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Maternal haemoglobin levels in pregnancy and child DNA methylation: a study in the pregnancy and childhood epigenetics consortium

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ABSTRACT

Altered maternal haemoglobin levels during pregnancy are associated with pre-clinical and clinical conditions affecting the fetus. Evidence from animal models suggests that these associations may be partially explained by differential DNA methylation in the newborn with possible long-term consequences. To test this in humans, we meta-analyzed the epigenome-wide associations of maternal haemoglobin levels during pregnancy with offspring DNA methylation in 3,967 newborn cord blood and 1,534 children and 1,962 adolescent whole-blood samples derived from 10 cohorts. DNA methylation was measured using Illumina Infinium Methylation 450K or MethylationEPIC arrays covering 450,000 and 850,000 methylation sites, respectively. There was no statistical support for the association of maternal haemoglobin levels with offspring DNA methylation either at individual methylation sites or clustered in regions. For most participants, maternal haemoglobin levels were within the normal range in the current study, whereas adverse perinatal outcomes often arise at the extremes. Thus, this study does not rule out the possibility that associations with offspring DNA methylation might be seen in studies with more extreme maternal haemoglobin levels.

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Supplemental data for this article can be accessed here.

Background

Maternal haemoglobin is routinely monitored throughout pregnancy as altered haemoglobin levels have been associated with adverse perinatal outcomes such as preterm delivery and intrauterine growth restriction [1–6]. Low maternal haemoglobin is estimated to affect 38% of all pregnancies worldwide translating to 32 million mothers annually [7]. During pregnancy, maternal haemoglobin levels normally decrease until about 20 weeks of gestation, mainly due to dilution because of an increase in plasma volume. Haemoglobin levels then rise to around 30 weeks of gestation due to increased red blood cell production; thereafter, they remain relatively stable [1]. Low maternal haemoglobin levels may relate to insufficient oxygen and/or nutrient delivery to the fetus, whilst high levels may indicate incomplete haemodilution resulting in high blood viscosity which may lead to fetal hypoxia due to impairment of maternal-fetal exchange [8].

A potential mechanism underlying the associations between maternal haemoglobin levels and adverse perinatal outcomes could include DNA methylation [9,10]. Methylation at cytosine-guanine dinucleotides (CpGs) in the DNA is the most widely studied epigenetic modification and its genome-wide pattern is highly determined during intrauterine development, partly due to environmental factors [11]. DNA methylation has been suggested as a mechanism underlying known associations of early-life exposures with later-life health outcomes. While associations of a number of maternal pregnancy characteristics and outcomes, including maternal BMI [12], maternal smoking [13], hypertensive disorders of pregnancy [14], gestational age [15] and childbirth weight [16], with offspring DNA methylation have been explored, it is unknown if maternal haemoglobin levels are associated with offspring DNA methylation.

Thus, in this epigenome-wide association study (EWAS), we meta-analysed harmonized cohort-specific associations between maternal haemoglobin level and DNA methylation in the offspring at birth, in childhood, andadolescence, using data from 10 studies in the Pregnancy And Childhood Epigenetics (PACE) Consortium.

Material and methods

Participating cohorts

Ten studies participated in the current meta-analyses. Details of cohort-level characteristics and methods are shown in the Supplementary Methods. We included seven cohorts in the meta-analysis of maternal haemoglobin levels and newborn (cord blood) DNA methylation: the Avon Longitudinal Study of Parents and Children (ALSPAC [17,18]), the Mother-child Cohort on the Prenatal and Early Postnatal Determinants of Child Health and Development (EDEN [19]), the Finnish Gestational Diabetes Study (FinnGeDi [20,21]), the Generation R Study (Generation R [22]), the Environment and Childhood Project (INMA [23]), the Prediction and Prevention of Preeclampsia and Intrauterine Growth Restriction Study (PREDO [24]), and the Programming Research in Obesity, Growth Environment, and Social Stress Study (PROGRESS [25,26]). Five cohorts participated in the meta-analysis of maternal haemoglobin and childhood (cohort mean age 4–7 years) DNA methylation: ALSPAC, EDEN, Generation R, INMA, and the Postpartum Outcomes in Women with Gestational Diabetes and Their Offspring Study (POGO [27]) and three cohorts in the meta-analysis of maternal haemoglobin and adolescent (cohort mean age 16–17 years) DNA methylation: ALSPAC, the Northern Finland Birth Cohort 1986 (NFBC1986 [28]) and the Raine Study [29]. All cohorts acquired ethics approval and informed consent from participants.

Maternal haemoglobin level during pregnancy

Where studies had more than one pregnancy maternal haemoglobin level, the value assessed at the oldest gestational age was used because, in previous studies, extreme maternal haemoglobin level at late pregnancy was more often associated

with adverse pregnancy outcomes (reviewed in [30]). Measurement methods and units varied between cohorts (see Supplementary Methods) so standardized maternal haemoglobin (i.e., each cohort centered and scaled the variable using their own study-specific mean and standard deviation = (maternal haemoglobin – mean (maternal haemoglobin)) / SD (maternal haemoglobin)) was used. Observations more than five standard deviations from the mean were excluded as outliers. According to this threshold, one observation was excluded from Generation R newborn analysis and one from ALSPAC childhood analysis.

DNA methylation data and quality control

DNA from cord or offspring peripheral blood underwent bisulphite conversion using the EZ-96 DNA Methylation kit (Zymo Research Corporation, Irvine, USA). DNA methylation was measured either using the Infinium Human Methylation 450 K Bead Chip or the Methylation EPIC Bead Chip platform (Illumina, San Diego, USA). Cohorts performed quality control and normalization using their own-preferred method, indicated in Supplementary Methods. Untransformed beta values representing the level of methylation and ranging from 0 to 1 were used in all analyses. We excluded DNA methylation values below the 25th percentile minus 3 times the interquartile range (IQR) and values above the 75th percentile plus 3 time IQR.

Cohort-specific statistical analyses

The association of maternal haemoglobin and offspring DNA methylation was analysed using robust linear regression separately for each methylation probe. Robust regression with White's covariance matrix estimator for calculating standard errors was chosen because of possible heteroscedasticity in the DNA methylation beta values [31]. Association analyses were performed in the following age categories: newborns (cord blood), children (age 4–7 years), and adolescents (age 16–17 years). Cohort-specific analyses were performed using *the rlm* function in *the MASS* package [32] for R [33]. P-values and standard errors were estimated using *coeftest* function with the function *vcovHC* from *sandwich* package [34,35] for White's type of covariance matrix. Newborn and childhood initial models were adjusted for gestational week at maternal haemoglobin measurement, child sex, DNA methylation batch, and white blood cells estimated with a Bakulski et al. reference panel [36] for newborn samples and with a Houseman et al. reference panel [37] for childhood and adolescent samples provided by the *minfi* package [38] for R [33]. Main analyses further adjusted for maternal parity, education, and smoking and gestational age at birth, and child age at the time of DNA methylation measurement (in the analyses of child and adolescent DNA methylation). Gestational age at maternal hemoglobin measurement was not available in the Raine Study and only for a subsample in NFBC1986 so this covariate was not included in the adolescent models. One case–control study (FinnGeDi study) was included in the newborn meta-analysis and for this, also selection factor (control vs. gestational diabetes case) was included in the models to account for the design. Each cohort used their own categorization for maternal education. Parity was defined as a dichotomous variable (nulliparity/multiparity) and maternal smoking as a three-level categorical variable (never smoked/stopped in early pregnancy/smoked throughout pregnancy). The FinnGeDi study only included non-smokers and therefore did not adjust for smoking. Only six women in PROGRESS reported smoking during pregnancy and were removed from the analysis. Non-smoking vs. smoking environment was included instead in the PROGRESS analysis. Cohort characteristics are presented in Table 1 and detailed information of all variables is summarized in Supplementary Table 1 and the Supplementary Methods.

Meta-analyses

Cohort-specific results were meta-analysed with METAL [39], using inverse-variance weighting. Multiple testing was accounted for using the Bonferroni correction with 0.05/number of analysed CpG sites as P-value cut off for statistical significance. Bonferroni-corrected P-values were considered as the primary indicators for statistical

Table 1. Characteristics of the cohorts involved in the meta-analyses. N, sample size; SD, standard deviation from mean; mHb, maternal haemoglobin; GA, gestational age; DNAm, DNA methylation; NA, not available.

Life-stage	Cohort	N	Females, %	mHb, g/L mean (SD)	GA at mHb, weeks mean (SD)	GA at birth, weeks mean (SD)	Child age at DNAm, years mean (SD)
Newborn Cord blood	ALSPAC	688	52.3	124.5 (9.0)	9.7 (2.4)	39.6 (1.5)	0
	EDEN	123	41.5	119.3 (10.5)	27.2 (1.1)	39.4 (1.5)	0
	FinnGeDi	484	51.4	123.8 (9.6)	36.6 (3.0)	39.9 (1.3)	0
	Generation R	1,205	49.5	124.6 (8.7)	14.9 (3.7)	40.2 (1.5)	0
	INMA	363	49.0	115.1 (9.9)	32.2 (4.3)	39.8 (1.3)	0
	Predo	709	47.7	121 (12.7)	30.3 (7.6)	39.8 (1.6)	0
	PROGRESS	395	45.6	128.2 (9.3)	31.6 (1.0)	38.5 (1.5)	0
Childhood 4 to 7 years	ALSPAC	749	51.3	124.4 (8.9)	9.7 (2.4)	39.6 (1.5)	7.4 (0.1)
	EDEN	121	41.3	119.1 (10.5)	27.2 (1.1)	39.4 (1.5)	5.7 (0.1)
	Generation R	429	53.4	124.2 (8.7)	14.8 (3.7)	40.2 (1.6)	6.0 (0.4)
	INMA	185	48.1	115.0 (10.1)	32.6 (3.7)	39.9 (1.3)	4.4 (0.2)
	POGO	71	49.3	123.8 (11.1)	34.7 (4.9)	38.5 (2.0)	7.6 (3.0)
Adolescence 16 to 17 years	ALSPAC	750	52.4	124.6 (8.8)	9.7 (2.4)	39.6 (1.5)	17.1 (1.0)
	NFBC1986	451	61.9	131.4 (10.2)	10.7 (2.9)	40.1 (1.3)	16.1 (0.4)
	Raine Study	761	49.3	122.8 (9.0)	NA	39.6 (1.7)	17.1 (0.3)

significance, but less stringent false discovery rate (FDR)-adjusted P-values [40] with 0.05 as cut off for statistical significance were also reported for comparison. Cross-reactive probes [41,42], as well as probes for which results from only one study, were available, the sample size was below 20 and those mapped to X or Y chromosomes were excluded from the meta-analyses and the subsequent analyses. Polymorphic CpG sites, i.e., sites located near genetic variants were flagged in the results because the adjacent variant might affect the methylation status of the CpG site [41]. The meta-analyses were conducted by two research groups independently and the results were compared.

Differentially methylated regions

Differentially methylated regions were analysed with *comb-p* [43] and *DMRcate* [44]. In short, *comb-p* use methylation probes' P-values to define differentially methylated regions. Regional P-values are calculated first using the Stouffer-Liptak-Kechris correction that accounts for autocorrelation and then adjusted for multiple testing with a one-step Šidák correction. *DMRcate* analysis was performed using the t-statistics from meta-analysis results as input. The program applies Gaussian kernel smoothing for t-statistics using a bandwidth lambda. P-values for regions are calculated based on the Satterthwaite method and corrected with FDR. Parameter settings for *DMRcate* and *comb-p* were chosen according to the results presented in [45]. In this paper, Mallik et al. evaluated power, precision, area under the precision-recall curve (AuPR), Matthews correlation coefficient, F1 score, and type I error rate from four different DMR analysis methods, including DMRcate and comb-p. Settings for best performance were defined as the parameters yielding the highest AuPR value and were set for *comb-p* as seed = 0.05, dist = 750, and for *DMRcate* as lambda = 500, C = 5. Differentially methylated regions that were identified with both programs, were accepted to be significant. The partial overlap between regions identified by both programs was accepted.

Study heterogeneity

Inter-study heterogeneity (I^2) statistic was used to assess between-study heterogeneity of the associations between maternal hemoglobin and offspring DNA methylation. I^2 represents the percentage of total variation across studies due

to heterogeneity. I^2 value of 50% or above indicated high heterogeneity.

Sensitivity analyses

Given the influence of gestational age on maternal haemoglobin levels and the variation in gestational age at which blood for maternal haemoglobin was collected, we also repeated the meta-analyses in two subgroups: those with maternal haemoglobin measured at early pregnancy and those who had it measured at late pregnancy. Of the seven studies contributing to the analysis of newborn DNA methylation, two studies, reflecting 48% (1,893/3,967) of participants, assessed maternal haemoglobin levels at a mean gestational age of 15 weeks or less and five studies, reflecting 52% (2,074/3,967) of participants, assessed maternal haemoglobin levels at a mean gestational age of 27 weeks or more (Table 1). For childhood DNA methylation, two of five studies (77% of the participants, 1,178/1,534) assessed the levels at 15 weeks or less and three of them (23% participants, 356/1,534) assessed maternal haemoglobin levels at 27 weeks or more. For the analyses with childhood DNA methylation, numbers for late pregnancy maternal haemoglobin were too small for subgroup analyses, and for the analysis with adolescent DNA methylation, only two studies had the information about gestational age at maternal haemoglobin measurement, and both had mean maternal haemoglobin measurement at 10 weeks of gestation.

Results

Study characteristics

Total sample sizes were 3,967 for the newborn analyses, 1,534 for childhood analyses, and 1,962 for adolescent analyses. Cohort-specific study characteristics are presented in Table 1. Detailed information on all characteristics used in the models is shown in Supplementary Table 1.

Epigenome-wide association studies

Table 2 shows a summary of cohort-specific EWAS results. The newborn and childhood

Table 2. Summary of cohort-specific and meta-analysis results for offspring EWAS on maternal haemoglobin during pregnancy. N, sample size; hits, statistically significant CpG sites after Bonferroni correction; probe N, number of CpG sites analysed.

			Initial model [1]			Main model [2]		
Life-stage	Cohort	N	Lambda	Hits	Probe N	Lambda	Hits	Probe N
Newborn Cord blood	ALSPAC	688	0.96	0	468,622	0.96	0	468,622
	EDEN	123	1.68	33	439,306	1.59	21	439,306
	FinnGeDi	484	1.06	0	687,640	1.01	0	687,640
	Generation R	1,205	1.04	0	450,068	1.03	0	450,116
	INMA	363	1.57	0	465,930	1.62	0	465,930
	Predo	709	0.88	0	428,619	0.88	0	428,603
	PROGRES	395	1.44	1	846,258	1.49	2	846,257
	Meta-analysis	**3,967**	**1.24**	**0**	**737,758**	**1.24**	**0**	**738,318**
Childhood 4 to 7 years	ALSPAC	749	1.06	1	471,078	1.07	0	471,078
	EDEN	121	1.83	62	439,306	1.73	47	439,306
	Generation R	429	1.02	0	457,863	1.01	0	457,866
	INMA	185	0.74	0	465,930	0.80	0	465,929
	POGO	50	0.84	0	845,824	0.84	0	845,699
	Meta-analysis	**1,534**	**1.12**	**1**	**424,780**	**1.16**	**1**	**425,188**
Adolescence 16 to 17 years	ALSPAC	750	1.10	0	470,334	1.10	0	470,334
	NFBC1986	451	0.83	0	466,289	1.28	0	466,284
	Raine Study	761	0.82	0	462,927	0.85	0	462,927
	Meta-analysis	**1,962**	**0.98**	**0**	**418,039**	**0.98**	**0**	**418,438**

[1] Initial model for newborn and childhood data is adjusted for gestational week at haemoglobin measurement, child sex, DNA methylation batch, selection factor in the case of randomized controlled trial and white blood cell estimates. Adolescence model is initial model without adjustment for gestational week at maternal haemoglobin measurement.

[2] Main model for newborn and childhood data is initial model adjusted for maternal parity, maternal education, maternal smoking, gestational age at birth and child age at the time of DNA measurement. Adolescence model is main model without adjustment for gestational week at maternal haemoglobin measurement.

models in the individual studies showed minimal inflation of associations with low P-value under the global null hypothesis (meta-analysis lambdas 1.24 and 1.16, respectively), whereas in the adolescent analyses there was little evidence of departure from the global null (lambda 0.98, Table 2, Figure 1 and Supplementary Figure 1). The number of analysed CpG sites was 738,318 in

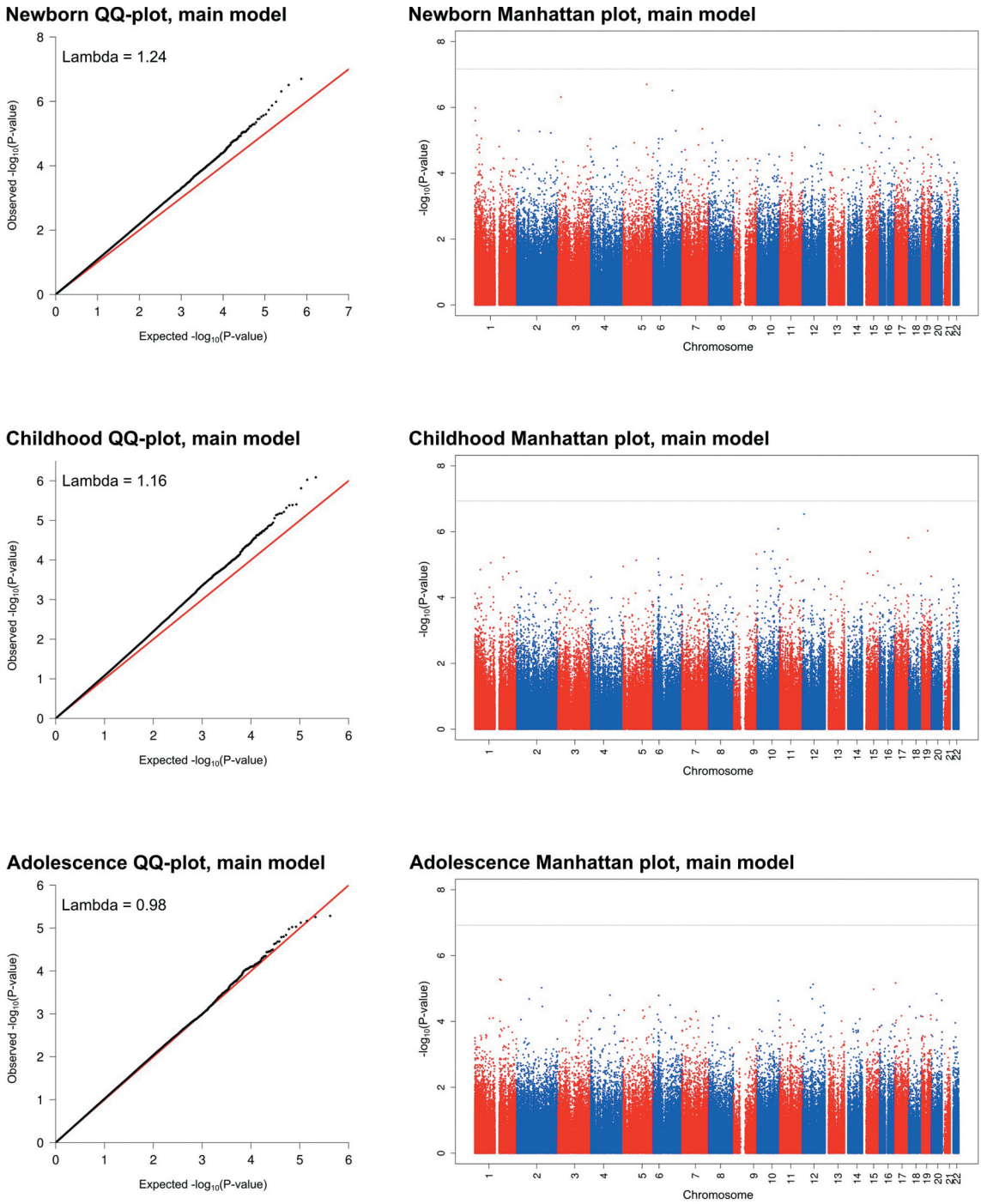

Figure 1. Maternal haemoglobin during pregnancy and offspring DNA methylation at birth, childhood and adolescence main models. Fully adjusted main model for newborn and childhood data is adjusted for gestational week at maternal haemoglobin measurement, maternal parity, maternal education, maternal smoking, child sex, gestational age at birth, child age at time of DNA methylation measurement, DNA methylation batch and white blood cells estimates. Adolescence model is fully adjusted model without adjustment for gestational week at maternal haemoglobin measurement. The grey line in the Manhattan plot corresponds the threshold of significant P-value after Bonferroni correction for multiple testing.

newborn, 425,188 in childhood, and 418,438 in adolescent models. I^2 values were below 50%, i.e., they did not indicate high inter-study heterogeneity in 602,276 (81.6%), 371,919 (87.5%) and 347,638 (83.1%) CpG sites in the newborn, childhood, and adolescent models, respectively.

After Bonferroni correction for 738,318 tests (P-value <6.77E-08), there were no significant associations of maternal haemoglobin levels with offspring DNA methylation at any CpG sites in newborns. The 40 CpG sites with the lowest P-values for the main model are shown in Table 3 and for the minimally adjusted model in Supplementary Table 2. Similarly, there was no statistical support for associations of maternal haemoglobin levels and DNA methylation in childhood (Bonferroni correction for 425,188 tests, P-value <1.18E-07) or in adolescence (Bonferroni correction for 418,438 tests, P-value <1.19E-07). Volcano plots of the meta-analysis results are in Supplementary Figure 2. The 40 CpG sites

Table 3. CpG sites with the lowest P-values in a meta-analysis of associations between maternal haemoglobin during pregnancy and offspring DNA methylation at birth. There are no significant CpG sites after Bonferroni correction (P-value <6.77E-08). A fully adjusted model for newborn and childhood data was adjusted for a gestational week at maternal haemoglobin measurement, maternal parity, maternal education, maternal smoking, child sex, gestational age at birth, child age at the time of DNA methylation measurement, DNA methylation batch, selection factor in the case of randomized controlled trial and white blood cells estimates. The adolescence model is a fully adjusted model without adjustment for a gestational week at maternal haemoglobin measurement. CpG, cytosine-phosphate-guanine; Chr, chromosome; Regression coefficient, difference in offspring DNA methylation beta value per one SE unit increase in maternal haemoglobin; SE, standard error. Polymorphic CpG sites are indicated with an asterisk after the site name.

CpG site	Chr	Gene	Regression coefficient	SE for regression coefficient	P-value	FDR-corrected P-value
cg05470963*	5	*ARHGAP26*	0.0015	0.0003	2.00E-07	0.114
cg18479141	6	*HDAC2*	−0.0022	0.0004	3.08E-07	0.114
cg04181092	3		0.0013	0.0003	4.88E-07	0.120
cg24953596	1	*MEGF6*	−0.0043	0.0009	1.03E-06	0.190
cg04365443	15	*MPI*	−0.0005	0.0001	1.34E-06	0.198
cg00736299*	16	*MGRN1*	0.0027	0.0006	1.83E-06	0.225
cg20169893	1	*PRDM16*	−0.0018	0.0004	2.51E-06	0.238
cg06928695	17	*PITPNM3*	−0.0030	0.0006	2.73E-06	0.238
cg09126014	15	*SCAMP2*	0.0022	0.0005	2.99E-06	0.238
cg23912509	12	*MIR135A2*	0.0015	0.0003	3.47E-06	0.238
cg05454731	13		−0.0040	0.0009	3.55E-06	0.238
cg04140066	7		−0.0033	0.0007	4.47E-06	0.259
cg14801038	6	*TCF21*	−0.0023	0.0005	5.13E-06	0.259
cg15753546*	2		0.0015	0.0003	5.13E-06	0.259
cg02935826	2		0.0022	0.0005	5.40E-06	0.259
cg06522562	2	*FAM117B*	0.0006	0.0001	5.95E-06	0.259
cg08908586	14	*FBLN5*	−0.0010	0.0002	5.96E-06	0.259
cg13305114	1	*VPS13D*	0.0009	0.0002	6.94E-06	0.263
cg05924031	16	*CACNA1H*	0.0026	0.0006	7.38E-06	0.263
cg14500916	18	*LOC101927410*	0.0009	0.0002	7.92E-06	0.263
cg24542758	16		−0.0023	0.0005	8.67E-06	0.263
cg09364660	1	*MYCBP, RP5-864K19.4, RP5-864K19.6, RP5-864K19.7*	0.0007	0.0002	8.82E-06	0.263
cg02662362	6	*HLA-DPB2*	−0.0007	0.0002	8.94E-06	0.263
cg24392197	3	*RN7SL36P, XXYLT1, XXYLT1-AS2*	−0.0032	0.0007	8.97E-06	0.263
cg15520639	6		0.0011	0.0002	9.11E-06	0.263
cg23076906	19	*ZNF444*	−0.0011	0.0002	9.27E-06	0.263
cg10250335	8	*LOC101927040*	0.0057	0.0013	1.01E-05	0.275
cg19681474	5		−0.0019	0.0004	1.19E-05	0.289
cg20757478	6		0.0044	0.0010	1.19E-05	0.289
cg20794351*	8		−0.0039	0.0009	1.20E-05	0.289
cg08008938	14	*ADSSL1*	−0.0017	0.0004	1.21E-05	0.289
cg18878872	1	*MAN1C1*	0.0052	0.0012	1.34E-05	0.295
cg16815082	7		0.0035	0.0008	1.37E-05	0.295
cg09041485	3	*USP13*	−0.0009	0.0002	1.49E-05	0.295
cg21961202	1		−0.0006	0.0001	1.53E-05	0.295
cg04342176	4	*DCLK2*	−0.0007	0.0002	1.54E-05	0.295
cg03927133	15	*ITPKA*	−0.0008	0.0002	1.59E-05	0.295
cg12751042	12	*CDKN1B*	0.0019	0.0004	1.61E-05	0.295
cg03726569	19	*SAFB2*	0.0012	0.0003	1.62E-05	0.295
cg26556719	5	*AC005609.17, PCDHA1 – PCDHA13*	−0.0026	0.0006	1.66E-05	0.295

with the lowest P-values in the childhood and adolescent models are listed in Supplementary Table 3–6. CpG sites that were statistically significant in individual cohorts are listed in Supplementary Table 7. We also corrected for multiple testing using the less stringent false discovery rate (FDR) threshold by Benjamini and Hochberg [40] and found no statistical support for association ($P < 0.05$).

To investigate the effect of maternal haemoglobin measurement timing on the associations, we conducted sensitivity analyses by stratifying the newborn studies into those with early (mean maternal haemoglobin level measured before or at gestational week 15) and late (mean maternal haemoglobin level measured after gestational week 27) maternal haemoglobin measurements. Global P-values were not inflated for the early gestational age measurements (meta-analysis lambda 0.98) and there was minimal inflation for those with late maternal haemoglobin measurements (meta-analysis lambda 1.24). There was no statistical support for associations of maternal haemoglobin levels with newborn DNA methylation when analyses were conducted separately for early and late maternal haemoglobin measurement (Supplementary Figure 3).

Differentially methylated regions

Using *comb-p* [43], we found 12 differentially methylated regions in the newborn analyses, 27 in childhood, and 17 in the adolescence models (Table 4). None of the differentially methylated regions overlapped between all of the ages, but there was an overlap of one differentially methylated region annotated to *HOXA2* between newborn and adolescent models and a region annotated to *CHRNE* between childhood and adolescent models. We did not find any differentially methylated regions using *DMRcate* [44].

Discussion

In the current study, we analysed associations of maternal haemoglobin levels during pregnancy with offspring DNA methylation at birth, in childhood and adolescence. We meta-analysed EWAS summary statistics of 10 studies comprising 3,967 neonatal, 1,534 childhood, and 1,962 adolescent offspring DNA methylation samples and their maternal haemoglobin levels during pregnancy. We did not find statistical support for an association between maternal haemoglobin levels during pregnancy and offspring DNA methylation at any of the three ages.

We found some evidence of an association between maternal haemoglobin levels and differentially methylated regions in the offspring DNA using *comb-p* [43]. We identified one shared region on chromosome 7 between newborn and adolescent models and one on chromosome 17 between childhood and adolescent models. Of these, the specifically interesting locus is the one situated in the homeobox A2 (*HOXA2*) gene, which encodes a transcription factor that is important during embryonic development. *HOXA2* locates in chromosome 7, has a role in the development of the lower and middle part of the face and middle ear, and its deficiency have been associated with ear microtia (reviewed in [46]). *Comb-p* is a flexible tool specifically for meta-analysed EWAS summary statistics as it uses P-values by sliding windows and takes into account the correlation between near-by sites; however, *comb-p* has been shown to produce false-positive results, especially if the signal in the original data was weak [47]. As there is no consensus on the best method for analysis of differentially methylated regions with meta-analysis data, we also analysed the results using *DMRcate* [44] which did not support the *comb-p* results. As the differentially methylated regions were identified by one method only, we conclude that the highlighted regions may be artifacts and should be cautiously interpreted.

The large sample size covering the newborn, childhood, and adolescent age periods was a major strength of the current study. Nearly 80% of the meta-analysed CpG sites show only a little or moderate evidence for between-study heterogeneity suggesting that the observed effects were reasonably consistent across cohorts. This is another strength, as lower heterogeneity improves the interpretability of the results. However, this study also had some technical limitations. Although the current method for epigenome-wide analysis of methylated CpG sites is arguably

Table 4. Differentially methylated regions in offspring DNA associated with maternal haemoglobin. Fully adjusted model for newborn and childhood data is adjusted for a gestational week at haemoglobin measurement, child sex, DNA methylation batch, white blood cell estimates, possible selection factor, gestational age at birth, child age at the time of DNA methylation measurement, maternal smoking, parity, and maternal education. The adolescence model is a fully adjusted model without adjustment for a gestational week at maternal haemoglobin measurement. The overlapping region in chromosome 7 between newborn and adolescence as well as in chromosome 17 between childhood and adolescence is highlighted. Chr, chromosome; N, number of CpG sites; P-value, Sidak-corrected P-value (significant when <0.05).

Life-stage	Chr	Gene	Start	End	N	P-value
Newborn	1	*PLEKHG5*	6,471,656	6,471,754	3	1.80E-02
Cord blood	3	*MBNL1-AS1, MBNL1*	152,268,820	152,269,011	6	4.75E-02
	3	*XXYLT1*	195,147,697	195,147,779	3	7.68E-03
	6	*LY6G5C*	31,682,957	31,683,502	18	1.41E-09
	7	***HOXA2***	**27,103,615**	**27,103,860**	**7**	**6.58E-03**
	7	*UPP1*	48,090,199	48,090,396	5	2.11E-05
	10	*MIR378C*	130,885,180	130,885,192	2	1.97E-02
	12	*LOC101593348, DIABLO*	122,227,440	122,227,666	8	6.68E-04
	15	*FOXB1*	60,002,198	60,003,114	5	2.46E-07
	16	*TEPP*	57,985,961	57,986,081	3	1.16E-02
	17	*TBC1D3P5*	27,380,401	27,380,510	2	2.87E-02
	19	*RPS9*	54,206,998	54,207,425	4	5.90E-05
Childhood	2	*GDF7*	20,670,326	20,671,642	8	1.35E-15
4 to 7 years	3	*LRRC15*	194,369,747	194,370,002	5	1.59E-06
	5	*FAM172A*	94,111,781	94,111,996	5	2.69E-02
	6	*PSORS1C3*	31,180,554	31,180,881	14	8.39E-03
	6	*VARS*	31,794,631	31,795,000	11	1.64E-02
	6	*HLA-DQB1*	32,664,553	32,665,387	16	9.01E-08
	6	*TAPBP*	33,312,274	33,312,678	12	3.02E-06
	6	*CRISP2*	49,713,464	49,713,679	7	2.03E-02
	7	*GPR146, C7orf50*	1,055,828	1,056,085	5	3.94E-02
	7	*HOXA-AS3, HOXA6*	27,147,752	27,147,942	6	1.60E-03
	10	*PRXL2A*	80,408,000	80,408,019	3	9.96E-05
	10	*GLRX3*	130,191,038	130,191,586	7	5.11E-08
	11	*PGGHG*	289,773	289,967	3	2.84E-02
	11	*IFITM5*	299,389	300,491	11	6.71E-08
	11	*TNNT3*	1,927,702	1,927,884	5	2.06E-02
	11	*ACY3*	67,650,634	67,650,935	11	3.55E-03
	12	*RIMBP2*	130,633,880	130,634,110	4	4.02E-03
	12	*ADGRD1*	131,132,498	131,132,548	3	1.24E-02
	14	*CDC42BPB*	103,058,561	103,058,653	3	5.11E-03
	17	***C17orf107, CHRNE***	**4,901,378**	**4,901,544**	**2**	**4.66E-02**
	17	*RAB34*	28,718,024	28,718,159	5	2.53E-02
	17	*NBR2*	43,126,117	43,126,364	7	1.64E-02
	17	*SEC14L1*	77,100,119	77,100,301	3	8.71E-03
	18	*SALL3*	78,506,264	78,506,438	3	1.19E-04
	19	*IZUMO1*	48,741,313	48,741,418	3	2.16E-02
	20	*CDH4*	61,773,104	61,773,352	3	4.89E-02
	20	*RTEL1-TNFRSF6B, TNFRSF6B*	63,696,614	63,696,742	3	2.30E-02
Adolescence	1	*RNU1-1, RNU1-3, RNVU1-18, RNU1-2, RNU1-4*	143,717,589	143,717,820	2	3.90E-05
16 to 17 years						
	1	*MIR5087*	148,328,899	148,329,313	3	3.52E-04
	3	*CACNA1D*	53,495,988	53,496,221	3	2.05E-02
	3	*COL6A6*	130,649,213	130,649,552	6	5.95E-05
	4	*CTBP1-DT*	1,250,060	1,250,299	7	3.57E-07
	4	*EXOC1L*	55,794,161	55,794,295	3	3.75E-03
	6	*LINC00533*	28,633,491	28,633,743	12	6.11E-03
	7	***HOXA2***	**27,103,615**	**27,103,860**	**7**	**8.72E-03**
	10	*GLRX3*	130,190,896	130,191,293	5	2.37E-04
	11	*KCNQ1*	2,807,294	2,807,549	4	1.50E-03
	15	*LOC100130111*	29,675,827	29,675,992	3	2.22E-02
	15	*TTC23*	99,249,416	99,249,651	5	7.89E-04
	17	***C17orf107, CHRNE***	**4,901,378**	**4,901,544**	**2**	**4.89E-05**
	19	*SMIM24*	3,480,364	3,480,675	5	1.57E-03
	22	*RFPL2*	32,203,523	32,203,662	4	3.44E-02

the best choice for high-throughput studies, the 450,000 or 850,000 sites analysed by the Illumina Infinium Methylation 450K and Methylation EPIC arrays, respectively, account for only 2% to 4% of the CpG sites in the whole genome. It is possible that DNA methylation at sites not covered on

either array could be related to maternal haemoglobin levels [48].

Furtermore, there is a large and ongoing proliferation of published methods for quality control, processing, and analysis of DNA methylation data. The optimal method may vary between cohorts based on technical issues prior to data analysis, such as bisulphite conversion efficiency, sample distribution on the chip, and the chip reading efficiency. In addition, the multitude of methods are often published with an insufficient evaluation of how these alter results or compare with other methods. Thus, we allowed each cohort, with their familiarity with how the samples were processed in their study, to assess the normalization method and apply their own correction. This might have influenced the downstream analysis. However, we have previously shown, that there are no large differences between a meta-analysis of cohorts that all used their own preferred normalization method and a meta-analysis of the non-normalized data of those same cohorts [13]. Due to the restrictions in data transfer permissions, we used a meta-analysis of summary statistics of individual studies, which is a standard practice in the PACE Consortium. Thus, the participating cohorts conducted their own EWAS locally and sent the summary statistics to the meta-analysis team, which then conducted the meta-analysis. This may also lessen the effect of differing normalization as the same normalization was always used within the cohort. That is, we would expect any true associations to be identified within the individual cohorts, regardless of the normalization method, and then to also come up in the meta-analysis.

Although the sample size in the current study was relatively large, it might have been insufficient to detect weak associations that might exist between the variation of maternal haemoglobin levels within the normal range and the offspring DNA methylation. Furthermore, maternal haemoglobin levels are routinely monitored during pregnancy, and if low haemoglobin was detected, it is likely that measures were taken in an attempt to increase levels by administration of iron supplements. This may have lowered the number of individuals with low maternal haemoglobin level in our analysis. In addition, we have used linear models in the current analyses, while the fact that both high and low maternal haemoglobin levels have been shown to associate with adverse pregnancy outcomes would support a nonlinear approach. There were not enough individuals in the cohort-specific strata of low/high maternal haemoglobin levels to make analyses in categories of low, normal, and high haemoglobin levels feasible. Future studies in populations with a higher prevalence of high or low maternal haemoglobin levels, such as those living at high altitudes [4] or in low-income countries [49], respectively, will provide insight into potential associations at more extreme maternal haemoglobin levels. The mean gestational age at which maternal haemoglobin levels were measured varied substantially between cohorts, from 9.7 to 36.6 weeks. During pregnancy, maternal haemoglobin levels normally decrease due to haemodilution until 20 weeks of gestation and begin to increase at around 30 weeks. We adjusted the models for gestational age at maternal haemoglobin measurement; however, this might not account for inter-cohort differences. To investigate this further, we conducted sensitivity analyses separately for studies that measured maternal haemoglobin levels during early and late pregnancy in newborn models and found no strong statistical support for associations in either of this strata.

One mechanism by which maternal haemoglobin levels could influence the DNA methylation of the offspring is through non-physiological intrauterine hypoxia [9]. Both low and high maternal haemoglobin levels may expose the fetus to hypoxia; low levels via insufficient oxygen availability and high levels via increased blood viscosity [8]. Hypoxia has been shown to increase methylation of approximately half of CpG sites that would in normoxic conditions become hypomethylated in the placental trophoblasts [10]. Nonphysiological hypoxia may affect the developing fetus either in a pre-placental, uteroplacental or post-placental manner [50]. From these, only pre-placental hypoxia influences both mother and fetus whereas uteroplacental and post-placental hypoxia may not be reflected in the maternal haemoglobin levels. Thus, the maternal haemoglobin levels investigated in the current study may represent only pre-placental hypoxia. Further mechanistic studies are warranted to fully understand the relationship between non-physiological intrauterine hypoxia and the offspring DNA methylation.

Conclusions

This study is the first to date to ascertain a possible association between maternal haemoglobin levels and DNA methylation in the offspring at three age ranges from newborns to adolescence. We did not find evidence to support epigenetic programming by physiological variations of maternal haemoglobin levels during pregnancy.

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Disclosure of interest

The authors report no conflict of interest.

Authors' contributions

AH, FV, EL, JR, JF, and SS designed, analysed, and interpreted the data. JR and AH were major contributors in writing the manuscript. All authors read and approved the final manuscript.

Disclosure statement

No, potential conflict of interest was reported by the authors.

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References

[1] Steer PJ. Maternal hemoglobin concentration and birth weight. Am J Clin Nutr. 2000;71(5):1285S–1287S.
[2] Jwa SC, Fujiwara T, Yamanobe Y, et al. Changes in maternal hemoglobin during pregnancy and birth outcomes. BMC Pregnancy Childbirth. 2015;15(1):80.

[3] Cordina M, Bhatti S, Fernandez M, et al. Association between maternal haemoglobin at 27-29weeks gestation and intrauterine growth restriction. Pregnancy Hypertens. 2015;5(4):339–345.
[4] Gonzales GF, Steenland K, Tapia V. Maternal hemoglobin level and fetal outcome at low and high altitudes. Am J Physiol Integr Comp Physiol. 2009;297(5):R1477–R1485.
[5] Tandu-Umba B, Mbangama AM. Association of maternal anemia with other risk factors in occurrence of great obstetrical syndromes at university clinics, Kinshasa, DR Congo. BMC Pregnancy Childbirth. 2015;15(1):183.
[6] Ronkainen J, Lowry E, Heiskala A, et al. Maternal hemoglobin associates with preterm delivery and small for gestational age in two finnish birth cohorts. Eur J Obstet Gynecol Reprod Biol. 2019;238:44–48.
[7] WHO (2015) WHO | the global prevalence of anaemia in 2011. *WHO*.
[8] Allen LH. Biological mechanisms that might underlie iron's effects on fetal growth and preterm birth. J Nutr. 2001;131(2):581S–589S.
[9] Ducsay CA, Goyal R, Pearce WJ, et al. Gestational hypoxia and developmental plasticity. Physiol Rev. 2018;98:1241–1334.
[10] Yuen RKC, Chen B, Blair JD, et al. Hypoxia alters the epigenetic profile in cultured human placental trophoblasts. Epigenetics. 2013;8(2):192–202.
[11] Marsit CJ. Influence of environmental exposure on human epigenetic regulation. J Exp Biol. 2015;218 (1):71–79.
[12] Sharp GC, Salas LA, Monnereau C, et al. Maternal BMI at the start of pregnancy and offspring epigenome-wide DNA methylation: findings from the pregnancy and childhood epigenetics (PACE) consortium. Hum Mol Genet. 2017;26(20):4067–4085.
[13] Joubert BR, Felix JF, Yousefi P, et al. DNA Methylation in newborns and maternal smoking in pregnancy: genome-wide consortium meta-analysis. Am J Hum Genet. 2016;98(4):680–696.
[14] Kazmi N, Sharp GC, Reese SE, et al. Hypertensive disorders of pregnancy and DNA methylation in newborns. Hypertens (Dallas, Tex 1979). 2019;74 (2):375–383.
[15] Merid SK, Novoloaca A, Sharp GC, et al. Epigenome-wide meta-analysis of blood DNA methylation in newborns and children identifies numerous loci related to gestational age. Genome Med. 2020;12(1):25.
[16] Kupers LK, Monnereau C, Sharp GC, et al. Meta-analysis of epigenome-wide association studies in neonates reveals widespread differential DNA methylation associated with birthweight. Nat Commun. 2019;10(1):1893.
[17] Boyd A, Golding J, Macleod J, et al. Cohort profile: the 'children of the 90s'–the index offspring of the avon longitudinal study of parents and children. Int J Epidemiol. 2013;42(1):111–127.
[18] Fraser A, Macdonald-Wallis C, Tilling K, et al. Cohort profile: the avon longitudinal study of parents and children: ALSPAC mothers cohort. Int J Epidemiol. 2013;42(1):97–110.
[19] Heude B, Forhan A, Slama R, et al. Cohort profile: the EDEN mother-child cohort on the prenatal and early postnatal determinants of child health and development. Int J Epidemiol. 2016;45(2):353–363.
[20] Mustaniemi S, Vääräsmäki M, Eriksson JG, et al. Polycystic ovary syndrome and risk factors for gestational diabetes. Endocr Connect. 2018;7(7):859–869.
[21] Keikkala E, Mustaniemi S, Koivunen S, et al. Cohort profile: the finnish gestational diabetes (FinnGeDi) study. Int J Epidemiol. 2020;49(3):762–763g.
[22] Kooijman MN, Kruithof CJ, van Duijn CM, et al. The generation R study: design and cohort update 2017. Eur J Epidemiol. 2016;31(12):1243–1264.
[23] Guxens M, Ballester F, Espada M, et al. Cohort profile: the INMA–INfancia y medio ambiente–(environment and childhood) project. Int J Epidemiol. 2012;41(4):930–940.
[24] Girchenko P, Lahti M, Tuovinen S, et al. Cohort Profile: prediction and prevention of preeclampsia and intrauterine growth restriction (PREDO) study. Int J Epidemiol. 2017;46(5):1380–1381g.
[25] Braun JM, Wright RJ, Just AC, et al. Relationships between lead biomarkers and diurnal salivary cortisol indices in pregnant women from Mexico City: a cross-sectional study. Environ Health. 2014;13(1):50.
[26] Burris HH, Braun JM, Byun H-M, et al. Association between birth weight and DNA methylation of IGF2, glucocorticoid receptor and repetitive elements LINE-1 and Alu. Epigenomics. 2013;5(3):271–281.
[27] Hummel S, Much D, Rossbauer M, et al. Postpartum outcomes in women with gestational diabetes and their offspring: POGO study design and first-year results. Rev Diabet Stud. 2013;10(1):49–57.
[28] Rantakallio P. The longitudinal study of the northern Finland birth cohort of 1966. Paediatr Perinat Epidemiol. 1988;2(1):59–88.
[29] Straker L, Mountain J, Jacques A, et al. Cohort profile: the Western Australian pregnancy cohort (raine) study- generation 2. Int J Epidemiol. 2017;46(5):1384–1385j.
[30] Kumar A, Rai AK, Basu S, et al. Cord blood and breast milk iron status in maternal anemia. Pediatrics. 2008;121(3):e673–7.
[31] White H. A heteroskedasticity-consistent covariance matrix estimator and a direct test for heteroskedasticity. Econometrica. 1980;48(4):817.
[32] Venables WN, Ripley BD. Modern applied statistics with s. *modern applied statistics with s*; fourth. New York: Springer; 2002.
[33] Core Team R (2019) R: A language and environment for statistical computing. R: A Language and Environment for Statistical Computing (2019).
[34] Zeileis A, Köll S, Graham N. Various versatile variances: an object-oriented implementation of clustered covariances in R. J Stat Softw. 2020;95(1):1–36.

[35] Zeileis A. Object-oriented computation of sandwich estimators. J Stat Softw. 2006;16(9):1–16.
[36] Bakulski KM, Feinberg JI, Andrews SV, et al. DNA methylation of cord blood cell types: applications for mixed cell birth studies. Epigenetics. 2016;11(5):354–362.
[37] Houseman EA, Accomando WP, Koestler DC, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC Bioinformatics. 2012;13(1):86.
[38] Aryee MJ, Jaffe AE, Corrada-Bravo H, et al. Minfi: a flexible and comprehensive bioconductor package for the analysis of Infinium DNA methylation microarrays. Bioinformatics. 2014;30(10):1363–1369.
[39] Willer CJ, Li Y, Abecasis GR. METAL: fast and efficient meta-analysis of genomewide association scans. Bioinformatics. 2010;26(17):2190–2191.
[40] Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B. 1995;57:289–300.
[41] Chen Y, Lemire M, Choufani S, et al. Discovery of cross-reactive probes and polymorphic CpGs in the illumina infinium humanMethylation450 microarray. Epigenetics. 2013;8(2):203–209.
[42] Naeem H, Wong NC, Chatterton Z, et al. Reducing the risk of false discovery enabling identification of biologically significant genome-wide methylation status using the HumanMethylation450 array. BMC Genomics. 2014;15(1):51.
[43] Pedersen BS, Schwartz DA, Yang IV, et al. Comb-p: software for combining, analyzing, grouping and correcting spatially correlated P-values. Bioinformatics. 2012;28(22):2986–2988.
[44] Peters TJ, Buckley MJ, Statham AL, et al. De novo identification of differentially methylated regions in the human genome. Epigenetics Chromatin. 2015;8(1):6.
[45] Mallik S, Odom GJ, Gao Z, et al. An evaluation of supervised methods for identifying differentially methylated regions in Illumina methylation arrays. Brief Bioinform. 2019;20(6):2224-2235.
[46] Si N, Meng X, Lu X, et al. Identification of loss-of-function HOXA2 mutations in Chinese families with dominant bilateral microtia. Gene. 2020;757:144945.
[47] Kolde R, Martens K, Lokk K, et al. seqlm: an MDL based method for identifying differentially methylated regions in high density methylation array data. Bioinformatics. 2016;32(17):2604–2610.
[48] Michels KB, Binder AM, Dedeurwaerder S, et al. Recommendations for the design and analysis of epigenome-wide association studies. Nat Methods. 2013;10(10):949–955.
[49] Rahman MM, Abe SK, Rahman MS, et al. Maternal anemia and risk of adverse birth and health outcomes in low- and middle-income countries: systematic review and meta-analysis. Am J Clin Nutr. 2016;103(2):495–504.
[50] Kingdom JC, Kaufmann P. Oxygen and placental villous development: origins of fetal hypoxia. Placenta. 1997;18(8):613–616.