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Sex-specific effects of early life cadmium exposure on DNA methylation and implications for birth weight

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Abbreviations: As, arsenic; BMI, body mass index; Cd, cadmium; CHR, chromosome; GW, gestational week; HDSS, health demographic surveillance system; HAZ, height-for-age z-score; ICPMS, inductively coupled plasma mass spectrometry; LMP, last menstrual period; MB-Cd, maternal blood cadmium; PCA, principal component analysis; SES, socioeconomic status; WAZ, weight-for-age z-score; U-As, urinary arsenic metabolites; U-Cd, urinary cadmium

Dietary cadmium exposure was recently found to alter DNA methylation in adults, but data on effects early in life are lacking. Our objective was to evaluate associations between prenatal cadmium exposure, DNA methylation and birth weight. In total 127 mother-child pairs from rural Bangladesh were studied. For comparison, we included 56 children at 4.5 years. Cadmium concentrations in mothers' blood (gestational week 14) and children's urine were measured by ICPMS. Global DNA methylation was analyzed by Infinium HumanMethylation450K BeadChip in cord blood and children's blood. Maternal cadmium exposure was associated with cord blood DNA methylation (p value < 10^{-16}). The association was markedly sex-specific. In boys, 96% of the top 500 CpG sites showed positive correlations (r_S values > 0.50), whereas most associations in girls were inverse; only 29% were positive (r_S > 0.45). In girls we found overrepresentation of methylation changes in genes associated with organ development, morphology and mineralization of bone, whereas changes in boys were found in cell death-related genes. Several individual CpG sites that were positively associated with cadmium were inversely correlated with birth weight, although none statistically significant after correction for multiple comparisons. The associations were, however, fairly robust in multivariable-adjusted linear regression models. We identified CpG sites that were significantly associated with cadmium exposure in both newborns and 4.5-y-old children. In conclusion, cadmium exposure in early life appears to alter DNA methylation differently in girls and boys. This is consistent with previous findings of sex-specific cadmium toxicity. Cadmium-related changes in methylation were also related to lower birth weight.

Introduction

The toxic metal cadmium (Cd) is ubiquitous in the environment. Human exposure occurs mainly via consumption of plant-derived foods, certain types of seafood and offal and via tobacco smoking.[1] Chronic health effects of Cd in adults are well documented[2] and there is increasing evidence that also early-life Cd exposure has detrimental effects on child health and development. Cadmium exposure during pregnancy has been associated with decreased birth weight,[3,4] which in turn is associated with future disease risk.[5,6] In a large longitudinal cohort study, we found a sex-specific association between maternal Cd exposure during pregnancy and infant size at birth, with inverse associations in girls, but little evidence of effects in boys.[7] The effects on children's size seemed to remain until 5 y of age.[8]

The mechanisms of early-life toxicity of Cd are not yet clear. Suggested mechanisms include disturbed zinc transfer to the fetus,[9] interference with glucocorticoid balance,[10] and the insulin-like growth factor (IGF) axis,[11] all of which may impair fetal growth. A further yet unexplored mechanism of Cd toxicity is interference with the epigenetic machinery, such as DNA methylation, processes that are crucial for early fetal development.[12,13] In particular early-life undernutrition have been liked to changes in epigenetic processes, with subsequent consequences for long-term illness.[14] In vitro studies have shown Cd-associated gene-specific DNA hypermethylation along with gene silencing, as well as global DNA hypomethylation.[15,16] In chick embryos Cd caused downregulation of gene expression of the DNA methyltransferases DNMT3A and 3B.[17] In a cross-sectional study of 202 women with low-level environmental Cd exposure from the

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Table 1. Characteristics of the 127 mother-child pairs in the present study, as well as all other women who were enrolled in the MINIMat trial from October 2002 through October 2003 and gave live birth (n = 1729)

Variable	Study sample[a] (n = 127)	All women[a] (n = 1729)
Maternal characteristics		
Maternal age (years)	25 ± 5.9	26 ± 5.9
BMI (kg/m^2; GW8)	20 ± 3.0	20 ± 2.7
Parity (no. of children)	1.1 ± 1.4	1.4 ± 1.4
0/ ≥ 1	54 (43%)/73 (57%)	548 (32%)/1179 (68%)
Socio-economic status		
Lowest	19 (15%)	315 (18%)
Lower middle	12 (9%)	349 (20%)
Middle	24 (19%)	353 (20%)
Upper middle	36 (28%)	350 (20%)
Highest	36 (28%)	362 (21%)
Betel chewing during pregnancy		
Yes/No	73 (58%)/52 (42%)	1150 (68%)/531 (32%)
Urinary Cd (μg/L; GW8)[b]	0.77 (0.25–2.4)	0.61 (0.21–2.3)
Urinary As (μg/L; GW8)[b]	68 (20–446)	68 (17–480)
Blood Cd (μg/kg; GW14)[c]	1.3 (0.54–3.1)	1.3 (0.65–2.8)
Newborn characteristics		
Sex		
Girls	65 (51%)	845 (49%)
Boys	62 (49%)	884 (51%)
Gestational age (weeks)	39 ± 1.8	39 ± 2.1
< 37/ ≥ 37	12 (10%)/112 (90%)	254 (15%)/1444 (85%)
Birth weight (g)	2780 ± 395	2709 ± 409

[a]Values are shown as mean ± SD, median (5–95th percentiles) or n (%). [b]Adjusted to the average specific gravity of 1.012 g/mL; urinary Cd n = 124 in the study sample and n = 412 of all women; urinary As n = 124 in the study sample and n = 973 of all women. [c]n = 117 in the study sample and n = 241 of all women.

diet, Cd in urine was associated with DNA hypomethylation of *LINE1* retrotransposon sequences, a crude marker for global methylation, in peripheral blood.[18] Also, in a study on the role of dietary factors for *LINE1* methylation in cord blood, the estimated maternal dietary Cd exposure was associated with *LINE1* hypomethylation.[19]

In this study our aim was to elucidate whether Cd exposure during pregnancy, assessed by individual biomarkers, is associated with altered DNA methylation in the newborn, and in turn, if this may affect the child's birth weight.

Results

Maternal blood Cd concentrations in gestational week (GW) 14 varied from 0.38 to 5.4 μg/kg (**Table 1**). Blood Cd concentrations in the study population did not differ from those among all women in the cohort, whereas the median Cd in maternal urine (GW8) was slightly higher in this subgroup compared with all women. Blood Cd concentrations in mothers of newborn boys (median 1.2 μg/kg; 5–95th percentile 0.52–3.1) were similar to the Cd concentrations in mothers of newborn girls (1.4 μg/kg; 0.56–3.0). The 4.5-y-old children had lower urinary Cd than the women (**Table S1**).

Cd exposure and DNA methylation in cord blood and 4.5-y-old children. Cd in maternal blood was associated with methylation levels in components 4 and 5 (**Fig. S1**). There was no association with Cd in maternal urine in any of the components and further analyses were therefore performed with Cd in blood only.

We first evaluated whether the Cd exposure was associated with global DNA methylation by analyzing all CpG sites in cord blood in all newborns in separate models vs. Cd in maternal blood. The analysis showed that small p values were more frequent than expected from a uniform distribution (Kolmogorov-Smirnov test p value < 10^{-16}). There were differences between sexes, in that the effect of Cd exposure seemed to be more pronounced in boys compared with girls (**Fig. 1**).

We then analyzed CpG-specific effects of Cd. Cadmium vs. DNA methylation showed correlations between r_s = −0.36 – 0.43 (range for the top 500 strongest correlations), and the lowest unadjusted p value was 2.6 × 10^{-6}. After adjusting for multiple comparisons all p values were > 0.05. The top five genes correlating with Cd (**Table 2**) were *HIST4L* (r_s = 0.43), *PAX9* (r_s = 0.42), *APBB3* (r_s = 0.41), *GAP43* (r_s = 0.41) and *PTPRN2* (r_s = −0.41).

We then stratified the analysis by sex, as we previously found sex-specific effects of Cd exposure during early life.[7] When all probes were considered, there were generally slightly stronger correlations between maternal blood Cd and DNA methylation in cord blood of boys than in girls (**Table 2**, showing the top ten CpG sites for all children and by sex). In boys, the lowest unadjusted p value observed among all CpG sites was 6.3 x 10^{-7} (r_s = 0.61), whereas in girls it was 0.72 × 10^{-7} (r_s = −0.57), although all p values adjusted for multiple comparisons were > 0.05. In boys, 96% of the top 500 CpG sites showed positive correlations between maternal Cd concentrations and methylation (all positive β-values had p values > 0.50), whereas in girls only 29% of the sites showed positive correlations (all positive r_s > 0.45) and the remaining were inverse (r_s > −0.41).

We performed sex-specific pathway analyses based on annotated genes among the 500 CpG sites with strongest correlations between maternal blood Cd and cord blood methylation. In the top five networks obtained, there were some overlap between boys and girls; e.g., cell morphology, cell cycle, cellular growth and proliferation (**Table S2**), but they were different in ranking. Sex-specific differences were found for specific functions. Girls showed the strongest associations between Cd and DNA methylation in CpG sites related to embryonic and organ development, especially connective tissue and skeletal development (bone mineralization and morphology of the bone; altogether around 40 genes; p value 1.5 × 10^{-5}). Boys, by contrast, showed the strongest associations between Cd and DNA methylation in genes involved in cell death (105 genes among the top 500; p value 3.9 × 10^{-6}). As mentioned above, CpG sites in those genes

showed more methylation in boys with increasing maternal blood Cd concentrations.

Among the 500 top CpG sites in cord blood (both sexes) that showed the strongest correlations between methylation and Cd in maternal blood, we selected those that were also significantly correlated in the same direction in the 4.5-y-old children (child blood CpG methylation vs. child urinary Cd; **Table 3**). Cadmium exposure was inversely correlated with methylation in the CpG site cg16001202, close to *SMOC2,* in both cord blood and children's blood, and the same direction of correlation, but non-significant, was found for two more sites in *SMOC2* (not shown in **Table 3**). Similarly, one CpG site each in *IL17RD*, *C4B*, *RAB23*, *MYPN*, *NODAL*, *PLCG2* and *NAA10* and one CpG-site each on chromosomes 8 (closest gene *RUNX1T1*), 10 (*CASC2*/*FAM204A*), and 13 in both cord blood and children's blood were significantly correlated in the same direction with Cd exposure. The function of these genes are shown in **Table S3** and some examples are shown in **Figure 2A–D**. There was no indication of sex-related differences between Cd and DNA methylation for these sites (data not shown). The effect estimates or the strength of associations did not change substantially after adjustments of other influential variables (**Table 3**). After adjustments, the associations for *C4B*, *RUNX1T1*, *MYPN* and the CpG site on chromosome 13 remained significant in 4.5-y-old children, and *ILI7RD, SMOC2, NODAL, PLCG2* were close to significant. We additionally adjusted for betel chewing and food and micronutrient supplementation in a separate analysis but it did not change the results (data not shown).

In general, the effect of Cd on degree of CpG methylation ranged between 0.5–1.4% for a doubling of blood Cd (μg/kg) in the mothers (based on significant effect estimates in both cord blood and 4.5-y-old children in **Table 3**).

Maternal Cd, DNA methylation and birth weight. Among the top 500 CpG sites in cord blood with strongest correlation between methylation and Cd in maternal blood, multiple sites also correlated significantly to birth weight (**Table 4**; unadjusted p values shown, none of which remained statistically significant when adjusted for multiple comparisons). We focused on relationships where: (1) DNA methylation was positively correlated with blood Cd and inversely correlated with birth weight, or (2) DNA methylation was inversely correlated with blood Cd and positively correlated with birth weight (**Table 4**). All associations found when considering both girls and boys were of type 1. We then stratified for sex and found for girls two CpG sites of interest: *TSH7DA* (cg07846874) on chromosome 7 and one site on chromosome X (cg11595135) that were positively associated with Cd (r_s = 0.51, unadjusted p = 0.00007 and r_s = 0.44, p = 0.00082, respectively) and inversely associated with birth weight (r_s = –0.35, p = 0.005; and r_s = –0.37, p = 0.003, respectively). In girls, we also found one site on chromosome 2 (cg00224807) that was inversely associated with maternal blood Cd (r_s = –0.43, p = 0.0012) and positively associated with birth weight (r_s = 0.26, p = 0.036). For boys, we found one site on chromosome 3 (cg19119945) that was inversely associated with maternal blood Cd (r_s = –0.48, p = 0.00016) and positively associated with birth weight (r_s = 0.27, p = 0.036).

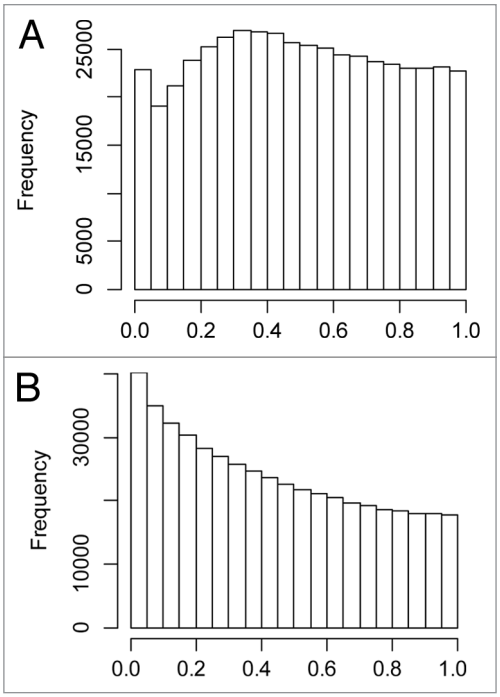

Figure 1. The histograms show the frequency distribution of the p values (x-axis) of the regression coefficients for cadmium from 482, 421 separate regression models, one for each CpG site, of DNA methylation in cord blood vs. cadmium in maternal blood for girls (**A**) and boys (**B**), respectively.

The multivariable-adjusted linear regression analyses for all newborns showed that the associations between Cd-related DNA methylation and birth weight were fairly robust (**Table 4**), and CpG sites in *PTTG1*, *THSD7A*, *BCCIP*, *TMEM179*, *SRP14* and *GDPD1* remained statistically significant after adjustment. We also adjusted for food and micronutrient supplementation (**Table S4**) and betel chewing in a separate analysis but it did not substantially change the results. For the CpG sites presented in **Tables 2–4**, only three out of 63 sites contained a SNP within ≤ 10 bp from the query site (**Table S5**). One of these SNPs has a minor allele frequency of 0.017, for the other two SNPs no allele frequencies were available. Ten out of 63 CpG sites showed a SNP > 10 bp from the query site, but for four of these SNPs no allele frequencies were available or the minor allele frequency was very low.

Discussion

This study indicates that low-level environmental Cd exposure during early pregnancy is associated with sex-specific alterations in DNA methylation in fetal blood. On a global level the effect of Cd was most evident in boys. Boys showed generally global hypermethylation. In contrast, the newborn girls showed markedly more hypomethylation. Interestingly, hypomethylation of repetitive sequences, a crude marker for global methylation, in relation to environmental Cd exposure has been reported for adult women.[18] Moreover, in rats exposed to Cd during pregnancy, CpG sites in the hepatic glucocorticoid receptor were

Table 2. Top 10 correlations (r_s) between maternal blood Cd concentrations (MB-Cd) and DNA methylation (CpG sites) in cord blood of all newborns as well as for girls and boys separately

CHR	Gene	CpG-site	r_s MB-Cd	p value[a]	Rank
All					
3	*GAP43*	cg19676835	0.410	8.66E-06	5
3	*C3orf23*	cg26130396	0.394	2.05E-05	9
5	[b]	cg16667631	0.424	4.00E-06	2
5	*APBB3*	cg21797405	0.413	7.35E-06	4
6	*HIST1H4L*	cg25479916	0.431	2.58E-06	1
6	*RAET1G*	cg01145232	0.404	1.19E-05	8
7	*PTPRN2*	cg06612016	−0.409	9.31E-06	6
14	*PAX9*	cg11194925	0.417	5.70E-06	3
15	*SRP14*	cg10636054	0.393	2.12E-05	10
19	*POLR2E*	cg07607583	0.406	1.10E-05	7
Girls					
2	*HDAC4*	cg26975040	−0.523	4.89E-05	6
2	*KLHL29*	cg09674093	−0.521	5.33E-05	7
4	*SORCS2*	cg19120695	−0.516	6.50E-05	10
5		cg22514112	−0.536	3.01E-05	5
7	*RBM33*[b]	cg03510186	−0.516	6.40E-05	9
8	*SLC45A4*[b]	cg02695267	−0.556	1.30E-05	2
10	*GPR123/TTC40*[b]	cg25679864	−0.569	7.20E-06	1
11	*ADAMTS8*	cg23886747	−0.517	6.31E-05	8
14	*GTF2A1*	cg02167732	0.541	2.38E-05	4
19		cg17335657	−0.542	2.35E-05	3
Boys					
5	*STK10*	cg11161755	0.592	1.57E-06	2
6	*STX7*	cg17201227	0.570	4.57E-06	9
8	*CDH17*	cg20987610	0.566	5.45E-06	10
11	*HRASLS2*	cg00156194	0.587	2.03E-06	3
11		cg10103906	0.585	2.22E-06	4
12	*MYO1H*	cg06955182	0.579	2.92E-06	6
17	*TBCD*	cg03955537	0.609	6.26E-07	1
17		cg13250566	0.572	4.04E-06	8
19	*CD70*	cg26737640	0.582	2.49E-06	5
19	*MATK*	cg13387994	0.577	3.27E-06	7

Sites are listed according to chromosome number. [a]None of the sites were statistically significant after adjustments for multiple comparisons. [b]If present, close gene/-s according to NCBI (www.ncbi.nlm.nih.gov/gene) are shown.

hypomethylated in relation to Cd in female fetuses but hypermethylated in male fetuses.[20] The sex differences in methylation in relation to Cd exposure were further highlighted in the analysis of specific gene functions and pathways; altered methylation of genes for bone morphology and mineralization was found in cord blood of girls, whereas hypermethylation of genes involved in cell death was found in boys. This is noteworthy in relation to the previous findings of the inverse associations of Cd exposure with fetal size, in particular size of the head and femur length, in girls, but not in boys.[21] Also, Cd is associated with osteoporosis and fractures particularly in women.[22] The observed sex-difference is noteworthy considering the emerging data on obvious sexual dimorphism in environmental epigenetic programming.[14]

We found methylation changes in CpG sites in genes associated with both Cd and birth weight when considering all children. The data could not clearly support our hypothesis that girls preferentially should have Cd-related methylation in genes regulating infant size. However, this might have been a question of low statistical power. The multivariable-adjusted analyses indicated a fairly large impact of DNA methylation in the selected sites on birth weight, for one percent-unit increment in DNA methylation; this would correspond to a decrease in birth weight of 7 g to 46 g. This emphasizes the notion that CpG methylation is very important for fetal development. Each doubling of μg/kg Cd in maternal blood resulted in an approximately 1% change in DNA methylation, which theoretically would correspond to up to a 46 g reduction in birth weight. This Cd-related reduction in birth weight is similar in size to what has been observed in girls, a reduction of 45 g per every 1 μg/L increment of Cd in maternal urine.[7]

We found similar Cd-related methylation changes in several specific CpG sites in both cord blood and blood of 4.5-y-old children. These findings suggest that some specific Cd-related changes persist at least to later in childhood, and may be important for later health effects. Thus, it is essential to follow these children to evaluate if the changes become modified later in life.

Some methodological aspects need to be commented upon. It is clear from our study that children's sex was influential for the Cd-related changes. Stratifying for sex markedly increased the effect sizes, largely because of the different directions of the Cd-related DNA methylation in boys and girls. The phenomenon of sex-specific effects on DNA methylation is not limited to exposure to Cd, but occurs for other exposures as well.[23] Sex-specific DNA methylation has been reported in whole blood and saliva samples from adults.[24] One can speculate that Cd might interfere with already existing sex-specific gene expression, or alternatively it causes sex-specific DNA methylation for genes that should not differ between the sexes. However, to our knowledge there is no study that has characterized sex-specific gene expression per se in cord blood to compare with our data. When comparing the sex-specific genes identified in the study with adults by Liu et al.[24] with our data, we found no overlap with the top genes for associations between Cd and DNA methylation that we report in our study.

We measured DNA methylation in cord blood mononuclear cells, which is a mixture of different cell types with partly different methylation patterns, in particular for cell type-specific immune functions[25] that may blur associations between DNA methylation and Cd exposure. We were not able to sort cells in the blood samples during the field studies. Houseman and coworkers[26] describe a method for inferring changes in the distribution of white blood cells between different subpopulations (e.g., cases and controls) using DNA methylation signatures. We did not apply this method since there were no clearly identified

Table 3. CpG sites that showed significant correlations (r_s) in the same direction both between Cd in maternal blood (MB-Cd) and DNA methylation in cord blood and between Cd in urine (U-Cd) and DNA methylation in blood from 4.5-y-old children

			MB-Cd at GW14					U-Cd at 4.5 y			
CHR	Gene	CpG-site[a]	r_s	p value	Beta[b]	p value	Rank	r_s	p value	Beta[c]	p value
3	*IL17RD*	cg13766687	−0.357	0.00013	−0.0093	0.009	94	−0.279	0.0372	−0.0055	0.059
6	*C4B*	cg19699291	−0.328	0.00046	−0.0064	0.009	496	−0.319	0.0166	−0.0044	0.018
6	*RAB23*	cg15068522	0.360	0.00011	0.0059	0.009	81	0.267	0.0467	0.0025	0.13
6	*SMOC2*[d]	cg16001202	−0.350	0.00018	−0.0085	0.034	138	−0.265	0.0480	−0.0056	0.059
8	*RUNX1T1*[d]	cg20309121	0.330	0.00043	0.0082	0.026	437	0.284	0.0342	0.0093	0.036
10	*MYPN*	cg09127607	0.332	0.00039	0.014	0.009	391	0.267	0.0463	0.011	0.026
10	*NODAL*	cg10850838	0.336	0.00034	0.0099	0.007	325	0.336	0.0112	0.0053	0.060
10	*CASC2*[d]	cg09439867	0.342	0.00025	0.018	0.014	219	0.266	0.0473	0.0094	0.12
13		cg25853960	−0.352	0.00016	−0.0063	0.063	117	−0.364	0.0058	−0.0068	0.012
16	*PLCG2*	cg07397481	−0.333	0.00037	−0.0051	0.020	367	−0.304	0.0227	−0.0037	0.055
X	*NAA10*	cg12521678	−0.350	0.00018	−0.0075	0.001	135	−0.315	0.0180	−0.00081	0.54

[a]None of the sites were statistically significant after adjustments for multiple comparisons. [b]Each CpG site vs. MBCd (log_2-transfomed), adjusted for maternal age, BMI (GW8), SES, gestational age, sex and maternal urinary As (GW8). [c]Each CpG site vs. children's U-Cd (log_2-transformed), adjusted for the children's concurrent age, HAZ, urinary As, sex and family SES.[d]The closest gene/-s according to NCBI are shown.

subpopulations in our study population (i.e., exposed and non-exposed newborns). Further, the DNA methylation signatures developed by Houseman et al.[26] were based on whole blood from different adult populations, which are not directly applicable here as they probably differ from cord blood. To note, Cd exposure is not associated with major changes in the blood cell profile, and although subtle changes cannot be ruled out, we consider it unlikely that our results represent shifts in leukocyte profile from Cd exposure. The consistency of several associations in cord blood and in 4.5-y-old children support that the findings indeed were true, and persist during early child development.

It should be noted that none of the correlations for specific CpG sites were statistically significant after adjustments for multiple comparisons and the specific genes identified here must be verified in new studies. Low power is a general problem when analyzing -omics data. Nevertheless, in the multivariable-adjusted linear regression analyses most associations were robust, also when taking other influential factors into account. For the pathway analysis the IPA software uses Fisher's exact test, which does not consider gene dependence structure and pathway hierarchical dependence structures. This might lead to too optimistic results.

Cadmium concentrations in maternal blood were more strongly associated with DNA methylation in cord blood than Cd in urine, which is reasonable, as the blood Cd to a larger extent represents the ongoing exposure, whereas Cd in urine represents the maternal body burden, mainly the accumulation in liver and kidneys.[2] We did not measure Cd in blood for the 4.5-y-old children, but in urine, which probably reflects, to a larger extent, ongoing Cd exposure when they are young, and thus, have not yet accumulated much Cd in their kidneys.

To our knowledge, none of the genes linked to CpG sites presented here have previously been associated with Cd exposure or Cd toxicity and the results need to be cautiously interpreted. One exception is the *PTTG1,* which was more expressed in liver cancer cells in response to Cd exposure in vitro.[27] In contrast, in the present study on healthy newborns *PTTG1* was more methylated with increasing Cd exposure. Methylation in *C4B*, *RUNX1T1* and *MYPN* was significantly associated with Cd-exposure both in newborns and in children at 4.5 y. *C4B* encodes the basic form of complement factor 4, part of the classical complement activation pathway. *RUNX1T1* is the gene for runt-related transcription factor 1; translocated to, 1 (cyclin D-related), a transcription factor that regulates critical processes in many aspects of hematopoiesis.[28] *MYPN* encodes myopalladin, a protein that regulates the formation of striated muscles in vertebrates.[29] In contrast to these three genes, few of the other genes that we found to be significantly associated with both Cd and birth weight have previously been related to developmental growth. BCCIP is important for structural stability of chromosomes and conditional *BCCIP* knock-down transgenic mice show growth retardation and impaired embryonic cell proliferation.[30,31] Thus, one could speculate that in humans increased methylation of *BCCIP* results in reduced growth of the developing child. To note, one CpG site was located in the HOX cluster, between *HOXC12* and *HOXC13*, on the long arm chromosome 12. The homeobox (HOX) family encodes transcription factors that serve as regulators in initiating developmental programs.[32] *THSD7A* was associated with birth weight both in the analyses for all children and for just girls. THSD7A is a protein described in zebrafish to be involved in angiogenesis by endothelial migration.[33]

In conclusion, Cd exposure in pregnancy alters fetal DNA methylation in a sex-specific manner. This may explain previous findings of differences in toxicity of Cd between girls and boys. Some Cd-related DNA methylation changes were also related to lower birth weight, but the consequences for child health and development remains to be elucidated.

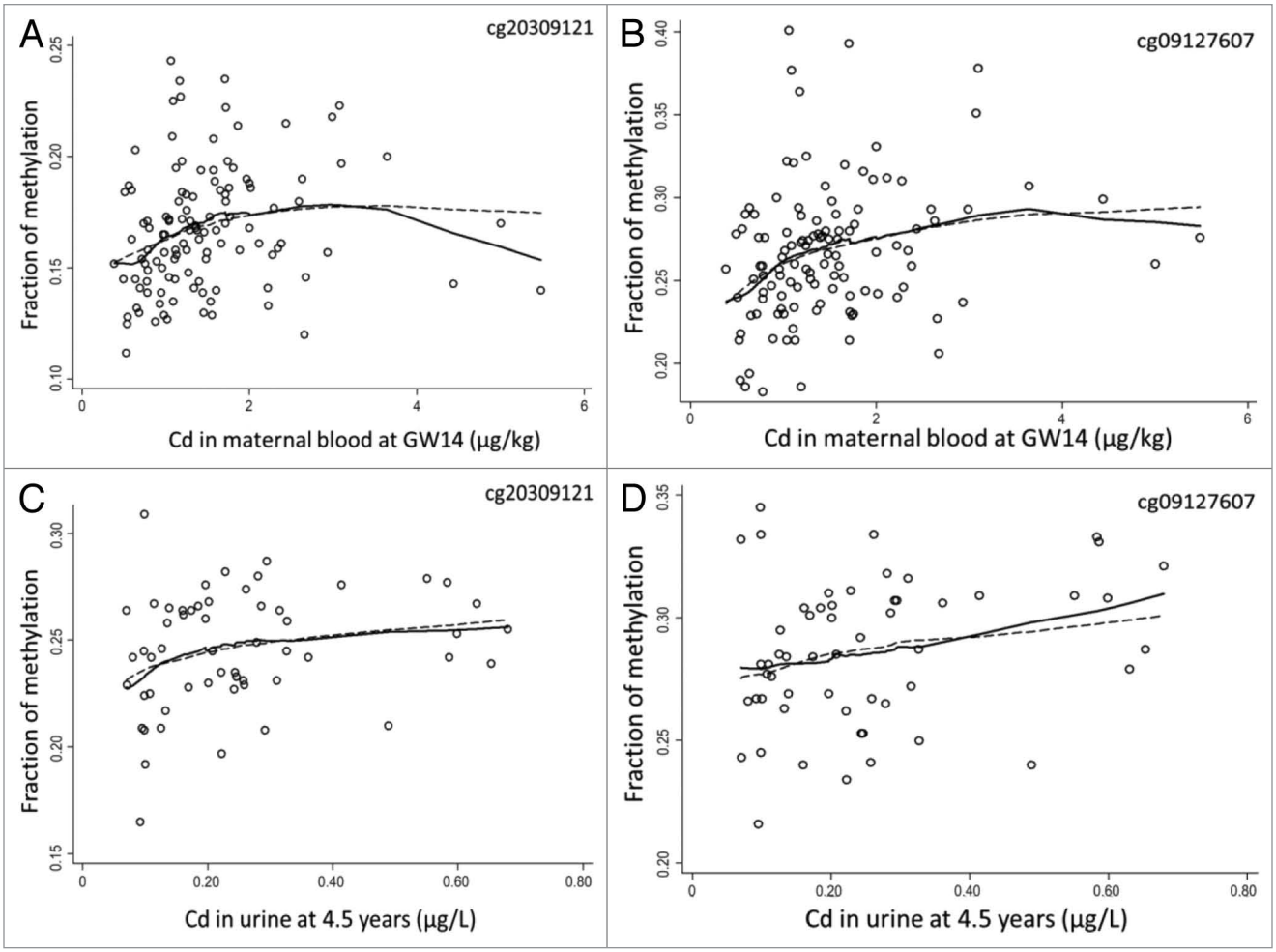

Figure 2. Scatterplots depicting (**A**) fraction of DNA methylation in cord blood for the CpG site cg20309121 in *RUNX1T1 vs.* maternal blood Cd at GW14; and (**B**) fraction of DNA methylation for cg20309121 in peripheral blood from 4.5-y-old children vs. their urinary Cd; (**C**) fraction of DNA methylation in cord blood for cg09127607 in *MYPN vs.* maternal blood Cd at GW14; and (**D**) fraction of DNA methylation for cg09127607 in peripheral blood from 4.5-y-old children vs. their urinary Cd. Solid lines represent Lowess-moving average curves; dashed lines represent fitted curves from the multivariable-adjusted regression analyses defined in **Table 3**.

Materials and Methods

Study area and subjects. Our studies on early-life Cd effects on fetal and child health and development were nested into a randomized population-based food and micronutrient supplementation trial in pregnancy (MINIMat trial)[34] involving 4436 pregnant women recruited from November 2001 through October 2003. Study area was the Matlab sub-district located approximately 50 km southeast of Dhaka, Bangladesh, where the International Centre for Diarrheal Disease Research, Bangladesh (icddr,b) runs a health and demographic surveillance system (HDSS), a hospital and four health clinics.

Pregnancy was initially identified by urine test, which was offered to women who reported that their last menstrual period (LMP) was overdue at the monthly home visit by community health research workers. In case of a positive result, the women were invited to participate in the MINIMat trial. The eligibility criteria for enrollment in the MINIMat trial included viable fetus, gestational age < 14 weeks, no severe illness, and consent for participation.[34] The intervention included randomized supplementation of both food (early invitation at GW9 or usual invitation at approximately GW20) and micronutrients (two groups with different combinations of iron and folic acid, one with 13 additional micronutrients), resulting in six different groups.[34,35]

For the present study of Cd exposure and DNA methylation we used a sub-sample of 127 women who were enrolled from October 2002 through October 2003, gave singleton birth at the health care facilities during early day-time, and had cord blood collected at delivery.[36] The main reasons for the low number of deliveries was the high frequency of home deliveries (> 60%)[37] and that many deliveries at the health facilities occurred in late afternoon or night, when the logistics didn't allow for processing and transporting of samples to the laboratory in Dhaka. In comparison to all other women who were enrolled in the MINIMat trial from October 2002 through October 2003 and gave singleton birth (n = 1729), these 127 women had slightly higher SES and were more likely to be primiparous (**Table 1**).

Table 4. CpG sites in cord blood that were significantly correlated (r_s) to both to Cd in maternal blood (MB-Cd) and birth weight

			MB-Cd at GW14					Birth weight (g)			
CHR	Gene	CpG-site[a]	r_s	p value	Beta[b]	p value	Rank Cd	r_s	p value	Beta[c]	p value
1	*CAMTA1*	cg05966431	0.344	0.00024	0.015	0.001	204	−0.199	0.02486	−19.5	0.086
3	*CACNA2D3*[d]	cg04075781	0.345	0.00022	0.018	0.002	187	−0.213	0.0161	−15	0.10
3	*FXR1*[d]	cg06507285	0.352	0.00017	0.014	0.014	124	−0.201	0.0233	−14	0.11
5	*UBE2QL1*	cg10935612	0.339	0.00029	0.013	0.013	266	−0.181	0.0420	−16	0.11
5	*PTTG1*	cg21784134	0.348	0.00020	0.0071	0.014	158	−0.230	0.0093	−35	0.049
7	*THSD7A*	cg07846874	0.346	0.00021	0.060	0.007	180	−0.295	0.0008	−7.6	0.001
7	*LRWD1*	cg20646995	0.359	0.00012	0.0052	0.002	87	−0.188	0.0347	−40.5	0.18
7	*LAMB1*	cg10064162	0.330	0.00043	0.0075	0.034	439	−0.194	0.0289	−27	0.062
8		cg25512848	0.334	0.00037	0.0070	0.022	365	−0.212	0.0169	−32	0.054
10	*UPF2*[d]	cg14136502	0.338	0.00031	0.011	0.0072	289	−0.187	0.0348	−21	0.088
10	*CXCL12*	cg18618334	0.344	0.00023	0.0071	0.010	197	−0.204	0.0216	−29.5	0.11
10	*BCCIP*	cg26735793	0.337	0.00031	0.0048	0.035	298	−0.239	0.0068	−46	0.037
10	*TTC40*[d]	cg23987897	0.329	0.00045	0.0071	0.001	469	−0.210	0.0176	−26	0.30
11	*TMEM9B*	cg26577738	0.332	0.00040	0.018	0.007	397	−0.219	0.0132	−13	0.081
12	*HOXC12/HOXC13*[d]	cg02066277	0.336	0.00034	0.015	0.003	327	−0.190	0.0325	−20	0.050
12	*FICD*	cg09475324	0.334	0.00036	0.0045	0.038	348	−0.194	0.0286	−22	0.36
14	*TMEM179*	cg22943329	0.347	0.00020	0.0088	0.044	166	−0.208	0.0188	−26	0.028
15	*SRP14*	cg10636054	0.393	0.00002	0.018	0.005	10	−0.183	0.0391	−19	0.026
17	*GDPD1*	cg06598597	0.329	0.00044	0.0066	0.017	458	−0.203	0.0223	−41	0.025
18	*DCC*	cg18841634	0.340	0.00028	0.0064	0.034	249	−0.176	0.0472	−22	0.20
19	*C19orf44*	cg10226967	0.350	0.00018	0.016	0.003	143	−0.243	0.0059	−17	0.072
21	*AIRE*	cg00495713	0.342	0.00025	0.0053	0.015	216	−0.179	0.0444	−41.5	0.076

[a]None of the sites were statistically significant after adjustments for multiple comparisons. [b]Each CpG site vs. MB-Cd (log_2-transfomed), adjusted for maternal age, BMI (GW8), SES, gestational age, sex and maternal urinary As (GW8). [c]Birth weight vs. each CpG site, adjusted for maternal age, BMI (GW8), SES, gestational age, sex, MB-Cd (GW14) and maternal urinary As (GW8). Effect size: change in birth weight (g) per percentage increment in DNA methylation. [d]The closest gene/-s according to NCBI are shown.

For comparison, we also studied Cd-related DNA methylation in blood mononuclear cells from 56 children at 4.5 y of age (**Table S1**). These children also originated from the MINIMat trial, but they were independent of the 127 pregnant women and were therefore used as an independent comparison group for follow-up of Cd-related DNA methylation later in life.

Participants gave written, informed consent, and the study was approved by the ethical review committees at icddr,b, in Bangladesh and at Karolinska Institutet, Sweden.

Exposure assessment. We measured Cd in maternal blood (erythrocyte fraction; hereafter referred to as blood Cd), a marker of ongoing exposure. We also measured Cd in maternal and child urine, which reflects long-term exposure. Maternal blood and urine samples were collected at GW8 and 14, respectively. Sample collection was performed either at the health care facilities or at home. Urine was collected in acid-washed plastic vials and blood in 5.5 mL Li-Heparin tubes.

Cadmium (Cd111) in urine and blood was measured with inductively coupled plasma mass spectrometry (ICPMS; model 7500ce; Agilent Technologies) at Karolinska Institutet with correction cell in helium mode. The sample preparation and details concerning the ICPMS analyses have been described in detail elsewhere.[38] No samples were below limit of detection (overall < 0.01 μg/L) and the quality control showed good agreement with the recommended concentrations. To compensate for variation in urine dilution, we adjusted for the average specific gravity of the urine (1.012 g/mL both in maternal and child urine).

DNA isolation and epigenetic analysis. DNA was isolated using QIAamp DNA Blood Mini kit (Qiagen) at icddr,b. DNA quality was evaluated on a NanoDrop spectrophotometer (NanoDrop Products) and a Bioanalyzer 2100 (Agilent) and showed good quality (260/280 nm > 1.80). One μg DNA (50 ng/μL) was bisulfite-treated using the EZ DNA Methylation kit (Zymo, D5001). Cord blood DNA samples were randomized for sex and maternal blood Cd concentrations on two 96-well plates for epigenetic analysis with the Infinium HumanMethylation450K BeadChip (lllumina). The samples from 4.5 y-old children were all positioned in one of the 96-well plates, but randomized for sex and Cd exposure within the plate. We also included four controls in duplicate, with each duplicate positioned on different 96-well plates and on different HumanMethylation450K BeadChips: three control samples were DNA extracted from blood and one sample was demethylated DNA (from Zymo). The SCIBLU facility used 200 ng

bisulphite-treated DNA, diluted with H_2O to 4 μl, for hybridization to the Infinium HumanMethylation450K BeadChip following the manufacturer's instructions. Unsupervised hierarchical cluster analysis showed that all duplicate clustered together despite being on different plates.

Birth weight and covariates. Birth weight was measured mostly within 24 h of delivery, using electronic scales (SECA pediatric scales) with precision of 10 g.[37] Gestational age was calculated by subtracting the date of LMP from the date of delivery. After delivery, mothers were asked about their smoking and betel chewing habits during pregnancy. None of the women reported smoking, whereas 58% reported betel chewing. For assessment of socioeconomic status (SES) an asset index, based mainly on house construction and household assets, was calculated using principal component analysis (PCA).[39] Height and weight of the children at 4.5 y of age were converted to age- and sex-standardized *z*-scores [weight-for-age (WAZ), and height-for-age (HAZ)], using the World Health Organization growth references].[40]

Because we have previously shown that the present study population is exposed to arsenic (As) via drinking water[41,42] and As exposure has been associated with DNA methylation,[43] we adjusted for concentrations of urinary As in the statistical models. Measurements of inorganic As and its methylated metabolites had previously been measured in maternal urine during pregnancy (GW8; hereafter referred to as As in urine) by hydride generation atomic absorption spectrometry,[42] and in children's urine at 4.5 y of age by high-performance liquid chromatography online with hydride generation and ICPMS.[41]

Statistical analysis. Methylation levels are specified by so-called β-values, which represent the fraction of methylation and hence range from 0 (unmethylated) to 1 (fully methylated). Beta-values were extracted from BeadStudio software (lllumina, San Diego, CA USA). Missing values (0.1%) were imputed using k-nearest neighbor imputation (k = 10).

Principal component analysis (PCA) captures the major directions of variation in the data. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component in turn accounts for as much of the variability possible given it is uncorrelated with the preceding components. For PCA, the R package "swamp" was employed. For each of the top principal components we fitted a univariate linear model with each of the sample annotations as regressor. The log10 p values of the models' F-statistics were plotted as a heat map (**Fig. S1**). PCA was run separately for newborns and 4.5-y-old children. The analysis for cord blood samples showed that analysis plate (two 96-well plates) was associated with cord methylation levels in the first ($p = 10^{-6}$) and third components ($p < 10^{-10}$). We removed the plate influence at each CpG site by using the residuals from the initial linear regression model of methylation with analysis plate as regressor. The residuals of the linear model added to the total mean before correction became the new data for each CpG site. In this way, the levels of each of the methylation sites were unrelated to analysis plate. No other variables had a major impact on general DNA methylation.

We evaluated whether the Cd exposure was associated with global DNA methylation by performing 482,421 separate linear regression models, one for each CpG site, where CpG methylation was the dependent variable and Cd concentrations the only independent variable. The range of Cd was rescaled to vary between 0 and 1. We tested whether the slope was statistically significant in all 482,421 models; if there was no effect of Cd on any CpG site, then the slopes would result from mere sampling error. In this case the p values would be distributed uniformly over the 0-to-1 range.

The associations of specific CpG site methylation levels with variables of interest were then evaluated by Spearman correlation. Resulting p values were corrected for multiple testing (n = 482,421) by the Benjamini-Hochberg method to obtain false discovery rates (FDR). Among the 500 top correlations of Cd in maternal blood with methylation in CpG sites in all newborns, we further analyzed those that also were correlated with birth weight. In addition, we compared the correlations to those in 4.5-y-old children. The associations with methylation in the CpG sites were subsequently evaluated using scatterplots and multivariable-adjusted regression modeling.

In general, associations of DNA methylation in different CpG sites with Cd exposure (maternal blood Cd and children's urinary Cd) and birth weight showed linear patterns, hence linear regression analyses were applied. Because the Cd concentrations in maternal blood and children's urine were left skewed (**Fig. 2A–D**) these variables were $\log_2$-transformed. We did not observed any genotype-related clustering of DNA methylation when visually inspecting associations between Cd and DNA methylation, and associations between birth weight and DNA methylation. When evaluating DNA methylation in cord blood in relation to maternal blood Cd concentrations, we adjusted for maternal age, BMI (GW8), SES, gestational age at birth, fetal sex and maternal urinary As (GW8). A similar strategy was applied when children's urinary Cd (4.5 y) was used as an exposure marker. We adjusted the model by children's concurrent age, HAZ, SES, children's concurrent urinary As, and sex of the child. When assessing the association between DNA methylation in cord blood and birth weight, we adjusted for maternal age, BMI (GW8), SES, gestational age at birth, fetal sex, maternal blood Cd and maternal urinary As. To evaluate potential sex differences, we also stratified the analyses by sex. In sensitivity analyses we tested if betel chewing or the food and micronutrient supplementation ingested during pregnancy had any impact on the above-mentioned associations.

For the bioinformatics analysis we used the Ingenuity Pathway Analysis Tool (IPA Tool; Ingenuity H Systems; www.ingenuity.com). We selected the top 500 CpG sites associated with Cd in maternal blood: that resulted in 390 gene-annotated (obtained from Illumina) CpG sites in boys, 348 annotated CpG sites in girls. In IPA, differentially methylated genes are mapped to genetic networks available in the Ingenuity database and then ranked by score. The Ingenuity Pathway Knowledge Base is derived from known functions and interactions of genes in the literature. The networks created are ranked depending on the number of significantly methylated genes they contain and also list diseases that were most significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/24401

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