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2 **TITLE PAGE**

3 Epigenome-wide contributions to individual differences in childhood phenotypes: A GREML
4 approach

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40 **ABSTRACT**

41 **Background:** DNA methylation is an epigenetic mechanism involved in human
42 development. Numerous epigenome-wide association studies (EWAS) have investigated
43 the associations of DNA methylation at single CpG sites with childhood outcomes.
44 However, the overall contribution of DNA methylation across the genome ($R^2_{Methylation}$)
45 towards childhood phenotypes is unknown. An estimate of $R^2_{Methylation}$ would provide context
46 regarding the importance of DNA methylation explaining variance in health outcomes.
47 **Methods:** We estimated the variance explained by epigenome-wide cord blood
48 methylation ($R^2_{Methylation}$) for five childhood phenotypes: gestational age, birth weight, and
49 body mass index (BMI), IQ and ADHD symptoms at school age. We adapted a genome-
50 based restricted maximum likelihood (GREML) approach with cross-validation (CV) to
51 DNA methylation data and applied it in two population-based birth cohorts: ALSPAC
52 (n=775) and Generation R (n=1382).
53 **Results:** Using information from >470,000 autosomal probes we estimated that DNA
54 methylation at birth explains 45% (SD_{CV} = 0.07) of gestational age variance and 16% (SD_{CV}
55 = 0.05) of birth weight variance. The $R^2_{Methylation}$ estimates for BMI, IQ and ADHD symptoms
56 at school age estimates were near 0% across almost all cross-validation iterations.
57 **Conclusions:** The results suggest that cord blood methylation explains a moderate to
58 large degree of variance in gestational age and birth weight, in line with the success of
59 previous EWAS in identifying numerous CpG sites associated with these phenotypes. In
60 contrast, we could not obtain a reliable estimate for school-age BMI, IQ and ADHD
61 symptoms. This may reflect a null bias due to insufficient sample size to detect variance
62 explained in more weakly associated phenotypes, although the true $R^2_{Methylation}$ for these
63 phenotypes is likely below that of gestational age and birth weight when using DNA
64 methylation at birth.

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65 **KEYWORDS**

66 DNA methylation; epigenetics; GREML; GCTA; child development; gestational age; birth
67 weight; BMI; ADHD; IQ

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68 # Background

69 DNA methylation (DNAm) is an epigenetic process, which involves the attachment of a
70 methyl group to cytosine bases, typically in the context of a cytosine-phosphate-guanine
71 dinucleotide (CpG) site. The methylation status of a CpG site can have an impact on gene
72 expression and downstream phenotypes (1). In turn, methylation levels are determined by
73 genetics, environment and stochastic processes (2,3). DNAm could therefore function as
74 mediator of many genetic and environmental determinants of human development,
75 functioning and pathology. A common research design to query the role of DNAm in these
76 processes is an epigenome-wide association study (EWAS). As a large number of CpG
77 sites are tested, to reliably identify relevant CpG sites, either large samples or big effect
78 sizes are required, which for most traits or CpG sites are not available or unlikely (4).

79 However, analogous to lessons learned from genome-wide association studies, no
80 matter the number of genome-wide significant CpGs identified in an EWAS, whether it be 0
81 or thousands, there is always a possibility that more CpGs are associated with a predictor
82 or outcome, but did not reach significance due to lack of power. Since an EWAS estimates
83 the associations of single CpG probes, no conclusions can be drawn about the overall
84 contribution of genome-wide DNAm towards a phenotype. Such an overall estimate of
85 variance explained by genome-wide DNAm ($R^2_{Methylation}$) would be highly informative for
86 several reasons: 1. $R^2_{Methylation}$ would provide a picture of how relevant DNAm levels are to
87 an outcome, either as causal determinant or predictor. 2. $R^2_{Methylation}$ would provide an upper
88 limit of how much the combined effects of CpG sites identified by an EWAS (e.g. poly-
89 epigenetic score) can explain. While estimates of $R^2_{Methylation}$ would be clearly useful, the
90 best approach to derive them is less clear. One option is to adapt the genomic restricted
91 maximum likelihood (GREML) (5) approach used in genetics.

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92 In genetics, the analogous measure of $R^2_{Methylation}$ is the single nucleotide
93 polymorphism heritability (SNP h^2), i.e. the variance explained by all measured SNPs. A
94 popular method to estimate SNP h^2 is through a GREML analysis which consists of two
95 steps: 1. The estimation of genetic relatedness values between participant pairs inferred
96 from their similarity in measured SNP genotypes. 2. Estimating how well genetic
97 relatedness predicts phenotypic similarity between participant pairs. While the GREML
98 approach has been developed for genetic data, the analysis can be applied to any high
99 dimensional data, such as genome-wide methylation data. First papers are now being
100 published using GREML and alternative methods to estimate the variance explained by
101 genome-wide DNAm. An early example is a study by Vazquez et al. (6), who used a
102 Bayesian variant of a GREML model to predict breast cancer survival. The authors found
103 that genome-wide DNAm is more predictive than the structural genome or traditional
104 covariates alone, explaining 16.2% of variance. More recently, Zhang et al. (7) tested the
105 validity of the GREML approach in methylation data using simulations and real data in a
106 sample of adults. The authors estimated that concurrent blood DNAm levels explained
107 6.5% of the variance in BMI but were not associated with height, when controlling for
108 genetic effects. In contrast, using a Bayesian approach not relying on similarity matrices,
109 Banos et al. (8) estimated the proportion of BMI variance explained by concurrent DNAm
110 to be 75.7% in adulthood. The CpG-level effects estimated by this model explained up to
111 30.8% in adult replications cohorts, but only 3.3%, 2.05% and 9.65% at birth, age 7 and
112 age 15 respectively, with BMI and DNAm measured at the same time-points. The results
113 suggest highly age specific effects depending on when both BMI and DNAm were
114 measured.
115 As previous studies focused on DNAm and outcomes in adults, the variance of
116 childhood outcomes explained by cord blood DNAm is unknown. In this study we aimed to

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117 use cord blood DNAm to estimate the $R^2_{Methylation}$ of five child outcomes, previously
118 addressed in EWAS studies: gestational age and birth weight, as well as BMI, IQ and
119 ADHD symptoms at school age. These outcomes were chosen because they represent
120 childhood outcomes in different areas (general health, cognition and psychopathology). In
121 addition, all of these have been studied in multi-center population-based EWAS before,
122 allowing for a comparison between $R^2_{Methylation}$ measures and EWAS findings. Two of the
123 phenotypes most robustly associated with DNAm in EWAS studies are gestational age and
124 birth weight. For gestational age, 8899 CpGs have been found to be significantly
125 associated in a previous EWAS at genome-wide significance (9). Prediction models based
126 on these CpGs were able to explain 50-80% of the gestational age variance in an
127 independent sample (10,11). In the case of birth weight, 914 sites were associated based
128 on an EWAS meta-analysis in 8,825 children (12). Cord blood has also the potential to
129 predict later development, e.g. nine CpG sites were associated with ADHD symptoms in
130 school-age according to a recent EWAS in 2,477 children (13) and one CpG site predicted
131 BMI in late childhood (n=4133) (14). In contrast, no genome-wide significant sites in cord
132 blood were identified for BMI in early childhood (14) nor IQ in school-age (n=3798) (15).
133 While the variance explained by specific sets of CpGs is known for some childhood
134 outcomes, the genome-wide contribution has not been studied before. The aim of this
135 study is to estimate the genome-wide contribution of cord blood DNA to various childhood
136 outcomes.

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137 # Methods

138 ## Participants

139 Participants for this study were drawn from two European population-based birth
140 cohorts: The ALSPAC Study and the Generation R study. ALSPAC had recruited 15,454
141 women with an expected delivery date between April 1991 and December 1992, who were
142 living in the former English county Avon, resulting in 15,589 foetuses. Of these 14,901
143 were alive at 1 year of age. The development of their children was subsequently studied at
144 multiple assessment waves. Cord blood DNAm was assessed for 1,018 children. To avoid
145 potential biases arising from shared family environment or population stratification, only
146 one sibling per family was included in the analyses sample, as well as only children whose
147 parents reported white ethnicity (analysis n=775). Full cohort descriptions have been
148 published previously (16,17). Please note that the study website contains details of all the
149 data that is available through a fully searchable data dictionary and variable search tool
150 (http://www.bristol.ac.uk/alspac/researchers/our-data/). Ethical approval for the study was
151 obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics
152 Committees. Consent for biological samples has been collected in accordance with the
153 Human Tissue Act (2004). Informed consent for the use of data collected via
154 questionnaires and clinics was obtained from participants following the recommendations
155 of the ALSPAC Ethics and Law Committee at the time.

156 Generation R invited all pregnant women living in the city of Rotterdam, the
157 Netherlands, with an expected delivery date between April 2002 and January 2006 to
158 participate in the study, of which 9,778 were enrolled. Cord blood DNA methylation was
159 assessed in a subgroup of 1396 children with parents of reported European national origin.

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160 After exclusion of siblings (one of each pair excluded), 1382 participants remained in the
161 analysis. Full study descriptions have been published previously (18), see also
162 https://generationr.nl/researchers/ for more information. All parents gave informed consent
163 for their children's participation. The Generation R Study is conducted in accordance with
164 the Declaration of Helsinki. Study protocols were approved by the Ethics Committee of
165 Erasmus MC.

166 Measures

167 DNA Methylation

168 DNAm was measured in cord blood at birth. Bisulfite conversion was performed with the
169 EZ-96 DNAm kit (shallow) (Zymo Research Corporation, Irvine, USA). DNAm levels were
170 then measured with the Illumina Infinium HumanMethylation450 BeadChip array (Illumina
171 Inc., San Diego, USA). Preprocessing in ALSPAC was performed with the meffil package
172 (19). Quality control check included mismatched genotypes, mismatched sex, incorrect
173 relatedness, low concordance with other time points, extreme dye bias, and poor probe
174 detection. In Generation R, pre-processing was performed with the CPACOR workflow
175 (20). Quality control exclusion criteria included failed bisulfite conversion, hybridization or
176 extension, sex mismatches and call rate <= 95%. Both cohorts were normalized using a
177 combined dataset, using meffil functional normalization with ten control probe principal
178 components and slide included as a random effect, see Mulder et al. (21) for further
179 details. To lessen the influence of methylation outliers while retaining a consistent sample
180 size, extreme values were winsorized. Per CpG site, DNAm levels exceeding three times
181 the interquartile range above the third or below the first quartile (3*IQR criterion) were
182 replaced by the maximum or minimum value, respectively, of the sample below the
183 exclusion criterion. Only autosomal probes were considered in this study for consistent

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184 interpretation of effects between sexes. This resulted in 470,870 and 473,864 CpG probes
185 in ALSPAC and Generation R, respectively, which were used for the computation of the
186 methylation similarity matrix.

187 Outcomes and covariates

188 Birth outcomes

189 In ALSPAC, birthweight was recorded by healthcare professionals at the time of
190 birth and extracted from birth records (12). Gestational age at delivery was also extracted
191 from birth records. Obstetric practice and antenatal care at the time means that for most
192 participants gestational age will have been estimated based on the last menstrual period,
193 supplemented by ultrasound scans and paediatric/obstetric assessment of the newborn at
194 birth.

195 In GenR, midwife and hospital registries were used to obtain information on birth
196 weight. Gestational age was based on ultrasound examinations for mothers who enrolled
197 in early or mid pregnancy, but based on last menstrual period for late pregnancy (22).

198 Childhood outcomes

199 In ALSPAC, measurements of height and weight, with the participant in light clothing
200 and without shoes, were obtained at clinic visits when the children were seven years of
201 age to calculate BMI. Non-verbal IQ at age 8 years was measured by the Wechsler
202 Intelligence Scale for Children WISC-III UK (23). ADHD symptomatology was assessed via
203 maternal ratings at age 7, with the Development and Well-Being Assessment interview
204 (DAWBA) (24).

205 In Generation R, when children were 6.0 (SD=0.15) years old, children’s height and
206 weight were measured at the research center without shoes or heavy clothing and used for

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207 the calculation of BMI (kg/m^2). Non-verbal IQ was assessed at the same age using the
208 Snijder-Oomen nonverbal intelligence test (25). ADHD symptoms were rated by a primary
209 caregiver (90% mothers) using the Conners' Parent Rating Scale-Revised (CPRS-R)
210 questionnaire at age 8.1 (SD=0.15) (26).

211 Covariates

212 In ALSPAC, mothers were asked about their smoking during pregnancy, and these
213 data were used to generate a binary variable of any smoking during pregnancy. Maternal
214 education was collapsed into whether they had achieved a university degree or not. Across
215 cohorts, white cell proportions were estimated with the Houseman method using the cord
216 blood specific Bakulski reference (27). In Generation R, maternal age was obtained at
217 enrollment. Maternal smoking was defined as either "Never smoked", "Quit smoking in
218 early pregnancy", "Continued smoking during pregnancy". Maternal education during
219 pregnancy was categorized as "no education", "primary education", "secondary education
220 first phase", "secondary education second phase", higher education first phase", higher
221 education second phase". See Table 1 for descriptive statistics of all variables.

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Table 1: Participant Characteristics

	ALSPAC		Generation R	
Characteristic	$n_{observed}$	Mean (SD)/ Proportion	$n_{observed}$	Mean (SD)/ Proportion
Girls	775	51.2%	1382	49.3%
Maternal Age in years	775	29.76 (4.4)	1382	31.7 (4.2)
Maternal Education				
Primary Education		79.1%	1362	1.9%
Secondary Education			1362	33.0%
Higher Education	775	20.9%	1362	65.1%
Smoking				
Continued smoking during pregnancy	767	12.5%	1378	13.4%
Quit smoking during pregnancy			1378	9.1%
Cell type composition				
CD8 T cells	775	8.9% (4.5%)	1382	13.1% (5.2%)
Natural killer cells	775	0.8% (1.8%)	1382	3.2% (2.9%)
CD4 T cells	775	17.8% (6.2%)	1382	16.1% (5.3%)
B cells	775	17.0% (4.4%)	1382	10.3% (2.8%)
Granulocytes	775	35.1% (9.7%)	1382	40.8% (10.7%)
Monocytes	775	1.3% (1.6%)	1382	9.2% (2.0%)
Nucleated red blood cells	775	19.9% (9.2%)	1382	11.8% (7.1%)
Outcomes				
Gestational Age in weeks	775	39.6 (1.5)	1382	40.1 (1.5)
Birth Weight in g	766	3490 (476)	1381	3545 (510)
BMI in kg/m^2	772	16.19 (2.0)	1183	15.9 (1.4)
ADHD	773	0.52 (0.90)	1060	7.5 (6.6)
IQ	747	102.6 (17.0)	1094	106.2 (14.3)

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222 **Statistical Analysis**

223 We adapted the GREML approach to estimate $R^2_{Methylation}$. The GREML procedure
224 consists of two steps: 1. Compute a genetic relatedness matrix (i.e. how genetically similar
225 two individuals are based on SNP data), 2. Regress the outcome similarity between
226 participants on the genetic relatedness (i.e. to establish whether greater genetic similarity
227 between individuals relates to greater phenotypic similarity).

228 We refer to a methylation similarity matrix (M) as opposed to a genetic relatedness
229 matrix (G). However, both M and G can be calculated with the same algorithm. First the
230 methylation in beta values were z-score standardized. The resulting matrix (X) of
231 methylation z-scores (columns: CpG sites, rows: participants) was then multiplied with the
232 transpose resulting in XX'. XX' was then standardized by dividing the matrix with the mean
233 of the diagonal, resulting in an average value of 1 for the diagonal of M. We used the R
234 package BGData 2.1.0 (28) to compute the similarity matrix.

235 The next step is to regress the outcomes on M and covariates using a mixed effects
236 model fitted with REML. Fixed effects covariates included several variables known to be
237 associated with DNAm levels: sex, maternal age, maternal smoking, maternal education,
238 cell type proportions, gestational age, birth weight (unless a variable was the outcome). M
239 and batch were defined as random effects.

240 The average of multiple imputations was used to avoid potential bias due to missing
241 data and to make analyses more comparable between outcomes by including the same
242 set of participants. We used the covariate and outcome variables to predict missing
243 variables in 100 imputations with 30 iterations using MICE (29) in R. Further analyses
244 were then performed using the average value across the imputations, or the most often
245 occurring category.

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246 According to power analyses with genetic data, to accurately estimate the variance
247 explained using GREML methods, large sample sizes are necessary. Especially with less
248 heritable traits sample sizes above 5,000 participants are recommended (30). Currently,
249 studies that have measured DNAm and child outcomes in more than 1000 participants are
250 rare. While the power requirements for DNAm data are unclear, there is nevertheless a
251 high risk of sampling variance, with results randomly changing heavily depending on a
252 particular sample composition. We attempted to reduce these risks by estimating $R^2_{Methylation}$
253 in two independent cohorts, as well as by performing cross-validation within cohorts.

254 Cross-validation (CV) was applied in the following way: 1. M was estimated across
255 all participants. 2. Eighty percent of the sample was randomly chosen as training sample
256 and the GREML model was fitted in this training sample. 3. Based on the results of the
257 training sample the best linear unbiased predictions (BLUP) were extracted for the test
258 sample. The BLUP estimates reflect the extent to which participants are predicted to have
259 above or below average outcome values, based on their similarity in genome-wide
260 methylation to other participants. 4. The outcome is predicted based on M and the
261 covariates 5. The predictions are correlated with the actual observed outcome and
262 squared to obtain the variance explained by the model. 6. The variance explained by a
263 covariate only model is subtracted to obtain the variance explained by DNAm beyond the
264 other tested variables ($\Delta R^2_{Methylation}$) 7. Step 1-6 are repeated to have results for 1000
265 random training-testing splits (Monte-Carlo cross-validation) 8. The mean estimate of
266 $\Delta R^2_{Methylation}$, with standard deviation across the cross-validation splits are extracted. 9.
267 Results of both cohorts are averaged, weighted by the inverse of the cross-validation
268 variance.

269 These analyses were run with the qgg package in R, which has implemented
270 GREML models with cross-validation (31). We wrote additional functions, which can be

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271 found in the omicsR2 package: https://github.com/aneumann-science/omicsR2. The
272 provided functions simplify the process of comparing the predictive performance of DNA
273 methylation compared to a covariates-only baseline model.

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274 # Results

275 DNAm explained 0 to 50% of the tested outcomes' variances. See Table 2 for full results
276 and Figure 1-4 for a graphical representation of the estimate distribution across cross-
277 validations.

278 Gestational age had the highest R^2 with 50.2% of the variance in gestational
279 variance explained by DNAm in cord blood independent of sex and batch. In a fully
280 adjusted model, 44.3% (SD_{CV} = 0.065) of variance was explained by DNAm. Notably, the
281 $\Delta R^2_{Methylation}$ was twice as large in GenR ($\Delta R^2_{Methylation}$ = 59.2%, SD_{CV} = 0.094) compared to
282 ALSPAC ($\Delta R^2_{Methylation}$ = 30.9%, SD_{CV} = 0.089). Across both cohorts 95% of cross-validation
283 results ranged from 16.3% to 70.4%, with 62.4% of values overlapping between the
284 Generation R minimum and ALSPAC maximum.

285 For birth weight, the variance explained was estimated at 15.9% (SD_{CV} = 0.051) with
286 basic adjustment and 12.2% (SD_{CV} = 0.038) with full covariate adjustment. Again, the
287 estimate was much larger in Generation R ($\Delta R^2_{Methylation}$ = 20.7%, SD_{CV} = 0.065) compared
288 to ALSPAC ($\Delta R^2_{Methylation}$ = 7.9%, SD_{CV} = 0.047). In the fully adjusted model, 95% of
289 estimates were between 0.8% and 31.4% and most cross-validation estimates overlapped
290 between these two cohorts (80.5%).

291 DNAm in cord blood did not explain variance in any of the childhood outcomes at
292 school age (BMI, ADHD and IQ). This result was consistent in both cohorts, in which all
293 cross-validation estimates were very close to 0, with the vast majority (97.5%) of estimates
294 being below 2% in both basic and fully adjusted model. Correspondingly, the cross-
295 validation standard deviations were below 0.1%, suggesting that no matter which

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296 participants were randomly assigned to training or validation, the estimated effect was
297 always near 0.

Table 2: Variance explained by genome-wide DNA methylation

		ALSPAC		Generation R		Pooled	
Outcome	Covariates	$\Delta R^2_{Methylation}$	SD_{CV}	$\Delta R^2_{Methylation}$	SD_{CV}	$\Delta R^2_{Methylation}$	SD_{CV}
Gestational Age	Basic	0.369 [0.169;0.556]	0.103	0.649 [0.367;0.795]	0.109	0.502 [0.184;0.783]	0.075
	Full	0.309 [0.137;0.475]	0.089	0.592 [0.352;0.719]	0.094	0.443 [0.163;0.704]	0.065
Birth Weight	Basic	0.109 [-0.016;0.241]	0.065	0.241 [0.073;0.398]	0.082	0.159 [0.004;0.375]	0.051
	Full	0.079 [0.000;0.179]	0.047	0.207 [0.077;0.334]	0.065	0.122 [0.008;0.314]	0.038
BMI	Basic	-0.005 [-0.052;0.022]	0.019	0.002 [-0.026;0.022]	0.011	0.000 [-0.040;0.022]	0.009
	Full	-0.005 [-0.037;0.008]	0.012	-0.001 [-0.010;0.002]	0.004	-0.001 [-0.026;0.004]	0.004
ADHD	Basic	0.000 [-0.025;0.026]	0.012	0.001 [-0.033;0.030]	0.014	0.000 [-0.030;0.029]	0.009
	Full	-0.001 [-0.024;0.012]	0.008	-0.001 [-0.020;0.010]	0.008	-0.001 [-0.022;0.010]	0.006
IQ	Basic	-0.001 [-0.037;0.020]	0.013	0.000 [-0.004;0.008]	0.005	0.000 [-0.024;0.018]	0.004
	Full	-0.001 [-0.014;0.002]	0.004	-0.001 [-0.006;0.002]	0.002	-0.001 [-0.010;0.002]	0.002

298 **Basic** sex and batch
299 **Full** sex, maternal age, maternal smoking, maternal education, cell type proportions, batch, gestational age*,
300 birth weight* (* not when outcome is gestational age or birth weight)
301 $\Delta R^2_{Methylation}$ Variance explained by genome-wide DNA methylation minus variance explained by covariates
302 [95% of values between lower;upper bound]
303 SD_{CV} Standard-deviation of cross-validation estimates

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304 # Discussion

305 This study is the first to report the extent to which childhood outcomes are
306 explained by cord blood genome-wide DNAm. We observed that methylation patterns
307 explained substantial variance for gestational age, moderate variance for birth weight and
308 no variance explained for prospective associations with BMI, IQ or ADHD symptoms at
309 school-age.

310 A strength of the study was the use of two cohorts, which are among the largest
311 samples of cord blood methylation currently available. Both cohorts are comparable in
312 many ways, for instance they represent populations of European ancestries living in
313 western European countries and similar outcome assessment ages. In addition, cord blood
314 DNAm assessment was very similar, as both cohorts used the same methylation array and
315 were normalized jointly.

316 The general trend of results regarding ranking from highest to lowest explained
317 outcomes agreed between the cohorts. The highest estimates across both cohorts were
318 found for gestational age, which is consistent with previous studies. Bohlin et al. tested a
319 prediction model based on 58-132 CpG sites in cord blood using similar covariates (sex,
320 maternal age, maternal smoking, cell composition) as in our study (10). The authors were
321 able to explain 50-65% of variance in a test sample of 685 participants from the MoBa
322 cohort. Since we modeled a much higher number of probes, we would expect at least
323 equal prediction performance in our study. The previous findings are consistent with the
324 Generation R estimate of 59% variance explained and suggests that adding more probes
325 from the Illumina 450k array would not increase performance of the prediction model.

326 However, the previous results are less consistent with the 31% estimate in
327 ALSPAC, indicating either a higher variability in lower powered samples or a potential bias

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328 towards null effects in lower sample sizes, as we will discuss later. Another contributor to
329 study heterogeneity may be the different methods used to estimate gestational age. Most
330 gestational age estimates in ALSPAC were based on the last reported menstrual period,
331 whereas in Generation R most estimates were based on ultrasound scans. The latter
332 method is expected to have less measurement error and thus higher variance explained
333 assuming constant methylation effects.

334 Genome-wide DNAm explained also explained variance in birth weight, albeit less
335 so than for gestational age. Interestingly, the estimate was again higher in GenR than
336 ALSPAC. In contrast to gestational age, there was no apparent noteworthy difference in
337 birth weight assessment, yet the estimates differed even more between cohorts than for
338 gestational age, so other potential causes for the observed study differences must be
339 discussed. One cause could be higher sampling variance in lower sample sizes. The
340 different estimates may hint that the $\Delta R^2_{Methylation}$ values at sample sizes of around 1000
341 samples or lower may be highly variable, with lower sample sizes more likely to over or
342 underestimate the true variance explained.

343 School-age outcomes showed a $\Delta R^2_{Methylation}$ near zero for BMI, IQ and ADHD
344 symptoms at age 6 in both cohorts. In contrast to gestational-age and birth weight, these
345 analyses present prospective associations over at least 6 years and have resulted in fewer
346 genome-wide significant findings in previous EWAS (13–15). This temporal component
347 together with perhaps lower contribution of DNAm may weaken associations and result in
348 lower variance explained estimates. While these factors lead to the expectation of a lower
349 variance explained estimate in prospective estimates as opposed to cross-sectional
350 analyses, estimates of 0% appear nevertheless unlikely. For instance, for ADHD, 9 CpG
351 sites have been identified in a meta-analysis, in which most participants were drawn from
352 ASLPAC and GenR (13). Both cohorts showed a high lambda in the EWAS, not accounted

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353 for by confounding, suggestive of a highly poly-epigenetic signal. Therefore, 0% variance
354 explained estimates in a subset of the data is implausible. Besides a true lower variance
355 explained for the school-age outcomes, a potential bias towards 0 values in underpowered
356 samples may be at play as well.

357 Assuming a high uncertainty of $\Delta R^2_{Methylation}$, we would expect a large standard
358 deviation in the cross-validation distribution, as some iterations will randomly show a
359 variance explained that is much too high or too low. However, in our study all analyses with
360 outcomes showing a 0% $\Delta R^2_{Methylation}$, had an estimate near 0% in almost all cross-
361 validation iterations. This resulted in very small cross-validation standard deviations, much
362 smaller compared to the gestational age or birth weight analysis. This is incompatible with
363 a high estimate uncertainty due to low sample size. Hence, we suspect that a bias towards
364 0 estimates is at play if outcomes, which are not very strongly associated with DNAm, are
365 analyzed in small samples. Such a behavior has been previously noted by GCTA author
366 Jian Yang in the context of GREML when applied to genetic data
367 (http://gcta.freeforums.net/thread/204/run-greml-analysis-small-sample). We therefore
368 speculate that the true $\Delta R^2_{Methylation}$ values for the school-age outcomes are likely to be
369 higher than 0% and below estimates found for gestational age and birth weight, which
370 themselves did not display a bias towards 0% estimates. Interestingly, early GCTA studies
371 indicated no SNP heritability for child psychiatric phenotypes (32), but later larger multi-
372 center GCTA (33), and LD-score regression studies (34) have since then repeatedly
373 demonstrated a SNP heritable component. Contrary to genetic studies, an additional
374 source of variability in DNA methylation is the assessment time point. Estimates for the
375 school-age outcomes are likely different for concurrent DNA methylation measures than
376 cord blood, but sample size was not sufficient for these analyses in the current study.

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377 A limitation of the current analyses is the coverage of the 450k methylation array.
378 The CpG sites measured by the array represent less than 2% of all CpG sites in the
379 genome. While neighboring CpG sites tend to be correlated, CpG sites may also represent
380 unmeasured CpG sites to a degree, but the correlations are not as stable or predictable as
381 correlations between single nucleotide polymorphisms in linkage disequilibrium. Thus, the
382 variance explained by array DNAm is unlikely the maximum which can be explained by all
383 DNAm variation in humans. That said, the estimates do in theory represent the maximum
384 that can be explained by the effects found in an EWAS using the same array, as it
385 represents the joint effect of all measured CpG sites.

386 This study adjusted for a number of potential confounders, such as maternal
387 smoking and education, as well as cell type proportions. Nevertheless, the observational
388 nature of the study design makes it unclear whether the strong association between
389 DNAm and gestational age represent direct effects of DNAm on gestational age, the
390 effects of gestational age on DNAm, or the effect of unmeasured confounding.
391 Furthermore, we only measured DNAm in a single tissue (cord blood). As DNAm can be
392 tissue-specific, other tissue may show higher associations with studied outcomes, e.g.
393 adipose tissue and body weight.

394 Despite the current limitations due to sample size, the results of the gestational age
395 analysis demonstrate that GREML methods are applicable to studies of DNA methylation.
396 We expect that increases in sample size will make this analytical approach more reliable
397 for outcomes less strongly associated with DNAm. An increase in sample size would also
398 allow for more complex questions to be answered. For example, as the method we utilized
399 enables one to fit multiple similarity matrices, it is in principle possible to estimate
400 $\Delta R^2_{Methylation}$ adjusted for genetic effects or to estimate the genome-wide interaction between
401 genetic and epigenetic effects. Answers to these questions would not only be helpful in

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402 further understanding of how DNAm relates to development and health, but would also
403 inform the design of future EWAS. For instance, EWAS might need to model interactions
404 between genetics and methylation levels, if interactions on a genome-wide level are
405 substantial (35).

406 In summary, we showed that genome-wide DNAm in cord blood explains almost
407 half of the variance in gestational age. DNAm was also associated to a lesser degree with
408 birth weight. DNAm at birth, however, did not explain variance in child BMI, IQ and ADHD
409 symptoms at school-age. The GREML approach holds promise for elucidating the
410 relationship between genome-wide DNAm, child development and health outcomes, but
411 increases in sample sizes are required to accurately estimate outcomes that are less
412 strongly associated with DNAm and to explore more complex models, which can integrate
413 different highly dimensional data.

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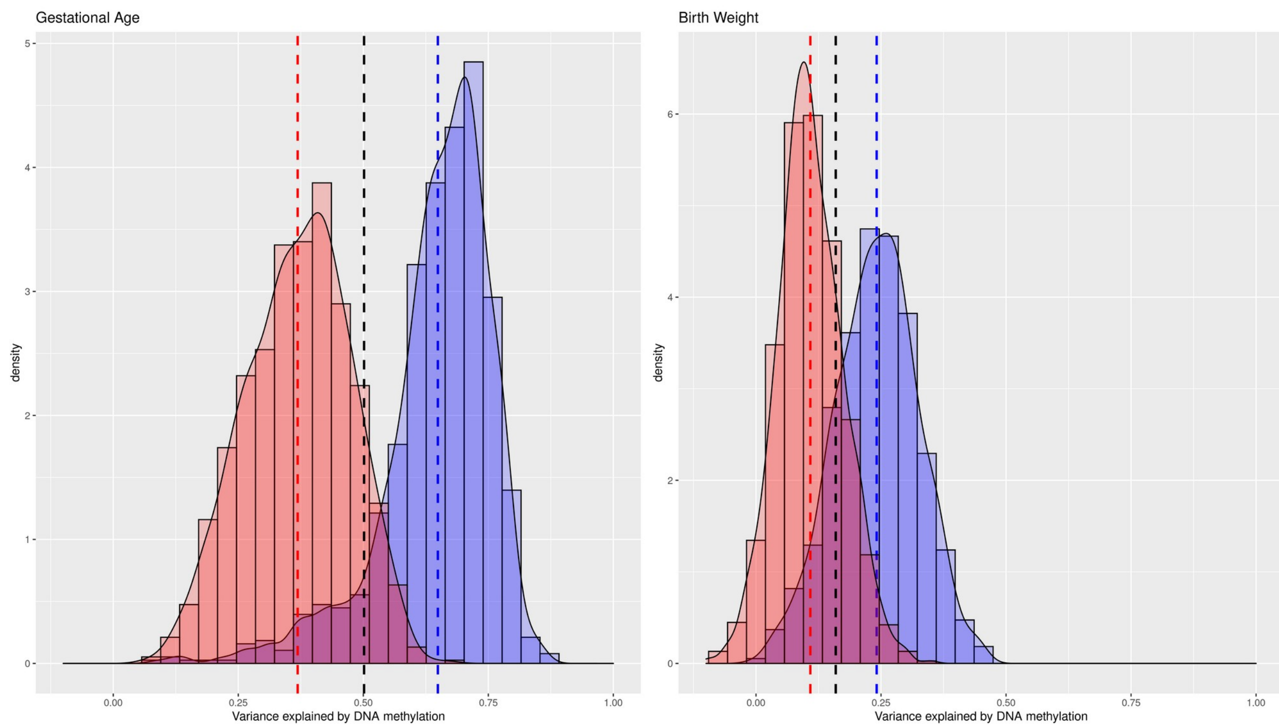
414 # Figures

415 **Figure 1: Variance explained in birth outcomes by cord blood DNA methylation**
416 **(basic adjustment).** Cross-validation distribution of $\Delta R^2_{Methylation}$, the variance explained by
417 genome-wide DNA methylation minus variance explained by covariates (sex and batch) in
418 ALSPAC (red) and Generation R (blue). Vertical lines indicate mean $\Delta R^2_{Methylation}$ in ALSPAC
419 (red), Generation R (blue) and a pooled estimate (black).



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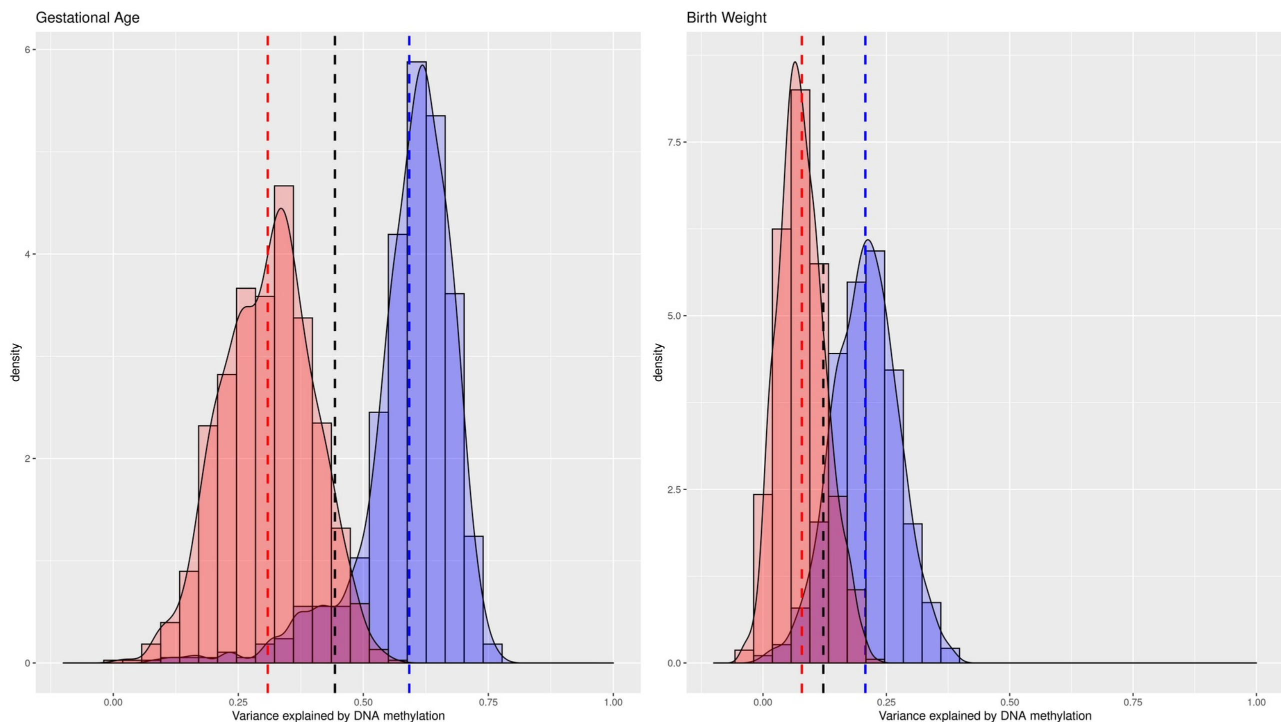
420 **Figure 2: Variance explained in birth outcomes by cord blood DNA methylation (full**
421 **adjustment).** Cross-validation distribution of $\Delta R^2_{Methylation,}$ the variance explained by
422 genome-wide DNA methylation minus variance explained by covariates (sex, maternal
423 age, maternal smoking, maternal education, cell type proportions, batch, gestational age*,
424 birth weight* (* not when outcome is gestational age or birth weight)) in ALSPAC (red) and
425 Generation R (blue). Vertical lines indicate mean $\Delta R^2_{Methylation}$ in ALSPAC (red), Generation
426 R (blue) and a pooled estimate (black).



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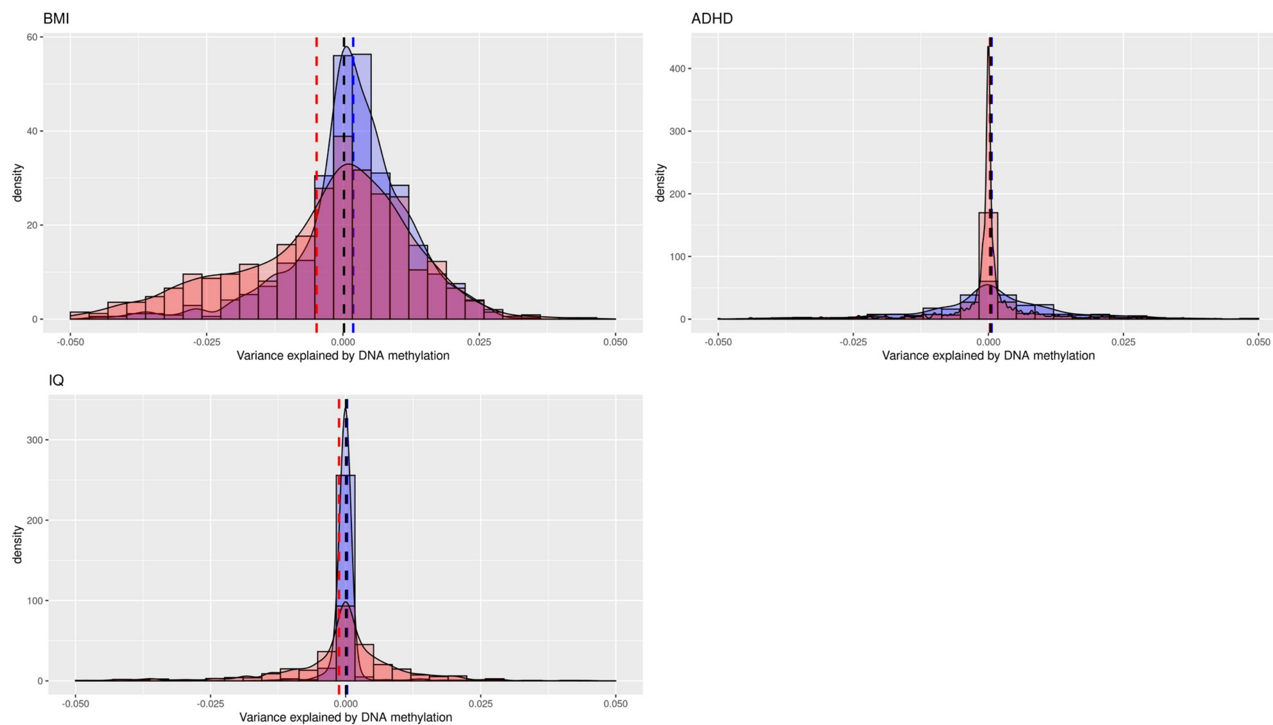
427 **Figure 3: Variance explained in childhood outcomes by cord blood DNA methylation**
428 **(basic adjustment).** Cross-validation distribution of $\Delta R^2_{Methylation}$, the variance explained by
429 genome-wide DNA methylation minus variance explained by covariates (sex and batch) in
430 ALSPAC (red) and Generation R (blue). Vertical lines indicate mean $\Delta R^2_{Methylation}$ in ALSPAC
431 (red), Generation R (blue) and a pooled estimate (black).



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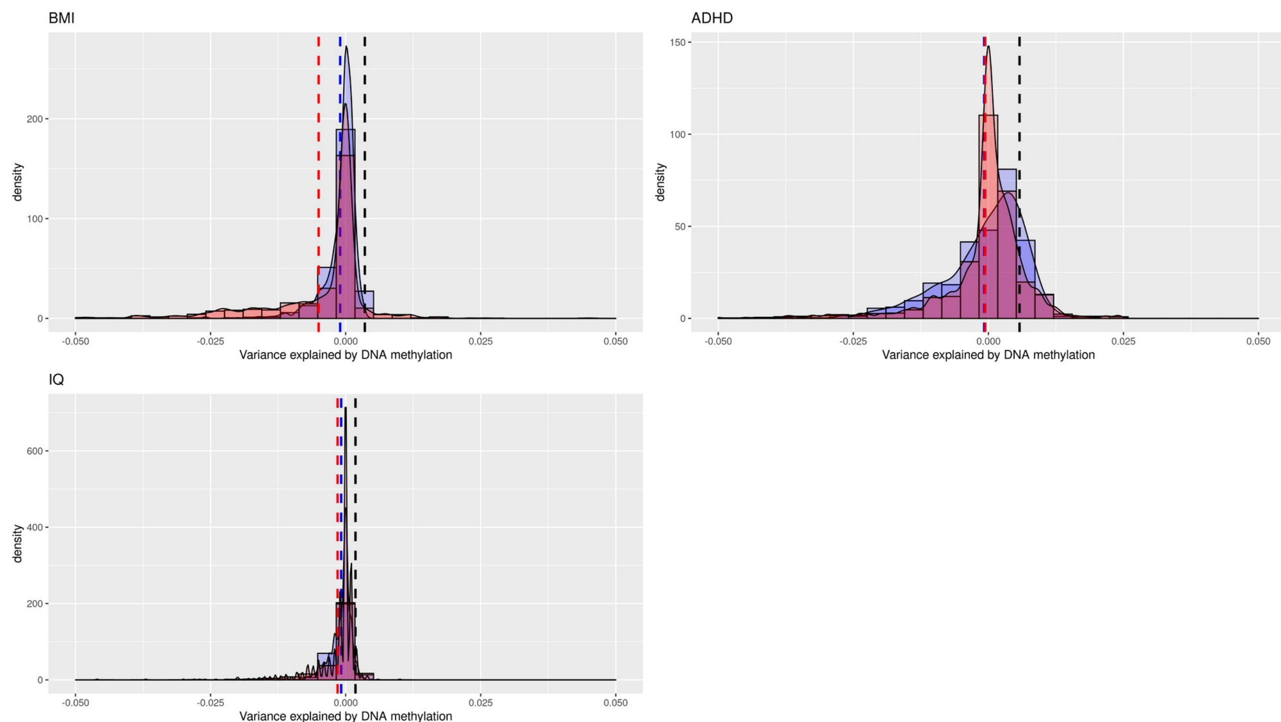
432 **Figure 4: Variance explained in childhood outcomes by cord blood DNA methylation**
433 **(full adjustment).** Cross-validation distribution of $\Delta R^2_{Methylation,}$ the variance explained by
434 genome-wide DNA methylation minus variance explained by covariates (sex, maternal
435 age, maternal smoking, maternal education, cell type proportions, batch, gestational age,
436 birth weight) in ALSPAC (red) and Generation R (blue). Vertical lines indicate mean
437 $\Delta R^2_{Methylation}$ in ALSPAC (red), Generation R (blue) and a pooled estimate (black).



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438 # List of abbreviations

439 Epigenome-wide association studies (EWAS)

440 Body mass index (BMI)

441 Cross-validation (CV)

442 DNA methylation (DNAm)

443 Methylation similarity matrix (M)

444 Genetic relatedness matrix (G)

445 # Declarations

446 ## Ethics approval and consent to participate

447 Ethical approval for the study was obtained from the ALSPAC Ethics and Law
448 Committee and the Local Research Ethics Committees. Consent for biological samples
449 has been collected in accordance with the Human Tissue Act (2004). Informed consent for
450 the use of data collected via questionnaires and clinics was obtained from participants
451 following the recommendations of the ALSPAC Ethics and Law Committee at the time.

452 All parents gave informed consent for their children's participation. The Generation
453 R Study is conducted in accordance with the Declaration of Helsinki. Study protocols were
454 approved by the Ethics Committee of Erasmus MC.

455 ## Consent for publication

456 Not applicable

457 ## Availability of data and materials

458 The datasets generated and analyzed during the current study are not publicly available to
459 ensure participant privacy and compliance with Dutch and UK law, but are available on

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460 reasonable request. For Generation R data, please contact management
461 (datamanagementgenr@erasmusmc.nl) and the corresponding author. For ALSPAC,
462 please see http://www.bristol.ac.uk/alspac/researchers/ for instructions on data access.

463 Competing interests

464 The authors declare that they have no competing interests

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482 findings. www.closer.ac.uk.

483 GENR

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505 Contributions

506 AN, EW and CC developed the study design and drafted the manuscript. AN and EW
507 performed statistical analysis on the GenR and ALSPAC data respectively and wrote the
508 omicsR2 package. EW and CC supervised the study and share senior authorship. JFF,

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509 JBP and HT advised on research design and statistical analysis. JFF manages the DNA
510 methylation data in GenR. VWJD is GenR director and oversaw data collection. All authors
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