 (0.95) | 0.187 (0.93) |
| Asthma | 0.147 (1.04) | 0.155 (1.05) |
| Dermatitis | 0.002 (0.99) | −0.014 (0.99) |
| Rhinitis | 0.023 (0.97) | 0.052 (0.95) |
| Total IgE | 0.083 (0.97) | 0.084 (0.97) |
| Family history of allergic diseases, % (n) [n missing] | | |
| No parent | 29.06% (68) [24] | 28.19% (64) [24] |
| One parent | 42.74% (100) [24] | 43.17% (98) [24] |
| Both parents | 17.95% (42) [24] | 18.06% (41) [24] |
| Other known risk factors for allergy, % (n) [n missing] | | |
| Smoking during pregnancy | 7.26% (17) [7] | 7.05% (16) [6] |
| Breastfeeding in first 4 months | 83.33% (195) [1] | 83.70% (190) [1] |
| Older siblings | 49.57% (116) [0] | 48.90% (111) [0] |
| ETS[4] in first 4 years | 25.64% (60) [3] | 25.11% (57) [3] |
| Bronchitis infection in first 3 years | 64.96% (152) [2] | 66.96% (152) [2] |
| Cat or dog in first 4 years | 19.66% (46) [8] | 19.82% (45) [9] |

[1] defined as more than 10 years of education

[2] defined by a specific IgE threshold of >0.35 kU/L (Radio-Allergo-Sorbent-Test (RAST) class 1)

[3] z-scores

[4] *Environmental tobacco smoke exposure;* If time point of measurement is not mentioned, data were obtained from questionnaire data filled out by the LISA parents at birth, 1, 2 or 4 years of age.

**Table 2:**

Overview of included EWAS, their phenotypes (allergy-related outcome) and age groups.

| EWAS | Phenotype | No. of significant CpGs with FDR-corrected p-values <0.05[with raw p-values <0.01[1]] | Sample size (discovery + replication) | Age | Ethnicity | P-value threshold for best MRS[2] | No. of included CpGs (best MRS) | Cohorts | Chip |
|---|---|---|---|---|---|---|---|---|---|
| *Everson2015*[6] | Atopy status (IgE >= 200kU/L) | 13 | 367 + 464 | 18 | European | $1\times10^{-8}$ | 2 | IoW + BAMSE | 450K |
| *Chen2017*[7] | Log10(IgE) | 200 [25 089] | 879 (meta) | 6 to 22 | Hispanic | $1\times10^{-8}$ | 3 | PR-GOAL, GALA II | 450K |
| *Peng2019*[8] | Environmental allergen sensitization (>= 0.35 IU/ml to common aeroallergens) | 395 | 739 (total) | mean: 7.7 & 9.8 | Multi-ethnic | $1\times10^{-13}$ | 5 | Project Viva, Generation R | 450K |
| *Zhang2019*[9] | Atopic status (>= 3mm grater than negative control in SPT or IgE >= 0.35 kU/L for mix inhalant of food allergens) | 35 [775] | 376 + 267 | 10 & 18 | European | $1\times10^{-4}$ | 24 | IoW + BAMSE | 450K/ EPIC |
| *Reese2019*[10] | Childhood asthma | 179 | 3493 (meta) | mean range: 7.1 to 17.01 | Multi-Ethnic | $1\times10^{-8}$ | 9 | BAMSE, CHOP, GALA II, ICAC, NFBC 1986, PIAMA, RAINE, STOPPA | 450K |
| *Xu2021*[11] | Any allergic disease (asthma, eczema, rhinitis) PLUS sensitization against common aeroallergens (>= 0.35IU/ml) | 21 | 1457 + 1436 | 4 & 8 | European | $1\times10^{-9}$ | 18 | BAMSE, INMA, PIAMA + EDEN, ECA, INMA, PIAMA, Karelia | 450K |
| *Hannon2016*[34] | Schizophrenia (negative control) | 2519 | 675 + 847 | Adults | European | $1\times10^{-5}$ | 867 | UCL case-control + Aberdeen case-control, MZ twins cohort | 450K |

[1] some studies provided additional summary statistics for all CpG sites with p-values < 0.01, n/a indicates that these additional summary statistics were not provided

[2] best MRS per EWAS were selected based on the highest c-statistic in the cross-sectional model at six years across all p-value thresholds that were tested (see also Table S2, where all MRS are evaluated)

**Table 3:**

Overview of CpGs, which are present in more than one of the best performing MRSs and their association with allergy-related outcomes in the original EWAS (A) and in the LISA cohort (replication, B).

| CpG (gene) | Allergy-related outcome | cg04983687 (ZFPM1) | cg09249800 (ACOT7) | cg11699125 (ACOT7) | cg11988722 (intergenic) | cg12077460 (MFHAS1) | cg14011077 (intergenic) | cg17971251 (SEC16B) |
|---|---|---|---|---|---|---|---|---|
| **A. Effect direction (p-values [1]) from original EWAS** | | | | | | | | |
| Everson2015[6] | Atopy | − (6.46E-09) | − (8.52E-09) | | | | | |
| Chen2017[7] | IgE | − (1.50E-12) | − (2.60E-11) | − (6.70E-07) | | | | |
| Peng2019[8] | Aeroallergens | − (7.11E-15) | | − (7.28E-12) | − (6.51E-14) | | | − (4.03E-11) |
| Zhang2019[9] | Atopy | | − (1.17E-04) | − (4.34E-05) | − (9.04E-05) | − (2.98E-05) | − (2.83E-05) | − (1.46E-05) |
| Reese2019[10] | Asthma | − (1.33E-10) | − (1.19E-08) | − (7.54E-10) | | | − (7.02E-09) | − (9.52E-09) |
| Xu 2021[11] | Any allergy | | | − (5.84E-19) | | − (3.73E-13) | | |
| **B. Beta coefficients (p-values [2]) in LISA** | | | | | | | | |
| LISA: 6 years | Aeroallergen sensitization | −11.61* (1.62E-03) | −14.71* (1.53E-02) | −11.94* (3.14E-02) | −21.59* (6.31E-04) | −19.65* (3.17E-03) | −40.89* (1.02E-02) | −26.84* (5.19E-04) |
| LISA: 10 years | Aeroallergen sensitization | −3.93 (1.62E-01) | −9.77* (2.79E-03) | −8.41* (2.47E-03) | −10.04* (2.35E-02) | −6.61 (4.27E-01) | −14.67 (1.22E-01) | −12.90* (8.01E-03) |

In A, effect direction (+/−) is reported instead of effect estimates because of the different allergy-related outcomes that were used in the original EWAS, which do not allow a direct comparison of effect estimates.

[1] raw p-values corrected for confounding variables as per EWAS (check[6-11] for details)

[2] raw p-values given corrected for Houseman cell type estimates

* These CpGs were all successfully replicated in the LISA cohort (FDR threshold of 0.05)