Differentially methylated DNA regions in early childhood wheezing: an epigenome-wide study using saliva

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Affiliations

[1] Cancer Epidemiology Unit, Department of Medical Sciences, University of Turin; Centro di Ricerca in Medicina Sperimentale (CeRMS) and CPO Piemonte, Turin, Italy

[2] Italian Institute for Genomic Medicine (IIGM), Turin, Italy.

[3] Unit of Epidemiology, 'Anna Meyer' Children's University Hospital, Florence, Italy

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Corresponding author:

Maja Popovic

Department of Medical Sciences,

University of Turin

Via Santena 7

10126 Turin, Italy

E-mail: maja.popovic@unito.it

Phone: +39(0) 116334628

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Abstract

Background Epigenetics may play a role in wheezing and asthma development. We aimed to examine infant saliva DNA methylation in association with early childhood wheezing.

Methods A case-control study was nested within the NINFEA birth cohort with 68 cases matched to 68 controls by sex, age (between 6 and 18 months, median: 10.3 months) and season at saliva sampling. Using a bump hunting region-based approach we examined associations between saliva methylome measured using Illumina Infinium HumanMethylation450k array and wheezing between 6 and 18 months of age. We tested our main findings in independent publicly available datasets of childhood respiratory allergy and atopic asthma, with DNA methylation measured in different tissues and at different ages.

Results We identified one wheezing-associated differentially methylated region (DMR) spanning ten sequential CpG sites in the promoter-regulatory region of *PM20D1* gene (family wise error rate <0.05). The observed associations were enhanced in children born to atopic mothers. In the publicly available datasets, hypermethylation in the same region of *PM20D1* was consistently found at different ages and in all analysed tissues (cord blood, blood, saliva and nasal epithelia) of children with respiratory allergy/atopic asthma compared with controls.

Conclusion This study suggests that *PM20D1* hypermethylation is associated with early childhood wheezing. Directionally consistent epigenetic alteration observed in cord blood and other tissues at older ages in children with respiratory allergy and atopic asthma provides suggestive evidence that a long-term epigenetic modification, likely operating from birth, may be involved in childhood atopic phenotypes.

Key words: wheezing, asthma, children, epigenome-wide association study, saliva, epigenetics, infant, EWAS, DMR, PM20D1

70 **Introduction**

71 Over the past 20 years the concept of developmental origins of health and disease has become
72 widely accepted and expanded to many common complex conditions, including, but not
73 limited to, immunometabolic disorders, such as obesity, asthma, and cardiovascular diseases.[1]
74 This theory states that intrauterine and early postnatal exposures can induce biological
75 changes that influence later disease susceptibility. Alteration of the epigenome is one potential
76 mechanism by which environmental exposures may cause structural and/or functional
77 changes in cells, tissues, and organs of developing individuals.[2] Several in utero exposures,
78 such as maternal smoking, maternal body mass index (BMI), and exposure to air pollution
79 have been associated with alterations in infant DNA methylation, most often measured in cord
80 blood.[3-5] Most of these exposures are also known to be associated with childhood wheezing
81 and asthma.[6-8] In addition, postnatal environment, such as air pollution, early microbial
82 exposures, and child's growth, may increase the risk of childhood wheezing disorders and
83 asthma through epigenetic modifications.[9-11]

84 Although DNA methylation is the most studied epigenetic mechanism to date, post-
85 translational histone modifications have also been implicated in the T-cells differentiation and
86 airway remodelling, contributing to the epigenetic regulation of allergic phenotypes, including
87 asthma.[12,13,14]

88 While asthma is a diagnostic category typically used from school age onwards, the disease
89 often initiates much earlier, with wheezing episodes starting from early infancy.[15] Wheezing
90 in early childhood, especially if accompanied with allergic sensitization or other atopic
91 conditions, has been shown to be a strong predictor of asthma development and later lung
92 function,[16] and thus is often studied in the context of large epidemiological studies.

93 Several previous studies reported associations with childhood wheezing and asthma of DNA
94 methylation changes at a number of single methylation sites and differentially methylated

95 regions (DMRs).[17-24] A large cross-sectional EWAS of childhood asthma conducted within the
96 MeDALL consortium identified an altered peripheral blood DNA methylation at 35 CpG
97 sites; most of the associations were with school age asthma, while only one CpG site was
98 associated with asthma at preschool age.[23] Importantly, all the associations were largely
99 attenuated, but persisted, following adjustment for eosinophil count. In the same study, DNA
100 methylation patterns at 14 out of the 35 identified CpG sites were replicated in peripheral
101 blood of children with asthma from independent cohorts, while none of the identified CpG
102 sites was associated with asthma when DNA methylation was measured in cord blood at
103 birth.[23] A recent meta-analysis of epigenome-wide DNA methylation and school-age asthma
104 identified 9 CpG sites and 35 DMRs in cord blood associated with childhood asthma.[24] These
105 early DNA methylation markers were, however, not among much larger number of hits
106 identified in a cross-sectional analysis within the same study where both asthma and blood
107 DNA methylation were assessed in school-age children and adolescents.[24]

108 It is well established that DNA methylation is tissue-specific, and for diseases for which the
109 local effect might be more pronounced than their systemic effect, such as asthma, the tissue
110 selection for DNA methylation measurement is essential. Bronchial tissue is difficult to obtain
111 in population studies, and most of the studies assessing epigenetic markers of wheezing and
112 asthma focused on DNA methylation measured in blood samples. Some studies, however,
113 found asthma methylomic markers in buccal, nasal and airway epithelial cells.[17, 18, 20, 25] As
114 saliva is a candidate body fluid reflecting pathological changes in the airways during asthma
115 development,[26] we aimed at investigating the associations between infant saliva methylome
116 and early childhood wheezing within the Italian birth cohort study NINFEA.

117 **Methods**

118 *Study population*

The NINFEA (Nascita ed INFanzia: gli Effetti dell'Ambiente) study is an Italian web-based birth cohort that recruited approximately 7500 pregnant women during the period 2005-2016 (https://www.progettoninfea.it). Members of the cohort are children of mothers who had access to the Internet, enough knowledge of Italian to complete online questionnaires, and volunteered to participate at any time during the pregnancy. At enrolment women completed the baseline questionnaire, and children are then followed up with five questionnaires completed by mothers 6 and 18 months after delivery, and when children turn 4, 7 and 10 years of age. At the end of the 6-month questionnaire mothers were invited to donate their and their child's saliva samples using the Oragene™ DNA self-collection kits (OG-250, DNA Genotek, Inc., Ottawa, Ontario, Canada). Mothers who did not respond to this initial invitation were invited again at the end of the 18-month and 4-year questionnaire. The samples are stored in a biobank at -80°C. Additional information on recruitment, study advertisement and follow-up is available in the dedicated paper.[27]

We conducted a nested case-control study within the subset of the NINFEA children who met the following criteria: i) singleton child, ii) saliva sample collected between 6 and 18 months after birth (time window for the outcome assessment), iii) residence in the Province of Turin, and iv) born to mother who did not have asthma active during the index pregnancy.

Wheezing was assessed from the questionnaire completed 18 months after delivery using the standardized question from the International Study on Asthma and Allergies in Childhood (ISAAC), and was defined as at least one episode of wheezing or whistling in the chest occurred between 6 and 18 months of age.

At the time of sampling of cases and controls (database version 02.2014), there were 551 children meeting the aforementioned inclusion criteria, of whom 79 had at least one wheezing episode between 6 and 18 months of age. Of the 79 children with wheezing, seven samples

143 with the lowest DNA content were excluded, and the remaining 72 cases were matched to
144 controls by sex, season and age at saliva donation keeping a constant 1:1 ratio.

145 The NINFEA study protocol and subsequent amendments have been approved by the Ethical
146 Committee of the San Giovanni Battista Hospital—CTO/CRF/Maria Adelaide Hospital,
147 Turin, Italy (approval N.0048362). All women gave their informed consent at recruitment and
148 when donating saliva samples.

149 *DNA methylation data*

150 The methylation status of over 485,000 probes was measured using the Illumina Infinium
151 HumanMethylation450 BeadChip (Illumina, Inc., San Diego, CA). Details on pre-processing
152 of samples and data quality control can be found in the **Supporting Information file**
153 (Methods, *DNA methylation measurement, data pre-processing, and quality control***)**. DNA
154 methylation for each probe was expressed as beta value (ratio of methylated probe intensity to
155 overall intensity, representing 0 to 100% methylation at each probe). After quality control and
156 probes filtering 136 samples with DNA methylation measured in 421,782 probes remained for
157 the analyses.

158 *Statistical analyses*

159 To remove variance caused by batch and unmeasured technical effects, and to account for
160 cell-type heterogeneity we performed surrogate variable analysis using the R package sva.[28,29]
161 We conducted an exploratory epigenome-wide single-probe analysis using logistic regression
162 models adjusted for matching variables (child's sex, age and season at saliva sampling) and
163 for presence of siblings at birth, maternal age at delivery, maternal smoking after delivery as a
164 proxy for child's exposure to passive smoking and day-care attendance between 6 and 18
165 months of age. Bonferroni-corrected and Benjamini and Hochberg False-Discovery Rate
166 (FDR)-corrected p-values were calculated to account for multiple testing.

167 Differentially methylated regions associated with early childhood wheezing were analysed
168 using the R package *bumphunter*.[30] We clustered probes with a maximum distance of 1kb,
169 and then with a bumphunter function fitted a linear model for each site with wheezing as the
170 independent variable, adjusting for the aforementioned confounding and matching variables
171 used in the exploratory epigenome-wide association analyses. The estimated coefficients of
172 the case-control status were then smoothed within the clusters using running medians. We
173 performed 1000 random bootstrap iterations to estimate an empirical null distribution. The
174 regions of interest were chosen based on the cut-off threshold of 5% in the methylation beta
175 value difference between cases and controls, retaining only regions with at least three probes
176 within the region and with family-wise error rate (FWER) <0.05.
177 We further tested whether some of in utero exposures confounded the observed associations
178 by performing additional adjustments for maternal history of atopy (atopic dermatitis and/or
179 allergic rhinitis; note that mothers with history of asthma were a priori excluded from the
180 study), maternal depression and/or anxiety and maternal genito-urinary and respiratory
181 infections (otitis, sinusitis, throat infections, bronchitis and influenza) during the index
182 pregnancy.
183 Differences between cases and controls in the mean DNA methylation at each CpG site
184 mapping to a DMR identified by the bumphunting procedure were analysed using unpaired t-
185 test. To explore the role of maternal atopy in the observed associations, these analyses were
186 additionally stratified by maternal history of atopic disorders (allergic rhinitis and/or atopic
187 dermatitis).
188 All analyses were performed using the statistical computing software R version 3.5.1.
189 *Analysis of publicly available datasets*
190 To test our main findings in independent samples we identified five datasets from the Gene
191 Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo/). Details on the search of

192 datasets containing epigenome-wide DNA methylation data and phenotypes related to
193 childhood wheezing can be found in the **Supporting Information file** (Methods, *Search of*
194 *publicly available datasets*).
195 In particular, we used three datasets from the project "Epigenetic profiling of children with
196 respiratory allergy" (GSE110128), which includes children from a Belgian birth cohort with
197 available cord blood samples, and blood and saliva samples collected at age 11 years.[31] DNA
198 methylation from cord blood mononuclear cells (CBMCs), peripheral blood mononuclear
199 cells (PBMCs) and saliva was measured using the Infinium HumanMethylation450 BeadChip
200 array. This project includes processed DNA methylation data from 485,512 CpG sites for 233
201 samples: 90 cord blood samples (30 respiratory allergy cases and 60 controls), 90 blood
202 samples (29 respiratory allergy cases and 61 controls) and 53 saliva samples (33 respiratory
203 allergy cases and 20 controls). Respiratory allergy was assessed when children were 10 years
204 old and was defined as IgE sensitization to a mix of airborne allergens and self-report or
205 doctor's diagnosis of at least one of the following: asthma, hay fever, other types of rhinitis,
206 wheezing, or runny nose in the past year and ever.[31]
207 The remaining two datasets (GSE40576 and GSE65163) include partially overlapping
208 samples of African American or Hispanic with Dominican/Haitian background children from
209 the Inner-City Asthma Consortium (USA). Atopic asthma was defined as persistent physician-
210 diagnosed asthma with altered lung function parameters and positive skin prick test to at least
211 one of a panel of indoor aeroallergens.[19, 20] The GSE40576 dataset includes epigenome-wide
212 DNA methylation measured in PBMCs from 6-12 years old children with atopic asthma
213 (N=97) and healthy controls (N=97).[19] The GSE65163 dataset involves children with atopic
214 asthma (N=36) and healthy controls (N=36) aged 9-12 years and epigenome-wide DNA
215 methylation measured in nasal epithelial cells.[20] Both studies used Infinium
216 HumanMethylation450 BeadChip array to measure DNA methylation.

217 We selected only probes located within the DMR identified in our study, calculated the
218 differences in the mean DNA methylation between cases and controls (delta beta [Δbeta]),
219 and formally tested them using the unpaired t-test. We report one-sided p-values because the
220 alternative hypotheses were determined by the findings from the NINFEA sample. Given the
221 public nature of the datasets, we were not able to perform data quality checks and to correct
222 for cell types, technical covariates and other potential confounders.

223 **Results**

224 *Epigenome-wide DNA methylation analysis of early childhood wheezing*

225 Descriptive statistics of the study population are shown in **Table 1**. Maternal age, educational
226 level, nationality, and smoking after delivery were balanced between cases and controls.
227 Children with wheezing were more likely to be born to mothers with a history of atopic
228 disorders (p=0.09), to have siblings and to have attended day-care compared with controls
229 (both p-values=0.02). Median age at saliva sampling was 10.3 months (interquartile range
230 [IQR] 9.4-11.8 months).

231 In an exploratory epigenome-wide association analysis 25,042 (5.9%) probes were associated
232 with childhood wheezing at the conventional p-value<0.05, but none remained associated
233 after adjustment for multiple comparisons (Bonferroni p-value<1.19e-07 and Benjamini-
234 Hochberg FDR-corrected p-value<0.05). The Manhattan plot of the EWAS results is shown in
235 **Figure S1**.

236 Of the four DMRs identified by the bumphunting procedure (**Table S1)** only one region was
237 associated with wheezing with a FWER<0.05 (**Table 2**). This DMR remained associated with
238 childhood wheezing also after adjustment for maternal history of atopy, maternal depression
239 and anxiety and maternal infections during pregnancy (data not shown).

240 The identified DMR consists of ten probes located on the chromosome 1 and maps to the
241 promoter region of the *PM20D1* gene. All 10 probes were hypermethylated in cases compared

242 with controls, with seven probes having Δbeta from 4.3% to 9.6% (all p-values<0.05, **Table**
243 **3**, first column). The largest differences between cases and controls were identified in the
244 *PM20D1* promoter region and in the region of the first exon.
245 When we stratified analyses on single CpG sites by maternal atopic disorders (**Table 3**) DNA
246 methylation differences between cases and controls at 7 CpG sites located within the *PM20D1*
247 promoter region and the region of the first exon were particularly increased in children born to
248 atopic mothers, with Δbeta ranging from 10% to 20%. The same differences were diluted, but
249 still followed similar pattern, in children of non-atopic mothers.
250 *Analysis of the publicly available datasets*
251 The difference in the mean DNA methylation between cases and controls in 10 probes located
252 within the *PM20D1* calculated in the NINFEA sample and in the five public datasets are
253 reported in **Figure 1** and **Table 4**. The *PM20D1* probes are ordered according to their base
254 pair position. The seven most strongly associated probes in the NINFEA data were
255 consistently associated with respiratory allergy in 11-year old children when DNA
256 methylation was measured in CBMCs and PBMCs (CBMCs: Δbeta 8.4%-11.7%; PBMCs:
257 Δbeta 10.4%-13.6%; all one-sided p-values<0.05). An analogous direction of the associations,
258 though lower in magnitude, was observed for DNA methylation measured in saliva of 11-year
259 old children. It should be noted that the sample size of the saliva public dataset was small
260 (half of the other two public datasets from the same project) limiting the power of this
261 analysis.
262 Only two of the 10 probes from the *PM20D1* DMR were available in the nasal epithelia
263 dataset of persistent atopic asthma in African-American 9-12 years old children, where the
264 associations were even stronger (Δbeta 10%-11%, both one-sided p-values<0.005). Despite
265 the strong associations observed in nasal samples, the differences in PBMCs of children from
266 the same study were much smaller, although still in the same direction.

267 The three probes that had Δbeta below 1% (and a p-value>0.05) in the NINFEA sample had
268 the greatest distance from the transcriptional start site and the lowest difference in the mean
269 DNA methylation between cases and controls also in all public datasets (**Tables 3 and 4**).
270 As GSE40576 PBMCs dataset was accompanied with paired gene expression data
271 (GSE40732) we further examined whether differentially methylated sites impact *PM20D1*
272 expression in healthy children (**Supporting Information file,** Results, *Correlation of*
273 *differentially methylated sites with expression of PM20D1*). Although the *PM20D1* expression
274 level was not associated with persistent atopic asthma in this datatset (p-value=0.382), there
275 was a negative relationship between DNA methylation in all but one CpG site located within
276 the DMR and *PM20D1* expression, with Spearman's rho ranging from -0.014 to -0,126 (all p-
277 values>0.05, **Supporting Information file, Figure S2**).

278 **Discussion**

279 We assessed early life saliva genome-wide patterns of DNA methylation related to wheezing
280 between 6 and 18 months of age, and identified one DMR related to early childhood wheezing
281 located in the promoter-regulatory region of *PM20D1* gene. The observed associations were
282 particularly strong in children born to atopic mothers. Using a public dataset we also found
283 that DNA methylation of this region, measured at 10 corresponding probes in children's blood
284 and cord blood, was higher in respiratory allergy cases compared with controls, and that the
285 same pattern of the association, although lower in magnitude, was present when methylation
286 was measured in saliva samples of a subset of the same children. Another public dataset
287 confirmed the observed associations in nasal epithelium from 9-12 years old children with
288 atopic asthma, while the associations were weaker in PBMCs from a partially overlapping
289 sample from the same study.
290 Our study is based on DNA methylation measured in early infancy, an approach that differs
291 from most of the previous studies that measured methylation in cord blood or later in

292 childhood. Our exploratory epigenome-wide single site analysis showed no single CpG site
293 associated with early wheezing, which is consistent with the findings from the MeDALL
294 consortium, in which 14 CpG sites identified in the discovery study were replicated in
295 peripheral blood from children with school-age asthma, while none of the CpG sites was
296 replicated in analyses focused on early life, where DNA methylation was measured in cord
297 blood.[23] On the other hand, CpG sites and DMRs identified in cord blood of children with
298 school-age and adolescent asthma from the PACE consortium[24] do not include *PM20D1*,
299 which may reflect more heterogeneous outcome definition in the PACE consortium, which is
300 not strictly related to atopy. Our and the results from the PACE and MeDALL studies suggest
301 that early life regional, rather than single site, DNA methylation differences are more likely to
302 be associated with childhood atopic and asthmatic phenotypes. These findings also suggest
303 that epigenetic markers for wheezing and asthma tend to develop later in childhood as a
304 consequence of adverse postnatal exposures, or the disease itself, whose effects are not
305 present at birth and in early infancy.

306 The sample size of our study limited the possibility to detect small-magnitude effect sizes that
307 can have functional relevance for childhood and later adulthood health. In fact, the magnitude
308 of the associations for wheezing- and asthma-candidate CpGs identified in several previous
309 EWAS was also small.[22,23,31] As the power of our exploratory EWAS was relatively low, we
310 used a more powerful approach that allowed us to identify a wheezing-related differentially
311 methylated region located in *PM20D1* gene. This gene codes Peptidase M20 Domain
312 Containing 1 enzyme that regulates production of N-fatty-acyl amino acids, which are a large
313 family of more than 70 endogenous signalling molecules involved in pain and inflammation
314 regulation, and their metabolites are important mediators of chronic airway inflammation in
315 asthma.[32] Differential DNA methylation patterns at this gene have been previously associated
316 with adult respiratory allergy[33] and maternal asthma.[34] In the study of Langie et al.[33]

317 cg11965913 located in the promoter region of *PM20D1* was hypermethylated in adult
318 individuals with respiratory allergy compared with controls, with similar differential
319 methylation profiles in PBMCs and saliva. Furthermore, hypomethylation of *PM20D1* in
320 blood from 12-month old children was associated with maternal asthma and asthma
321 medication use, maternal atopic status and serum IgE level.[34] In the NINFEA study, on the
322 contrary, hypermethylation of *PM20D1* related to wheezing was much stronger in children
323 born to atopic compared with non-atopic mothers. Although apparently contrasting, findings
324 by Gunawardhana et al.[34] and our own findings both indicate that maternal atopy likely
325 modify offspring atopic phenotypes through alterations in *PM20D1* DNA methylation.

326 In our analyses we accounted for the most important determinants of childhood wheezing,
327 such as maternal age, smoking, child's siblings and day-care attendance. In particular, the
328 presence of older siblings and day-care attendance, the proxies of early childhood respiratory
329 tract infections and microbial exposure in general, were strongly positively associated with
330 wheezing in our sample, but did not confound the observed association.

331 We acknowledge that we did not perform a "real" replication analysis with a well-planned
332 replication group, and this results in important limitations. First, the phenotypes of the public
333 datasets were different from the one analysed in our sample. Respiratory allergy in three
334 public datasets covers several atopic conditions related to sensitization to airborne allergens,
335 including asthma and wheezing at 10 years of age, while atopic asthma in two other datasets
336 includes persistent asthma with impaired lung function and allergic sensitization. Although
337 our phenotype, namely wheezing in early childhood, is a heterogeneous condition, often
338 related to a relative narrowing of the airways during an acute viral infection,[35] it strongly
339 predicts later asthma development.[15] As all phenotypes in the public datasets have an atopic
340 characterization together with data on respiratory conditions, including wheezing and asthma,

341 we considered them as good candidates for replication of DNA methylation patterns related to
342 wheezing in early childhood. This is further supported by the fact that atopic phenotypes,
343 especially respiratory allergy and atopic asthma/wheezing, share multiple environmental and
344 genetic risk factors, as well as immunological features.[36]

345 Second, the age of the outcome assessment in our study was different compared with the
346 public datasets (18 months vs. 6-12 years). However, identification of wheezing-related
347 epigenetic markers in early childhood, especially if they overlap with markers of later asthma
348 or respiratory allergy, would allow identification of high-risk children, improve diagnostic
349 and therapeutic approaches, and would contribute to the understanding of the involvement of
350 epigenetics in asthma aetiology.

351 Albeit different traits analysed, the pattern of the association observed in **Figure 1** suggests
352 that DNA methylation differences related to preschool wheezing and school-age respiratory
353 allergy and atopic asthma is largely overlapping, which is not likely to be by chance. In fact,
354 consistently increased DNA methylation within the *PM20D1* region was observed in cases
355 compared with controls at different ages (at birth, 10 months in the NINFEA study, and 6-12
356 years in other four public datasets), as well as in different biological materials analysed
357 (CBMCs, saliva, nasal epithelium and PBMCs). Interestingly, very similar DNA methylation
358 differences related to childhood respiratory allergy were observed between cord blood and
359 blood at 11 years of age in the Belgian study, suggesting the stability of DNA methylation
360 patterns after birth. Although it has been shown that DNA methylation is influenced also by
361 postnatal environmental exposures, such as early microbial exposures (e.g. DNA methylation
362 of several asthma-candidate genes was strongly influenced by early farming exposure),[37] our
363 findings indicate that this is not a general rule. DNA methylation differences in *PM20D1*

364 related to atopic respiratory phenotypes seem to be determined already at birth and stable over
365 time, suggesting a rather weak impact of postnatal exposures.

366 One might question why *PM20D1* was not among the top genes identified by previous studies
367 which public datasets we used to replicate our findings.[19,20,31] However, this is not surprising,
368 as we based our analyses of public datasets on a candidate gene approach compared with the
369 epigenome-wide approach with multiple comparison adjustments reported by previous
370 studies.[19,20,31] On the other hand, it should be also noted that despite the smaller sample size,
371 the study by Langie et al.[31] and by Yang et al.[20] identified many more DMRs compared with
372 our study, and that these DMRs do not overlap with the one DMR identified in our study. One
373 possible explanation could be that the methylation-outcome associations were explored at
374 older ages in the previous two studies which public data was used in our study;[20,31] this
375 enhanced the possibility to identify a larger number of DMRs, which are also more likely to
376 be a result either of the disease itself or of the accumulating environmental exposures in the
377 first 12 years of life.

378 One of the limitations of our study is the fact that the time-sequence between DNA
379 methylation measurement and wheezing cannot be clearly established as the period of saliva
380 sample collection (median age 10.3 months) partially overlaps with the period of wheezing
381 assessment (6-18 months). Although the *PM20D1* DMR could also be a consequence of
382 wheezing disorders or postnatal environmental influences, similar pattern of association
383 observed in public cord blood dataset speaks against reverse causation and supports the role
384 of prenatal factors.

385 Findings from previous studies suggest that the associations observed between DNA
386 methylation and asthma are largely driven by eosinophils[22,23] that are known to be involved in
387 airway inflammation present in T-helper-2-type asthmatic phenotypes.[38] However, we used a

388 quite robust method for cell composition adjustment and we believe that the differences
389 observed in *PM20D1* are not likely to be driven by eosinophil count. This is further supported
390 by the findings from the publicly available datasets in which the associations were observed
391 in CBMCs and PBMCs that do not contain eosinophils.

392 The type of tissue used for DNA methylation measurements is the most innovative element of
393 our study. In the NINFEA study infant samples were collected using saliva sponges at
394 approximately 10 months of age. This non-invasive method of genomic DNA collection is
395 becoming an alternative to the blood DNA collection in large-scale studies, especially if
396 performed on infants and young children. However, there are two aspects that need to be
397 considered when using saliva as a material for epigenetic studies. First, saliva has a
398 heterogeneous cell composition that, in addition to leukocytes, contains a large proportion of
399 epithelial cells that might influence DNA methylation measurements. So far, saliva cell type
400 composition is poorly characterized, and several reference-free methods have been developed
401 to correct for cell type heterogeneity in EWAS.[28,29] Using simulated scenarios, a study of
402 McGregor et al.[29] found that surrogate variable analysis had the best performance; thus we
403 used this method to account for cell type composition, batch effects and technical residual
404 confounding. We, therefore, believe that the association observed in our study is not driven by
405 differential cell type composition. Second, blood and peripheral material, such as saliva,
406 buccal or nasal epithelium, may not be representative of DNA methylation status at central
407 organs involved in a disease pathophysiology. However, various studies identified asthma-
408 and wheezing-related epigenetic marks in several tissues, including nasal and buccal
409 epithelium, whole peripheral blood, cord blood, as well as in mononuclear cells from cord and
410 peripheral blood.[17-24,39] While blood may be valuable for studying common immunologic
411 processes, nasal and saliva epithelium, being probably closer to the methylation status of the
412 bronchial mucosa than blood or cord blood DNA methylation, are likely better surrogate

413 tissues for studies focused on local inflammation and environmental influences in asthma.
414 These two tissues are also easily accessible and appear to reflect pathological changes in the
415 airways during asthma development.[26]

416 In conclusion, we have shown that higher DNA methylation in the promoter-regulatory region
417 of *PM20D1* from infant saliva was associated with the occurrence of early childhood
418 wheezing, especially in children of atopic mothers. Directionally consistent epigenetic
419 alteration observed in cord blood of children with respiratory allergy suggests that this
420 modification might operate from birth, while the confirmation in blood and nasal epithelia of
421 school-aged children indicates possibly long-term changes. The *PM20D1* promoter region
422 was previously implicated in adult respiratory allergy, suggesting its potential role in
423 aetiology of atopic phenotypes, including asthma.

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Table 1. Characteristics of the study population

Characteristics	**Cases** N (%)[†] or Mean (SD[‡])	**Controls** (N, %)[†] or Mean (SD[‡])	p-value[§]
Total number	68 (50.0)	68 (50.0)	-
Maternal age (years)	35.3 (3.8)	34.1 (4.4)	0.10
Highly educated mothers[¶]	47 (70.2)	51 (75.0)	0.53
Mother born outside Italy	3 (4.4)	3 (4.4)	1.00
Maternal atopic dermatitis and/or allergic rhinitis	21 (31.8)	13 (19.1)	0.09
Maternal depression and/or anxiety	9 (13.6)	10 (14.7)	0.86
Genito-urinary infections during pregnancy	10 (15.6)	19 (28.4)	0.08
Respiratory infections during pregnancy[††]	22 (34.4)	18 (26.9)	0.35
≥1 sibling	27 (40.9)	15 (22.4)	0.02
Child's exposure to passive smoking	7 (10.3)	5 (7.4)	0.55
Day-care attendance	45 (66.2)	31 (45.6)	0.02
Child's eczema 0-18 months	10 (14.7)	13 (19.1)	0.49
Matching variables			
Sex			
Males	38 (55.9)	38 (55.9)	
Season of saliva sampling			
Autumn or winter	32 (47.1)	32 (47.1)	
Age at saliva sampling (months)[‡‡]	10.3 (9.3-11.7)	10.4 (9.4-	

[†] Total numbers may vary due to missing data
[‡] SD – Standard Deviation
[§] Chi-square test for categorical variables, unpaired t test or Wilcoxon-Mann-Whitney test for continuous variables
[¶] University degree or higher
[††] Otits and/or sinusitis and/or throat infections and/or bronchitis and/or influenza during pregnancy
[‡‡] Median (Interquartile Range – IQR)

Table 2. Differentially methylated region (DMR) associated with early childhood wheezing

Cluster number	Chromosome	Start Position	End Position	Delta β[†]	Number of probes	p-value	FWER[‡]	Nearest gene[§]
12362	1	205818484	205819609	0.077	10	0.006	0.028	*PM20D1*

[†] The average difference in DNA methylation between cases and controls
[‡] Family-wise error rate from 1000 bootstraps
[§] Genome coordinates are given with respect to hg19 genome assembly

Table 3. Associations of DNA methylation in 10 probes, located within the differentially methylated region in *PM20D1* with childhood wheezing, stratified by maternal history of atopic disorders, ordered by the base pair position.

Probe	Base pair position[†]	Genic location[‡]	NINFEA (N=136)		Maternal atopic disorders[††]			
					No (N=100)		Yes (N=34)	
			Delta β[§] (%)	p-value[¶]	Delta β[§] (%)	p-value[¶]	Delta β[§] (%)	p-value[¶]
cg07157834	205819609	TSS1500	0.77	0.587	0,99	0,524	1,02	0,724
cg24503407	205819492	TSS1500	4.25	0.047	2,95	0,226	7,77	0,060
cg07167872	205819463	TSS200	5.19	0.018	3,52	0,156	10,58	0,014
cg11965913	205819406	TSS200	8.93	0.009	5,73	0,149	18,70	0,005
cg14893161	205819251	5'UTR;1stExon	9.15	0.009	5,68	0,156	19,27	0,005
cg14159672	205819179	1stExon	9.58	0.009	6,61	0,116	18,07	0,012
cg26354017	205819088	1stExon	9.14	0.004	5,38	0,144	20,33	0,002
cg17178900	205818956	Body	6.36	0.006	4,06	0,129	13,98	0,003
cg06815965	205818668	Body	0.40	0.576	0,54	0,545	0,91	0,351
cg03461704	205818484	Body	0.15	0.809	-0,06	0,938	0,67	0,667

[†] Genome coordinates are given with respect to hg19 genome assembly
[‡] According to UCSC reference gene information
[§] The difference in the mean DNA methylation between cases and controls (%)
[¶] P-values based on a two-sided Student's t-test for the difference in the mean DNA methylation between cases and controls.
[††] Maternal history of allergic rhinits and/or atopic dermatitis

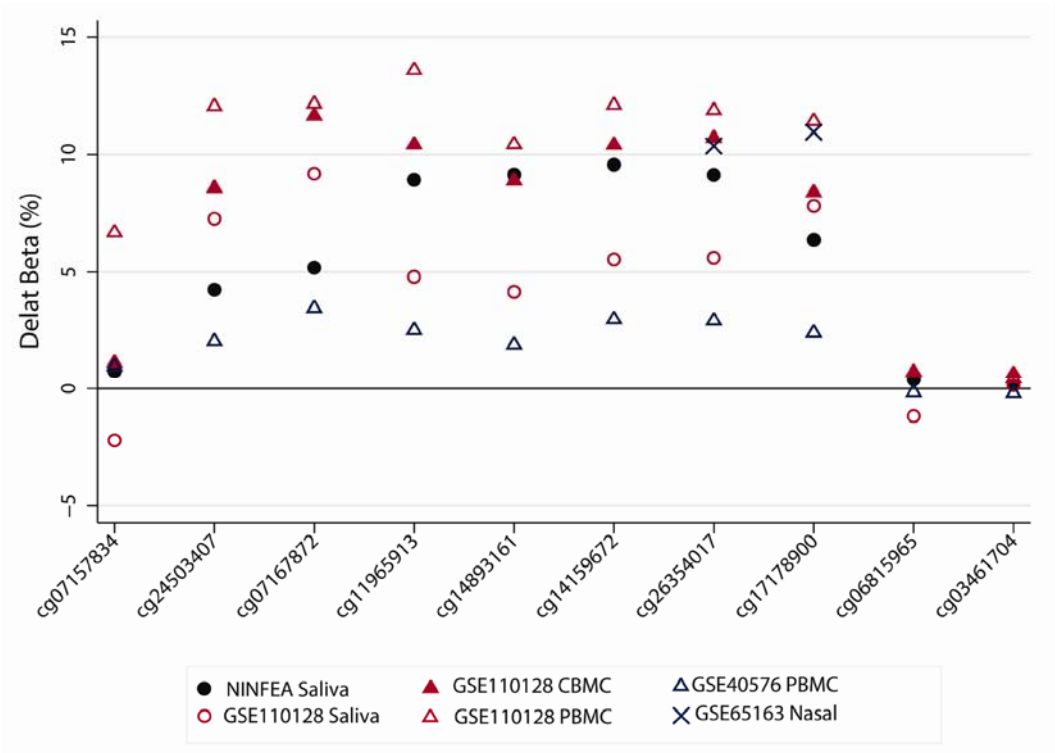
Table 4. Associations of DNA methylation in 10 probes, located within the differentially methylated region in *PM20D1*, with wheezing in the NINFEA sample and with childhood respiratory allergy/atopic asthma, in the five publicly available datasets, ordered by the base pair position.

Probe	NINFEA (wheezing)		GSE110128 (respiratory allergy)						GSE40576 (atopic asthma)		GSE65163 (atopic asthma)	
	Saliva 6-18 months (N=136)		Saliva 11 years (N=53)		Blood[§] 11 years (N=90)		Cord blood[¶] (N=90)		Blood[§] 6-12 years (N=194)		Nasal epithelia 9-12 years (N=72)	
	Delta β[†] (%)	p-value	Delta β[†] (%)	p-value[‡]	Delta β[†] (%)	p-value[‡]	Delta β[†] (%)	p-value[‡]	Delta β[†] (%)	p-value[‡]	Delta β[†] (%)	p-value[‡]
cg07157834	0.77	0.587	-2.22	0.761	6.69	0.004	1.14	0.074	0.98	0.181		*NA*
cg24503407	4.25	0.047	7.27	0.116	12.06	0.009	8.58	0.031	2.06	0.166		*NA*
cg07167872	5.19	0.018	9.19	0.066	12.17	0.005	11.66	0.006	3.47	0.062		*NA*
cg11965913	8.93	0.009	4.80	0.264	13.59	0.009	10.43	0.027	2.53	0.137		*NA*
cg14893161	9.15	0.009	4.16	0.276	10.44	0.024	8.89	0.037	1.92	0.157		*NA*
cg14159672	9.58	0.009	5.55	0.215	12.12	0.012	10.42	0.021	3.00	0.094		*NA*
cg26354017	9.14	0.004	5.61	0.214	11.89	0.012	10.71	0.020	2.94	0.109	10.36	0.005
cg17178900	6.36	0.006	7.81	0.130	11.43	0.019	8.39	0.044	2.42	0.149	10.96	0.001
cg06815965	0.40	0.576	-1.18	0.997	0.73	0.016	0.75	0.051	-0.15	0.771		*NA*
cg03461704	0.15	0.809	0.15	0.464	0.44	0.362	0.67	0.339	-0.19	0.610		*NA*

[†] The difference in the mean DNA methylation between cases and controls (%)
[‡] P-values based on a one-sided Student's t-test for the difference in the mean DNA methylation between cases and controls.
[§] Peripheral blood mononuclear cell (PBMC) fraction
[¶] Cord blood mononuclear cell (CBMC) fraction

Figure 1. Associations of DNA methylation at 10 CpGs from the *PM20D1* DMR with early childhood wheezing in the NINFEA sample and with childhood respiratory allergy and atopic asthma from the public datasets. The corresponding CpGs are reported on the x-axis, while the y-axis represents DNA methylation difference between wheezing/respiratory allergy/atopic asthma cases and controls in the NINFEA sample (full black circle), public saliva dataset (hollow red circle), public blood datasets (blue and red hollow triangles), public cord blood dataset (red full triangles) and public nasal epithelia dataset (blue Xs).

Figure 1
Associations of DNA methylation at 10 CpGs from the PM20D1 DMR with early childhood wheezing in the NINFEA sample and with childhood respiratory allergy and atopic asthma from the public data sets. The corresponding CpGs are reported on the x-axis, while the y-axis represents DNA methylation difference between wheezing/respiratory allergy/atopic asthma cases and controls in the NINFEA sample (full black circle), public saliva data set (hollow red circle), public blood data sets (blue and red hollow triangles), public cord blood data set (red full triangles) and public nasal epithelia data set (blue Xs)



Differentially methylated DNA regions in early childhood wheezing: an epigenome-wide study using saliva

SUPPORTING INFORMATION

Methods

DNA methylation measurement, data pre-processing, and quality control

DNA was extracted from 144 saliva sponge samples using an automated protocol and the Oragene™ Purifier Solution (DNA Genotek, Inc., Ottawa, Ontario, Canada). Genomic DNA (500 ng) was bisulphite-converted using the EZ DNA Methylation-Gold™ Kit (Zymo Research, CA, USA) following the manufacturer's protocol.

The methylation status of over 485,000 probes was measured using the Infinium HumanMethylation450 BeadChip array (Illumina, Inc., San Diego, CA). The matched pairs were placed on the same chip to minimize confounding by batch. Data pre-processing steps were performed in R statistical computing software version 3.4.1[1] using the *minfi* package.[2] DNA methylation for each probe was expressed as beta-value (ratio of methylated probe intensity to overall intensity, representing 0 to 100% methylation at each probe).

Three samples with more than 1% of the CpGs with detection p-value>0.01 together with their respective matched pairs were excluded from the study (N=6). An additional matched pair was excluded because of sex mismatch (N=2).

To minimize the unwanted variation within and between samples the preprocessQuantile function was used.[3] This function implements a stratified quantile normalisation procedure that is applied to the methylated and unmethylated signal intensities separately, and takes into account the different probe types.[3] This procedure does not apply background correction, but very small intensities close to zero are thresholded using the *minfi* function fixMethOutliers.[2]

We filtered out probes that failed in one or more samples based on detection p-value (detection p-value>0.01; N=10,902), probes located on the sex chromosomes (N=10,831), all probes containing a single-nucleotide polymorphism at the CpG interrogation and/or at the single nucleotide extension for any minor allele frequency (using the *minfi* function dropLociWithSnps,[2] N=15,654), and polymorphic CpG probes that have been demonstrated

to map to multiple places in the genome[4] (N= 26,343 probes). The final data set included 136 samples with DNA methylation measured in 421,782 probes.

Search of publicly available datasets

On November 15th 2018 we searched publicly available datasets containing epigenome-wide DNA methylation data and phenotypes related to early childhood wheezing. In particular, we used the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) using the following field query "child* [All Fields] AND ("asthma" [All Fields] OR wheez* [All Fields] OR "allergy" [All Fields] OR atop* [All Fields]) AND ("Methylation profiling by genome tiling array"[DataSet Type] AND "Homo sapiens"[Organism])". Of the two available datasets of childhood respiratory allergy and asthma (GSE110128 and GSE85228, respectively), in order to have comparable CpG sites we excluded the dataset GSE85228 because of the different DNA methylation measurement platform compared with our study. The remaining GSE110128 dataset consists of three subsets that include cord blood, and peripheral blood and saliva[5] DNA methylation measured at 11 years of age in children with respiratory allergy and respective controls.

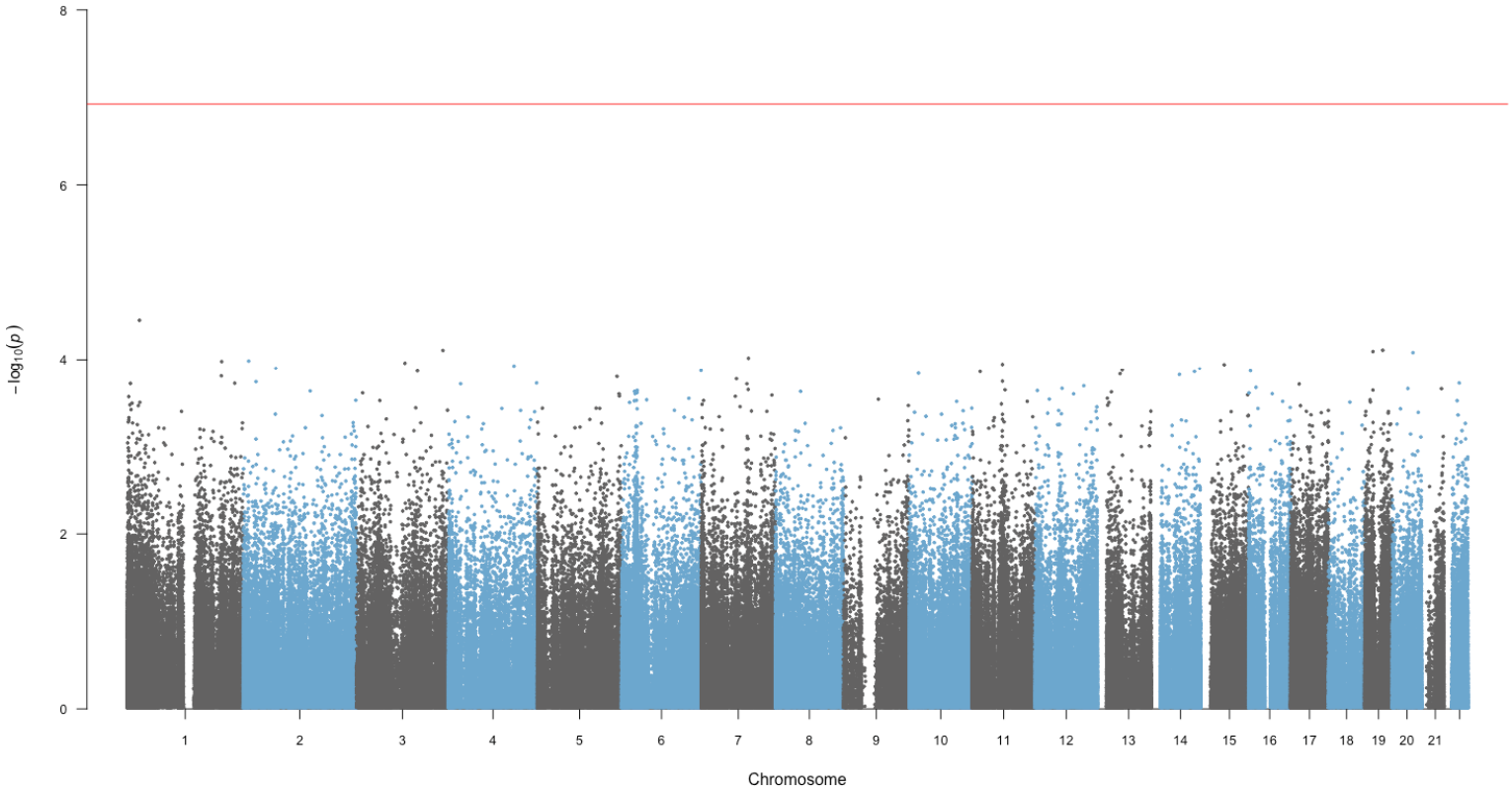
With further free text query ("(asthma OR wheez* OR allerg* OR atop*) AND "DNA methylation" AND child*") we identified additional three datasets based on Homo sapiens samples: two nasal epithelia datasets (one of childhood asthma GSE109446, and one of childhood atopic asthma GSE65163) and one peripheral blood dataset of childhood atopic asthma (GSE40576). The dataset GSE109446 was excluded from our analyses, as it is a sibling-control study,[6] which is particularly difficult to study in the context of DNA methylation.

Therefore, to test our main findings in independent samples we used five datasets: i) three GSE110128 datasets of European children (Belgium)[5] with childhood respiratory allergy at 10 years of age and their respective controls consisting of cord blood DNA methylation (N=90), and saliva (N=53) and peripheral blood (N=90) DNA methylation measured at 11 years of age, ii) one GSE65163 dataset (N=72) of African American children, with persistent

atopic asthma at 9-12 years of age and healthy controls, containing epigenome-wide DNA methylation measured from nasal epithelial cells,[7] and iii) GSE40576 dataset (N=194) of African American or Hispanic with Dominican/Haitian background children aged 6-12 years with persistent atopic asthma and healthy controls, with epigenome-wide DNA methylation measured from peripheral blood mononuclear cells (PBMCs).[8] Although not clearly specified in the original studies, the last two datasets include DNA methylation that was most likely measured at the same age as the outcome.[7,8]

Results

Figure S1. Manhattan plot of the early childhood wheezing EWAS analysis. The x-axis is the position of each CpG site by chromosome, and the y-axis is the negative log10 of the p-value for association of each CpG site with early childhood wheezing.The red line indicates the Bonferroni-corrected epigenome-wide treshold (p=1.19e-



07).

Table S1. Differentially methylated regions (DMRs) associated with early childhood wheezing at 5% in the methylation beta value difference between cases and controls

Cluster number	Chromosome	Start Position	End Position	Delta β [†]	Number of probes	p-value	FWER [‡]	Nearest gene [§]	Description	Distance [¶]
12362	1	205818484	205819609	0.077	10	0.006	0.028	PM20D1	Overlaps 5'	0
121572	6	30070074	30070109	-0.054	5	0.443	0.838	PPP1R11	Downstream	35142
57141	16	56659552	56659964	0.054	5	0.454	0.845	MT1M	Inside intron	8179
114918	5	102898223	102898729	0.052	5	0.521	0.885	NUDT12	Overlaps 5'	0

[†] Delta β - the average DNA methylation differences between cases and controls
[‡] Family-wise error rate from 1000 bootstraps
[§] Genome coordinates are given with respect to hg19 genome assembly
[d] The distance to the 5' end of the nearest transcript (base pairs)

Correlation of differentially methylated sites with expression of PM20D1

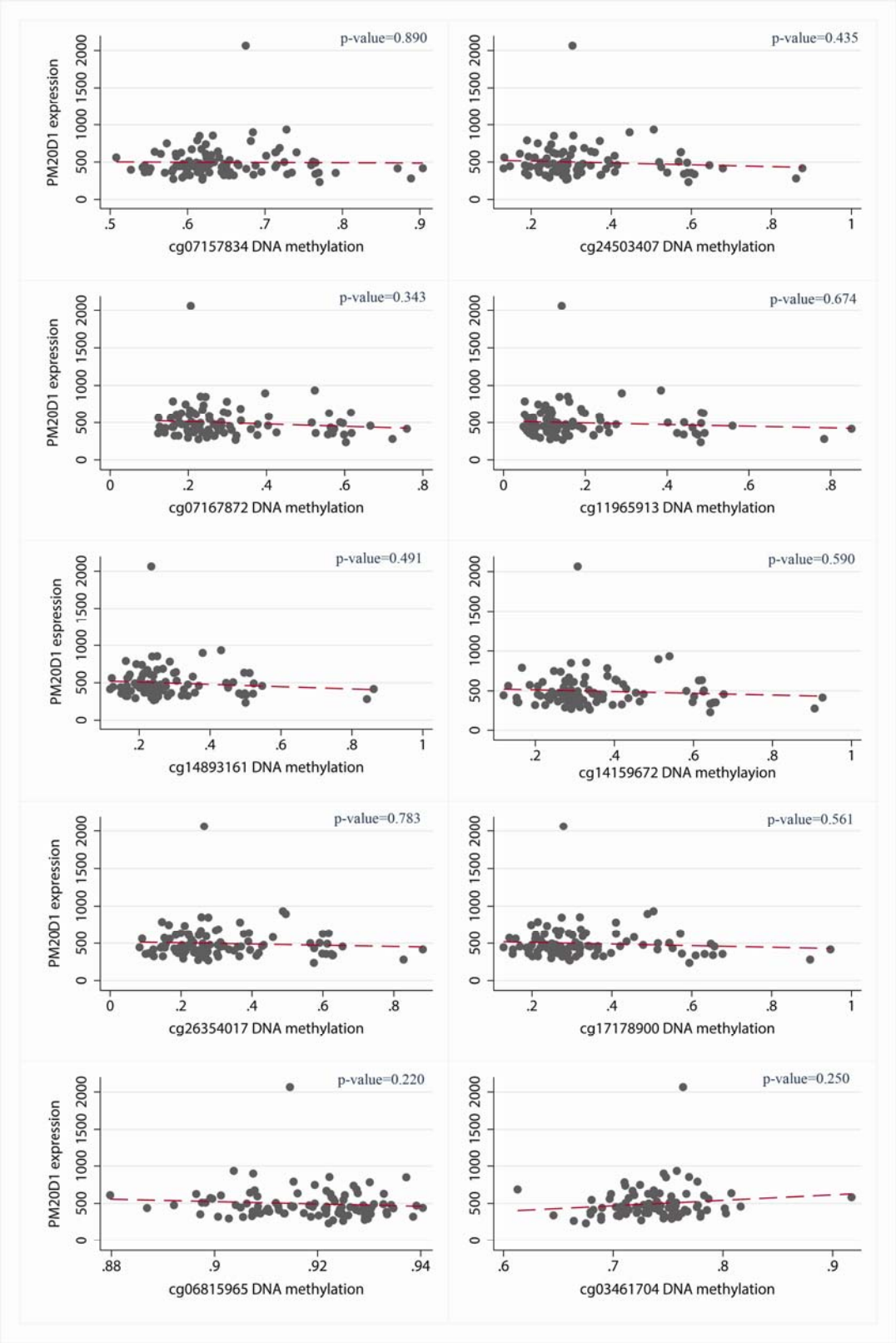
Two of the five public DNA methylation datasets that we used to replicate our differentially methylated region (GSE40576 and GSE65163) have publicly available paired gene expression data. The publicly available gene expression dataset (GSE65204), related to the GSE65163 DNA methylation data, do not contain the expression for the gene under study, and was thus not analyzed in our study.

The project "DNA Methylation Changes and Childhood Asthma in the Inner City" (SuperSeries GSE40736) includes paired DNA methylation and gene expression data from 6-12 years old children with atopic asthma (N=97) and healthy controls (N=97). DNA methylation GSE40576 dataset involves epigenome-wide DNA methylation measured in PBMCs using Infinium HumanMethylation450 BeadChip array, while GSE40732 dataset consists of gene expression data measured using Nimblegen Human Gene Expression array (12x135, Roche Nimblegen, Madison, WI).[7]

To explore to what extent DNA methylation influences *PM20D1* expression, we used only healthy controls (N=97) to relate DNA methylation at the identified region to the *PM20D1* expression using the Spearman correlation coefficients. In the same dataset we also tested the difference in the gene expression between atopic asthma and controls using Wilcoxon-Mann-Whitney test.

There was a negative relationship between DNA methylation in all but one CpG site located within the DMR and *PM20D1* expression levels, with Spearman's rho ranging from -0.014 to -0,126 (all p-values>0.05, **Figure S2**). Atopic status of children from the same dataset was not associated with the *PM20D1* expression levels (p-value=0.382).

Figure S2. Correlation between *PM20D1* expression and DNA methylation at 10 CpG sites overlapping the *PM20D1* DMR in healthy children (N=97) from the project "DNA Methylation Changes and Childhood Asthma in the Inner City" (SuperSeries GSE40736). Dashed lines indicate the best-fitting lines for the observed data.



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