Brief Report

Timing- and Dose-Specific Associations of Prenatal Smoke Exposure With Newborn DNA Methylation

Giulietta S. Monasso MD[1,2], Vincent W. V. Jaddoe MD, PhD[1,2], Johan C. de Jongste MD, PhD[1,2], Liesbeth Duijts MD, PhD[1,2], Janine F. Felix MD, PhD[1,2]

[1]The Generation R Study Group, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands;
[2]Department of Pediatrics, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands

Corresponding Author: Janine F. Felix, MD, PhD, Generation R Study Group (Na-2918), Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands. Telephone: +31-10-7043405; Fax: +31-10-7044645; E-mail: j.felix@erasmusmc.nl

Abstract

Introduction: Fetal changes in DNA methylation may underlie associations of maternal smoking during pregnancy with adverse outcomes in children. We examined critical periods and doses of maternal smoking during pregnancy in relation to newborn DNA methylation, and associations of paternal smoking with newborn DNA methylation.

Aims and Methods: This study was embedded in the Generation R Study, a population-based prospective cohort study from early pregnancy onwards. We assessed parental smoking during pregnancy using questionnaires. We analyzed associations of prenatal smoke exposure with newborn DNA methylation at 5915 known maternal smoking-related cytosine-phosphate-guanine sites (CpGs) in 1261 newborns using linear regression. Associations with false discovery rate-corrected *p*-values < .05 were taken forward.

Results: Sustained maternal smoking was associated with newborn DNA methylation at 1391 CpGs, compared with never smoking. Neither quitting smoking early in pregnancy nor former smoking was associated with DNA methylation, compared with never smoking. Among sustained smokers, smoking ≥5, compared with <5, cigarettes/d was associated with DNA methylation at seven CpGs. Paternal smoking was not associated with DNA methylation, independent of maternal smoking status.

Conclusions: Our results suggest that CpGs associated with sustained maternal smoking are not associated with maternal smoking earlier in pregnancy or with paternal smoking. Some of these CpGs show dose–response relationships with sustained maternal smoking. The third trimester may comprise a critical period for associations of smoking with newborn DNA methylation, or sustained smoking may reflect higher cumulative doses. Alternatively, maternal smoking limited to early pregnancy and paternal smoking may be associated with DNA methylation at specific other CpGs not studied here.

Implications: Our results suggest that quitting maternal smoking before the third trimester of pregnancy, and possibly lowering smoking dose, may prevent differential DNA methylation in the newborns at CpGs associated with sustained smoking. If the relevance of DNA methylation for clinical outcomes is established, these results may help in counseling parents-to-be about quitting smoking.

Introduction

Maternal smoking during pregnancy is associated with adverse child health outcomes.[1,2] There is little information on critical time-windows or critical doses for prenatal smoke exposure. Observational studies suggest that smoking restricted to early pregnancy and low doses may have limited impact on newborn development.[2–4]

DNA methylation may underlie associations of prenatal smoke exposure and adverse outcomes. A large meta-analysis of epigenome-wide association studies (EWAS) showed associations of sustained smoking in pregnancy with cord blood DNA methylation at 6073 cytosine-phosphate-guanine sites (CpGs).[5] Only few studies explored associations of timing and dose of prenatal smoke exposure with newborn DNA methylation, either epigenome-wide or at specific candidate CpGs.[6–10] These suggested effects of timing or dose on DNA methylation, but maternal smoking timing was often not assessed in detail. Specifically, differentiating between mothers who never smoked, quit before pregnancy, or quit early in pregnancy could be informative. Associations of paternal smoking with new-born DNA methylation could reflect either transmission through the sperm epigenome or in utero exposure to maternal second-hand smoking. One previous study examined paternal smoking at 26 maternal smoking-related CpGs, but found no associations.[10]

In this hypothesis-generating study, we focused on the 6073 CpGs with robust evidence for association with sustained maternal smoking in pregnancy from a previous meta-analysis.[5] We categorized maternal smoking in well-defined time windows. We examined dose effects among sustained smokers only. We additionally explored associations of paternal smoking with DNA methylation at these maternal smoking-associated CpGs.

Methods

Study Design

The analyses were embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards.[11] The Medical Ethical Committee of Erasmus MC approved the study (MEC 198.782/2001/31). Pregnant women with an expected delivery date between April 2002 and January 2006 living in Rotterdam were eligible to participate and 9778 women were included. We obtained written informed consent for all participants. In a subgroup of 1396 newborns, we measured cord blood genome-wide DNA methylation.

Here, we included newborns with complete information on DNA methylation, parental smoking during pregnancy and covariates. For the 15 mothers with two (nontwin) children in the analysis, we excluded one child by selecting on data completeness ($N = 5$) or, if both complete ($N = 10$), randomly (Supplementary Figure 1).

Parental Smoking

As described previously, information on maternal smoking before and during pregnancy was collected through three questionnaires.[3] Mothers reported on smoking timing and daily cigarette dose, in six categories ranging from <1 to >20 per day, at gestational ages <18 weeks (early pregnancy), 18–25 weeks (midpregnancy), and ≥25 weeks (late pregnancy). We constructed one variable describing maternal smoking behavior based on the answers in the three questionnaires: never smoker (reference); former smoker (quit smoking at any time before pregnancy); early quitter (quit smoking when pregnancy was known); and sustained smoker (smoked in second and/

or third trimester). Among sustained smokers, we recategorized the reported daily cigarette dose in the third trimester into <5 and ≥5. We constructed one variable describing paternal smoking (biological fathers only) based on maternal report on the question if the father smoked. Previous analyses showed a good agreement with paternal self-report.[12]

DNA Methylation

We used the salting-out method to extract DNA from cord blood samples. Five-hundred nanograms of DNA was bisulfite converted using the EZ-96 DNA Methylation kit (Shallow) (Zymo Research Corporation, Irvine, CA). Samples were processed with the Illumina Infinium HumanMethylation450 BeadChip (Illumina Inc., San Diego, CA). Quality control and normalization were performed using the CPACOR workflow.[13] Probes with a detection *p*-value ≥ 1E-16 were set to missing. Intensity values were quantile normalized. Arrays with technical problems, a call rate ≤ 95%, or a mismatch between sex of the proband and sex determined by chromosome X and Y probe intensities were removed. The final DNA methylation data set contained information on 458 563 CpGs, including 5915 of the 6073 CpGs associated with sustained smoking in the published meta-analysis.[5] We used untransformed beta-values as measures of DNA methylation.

Covariates

Directed acyclic graphs are presented in Supplementary Figure 2a and b. We obtained information on parental age, parental pre-pregnancy body mass index (BMI), maternal education, and parity from questionnaires, and newborn sex and birthweight (sex and gestational age-adjusted standard deviation scores) from midwife and hospital records.[14] We compared newborns included in the study with those not included.

Statistical Analysis

We used linear regression models to analyze associations of prenatal smoke exposure (maternal smoking: timing and dose; paternal smoking: any) and cord blood DNA methylation at the 5915 previously identified maternal smoking-related CpGs available in our data set.[5] Analyses of smoking dose were conducted among sustained smokers only, comparing high (≥5) to low (<5) daily cigarette doses during the third trimester. In a trend test, we analyzed dose as a continuous variable, using never smokers, former smokers, and early quitters combined as the reference. We studied paternal smoking during pregnancy (biological fathers only), first among newborns with nonsmoking mothers. Second, to ensure that this selection did not bias the results, we included all newborns, adjusting for maternal smoking status.

We ran multiple models using R 3.6.1 (R Core Team, 2013). First, crude models were adjusted for batch (plate number) and white blood cell types (CD4+ and CD8+ T-lymphocytes, natural killer cells, B-lymphocytes, monocytes, granulocytes) estimated by the cord-blood specific "Gervin" reference panel.[15,16] Second, the main models were additionally adjusted for maternal age and education, parity, and sex.[5] The paternal smoking model was additionally adjusted for paternal age, and in the analysis among all newborns also for maternal smoking (sustained vs. nonsustained). Third, the main models were additionally adjusted for maternal BMI and the paternal model also for paternal BMI. Fourth, the main models were additionally adjusted for birthweight.[14]

DNA methylation levels differ per cell type; therefore, correction for estimated white blood cell subtypes is needed. There is no consensus on which cord-blood specific reference panel should be used for cell type estimation. To ensure robust results, independent of the reference panel, we repeated the main timing analysis using the "Bakulski"[17] rather than the "Gervin" panel,[15] as well as without cell-type adjustment. The "Bakulski" panel includes the six white blood cell subtypes from the "Gervin" panel plus nucleated red blood cells.

For all analyses, we calculated the correlations between the effect estimates of the main models versus all other models. For the significant CpGs of the main model, we calculated the change in effect estimates in the BMI and birthweight model.

We accounted for multiple testing by applying the false discovery rate (FDR) method of Benjamini and Hochberg.[18] Associations with FDR-corrected *p*-values of <.05 were taken forward.

Results

Participant Characteristics
Participant characteristics are shown in Table 1 and Supplementary Table 1. Newborns included in the analyses less often had a sustained smoking mother or smoking father, compared with those not included. Furthermore, they had older parents and higher educated mothers with slightly lower BMI (Supplementary Table 2).

Timing
In the main model, sustained maternal smoking during pregnancy was associated with newborn DNA methylation at 1391 CpGs, compared with never smokers (Table 2; Supplementary Table 3a).

Of these, 941 CpGs (67.6%) had lower DNA methylation levels. Cg05575921 (*AHRR*) showed the strongest association, with a 6.9% lower DNA methylation level (standard error = 0.4, FDR *p*-value = 2.11E-67). Neither quitting smoking early in pregnancy (smallest FDR *p*-value = .08) nor former smoking (smallest FDR *p*-value = .63) was associated with newborn DNA methylation, compared with never smoking in any of the models (Supplementary Table 3b and c).

When compared with the main model, additional adjustment for BMI or birthweight decreased the number of associated CpGs to 988 and 1206, respectively. For the 1391 CpGs associated with sustained maternal smoking, the mean change in effect estimates was 9.5% in the BMI model and 5.1% in the birthweight model. Results of the Bakulski-adjusted model and model without cell-type adjustment were very similar (Supplementary Table 3a–c). For all analyses, there were strong correlations between the effect estimates of the sensitivity models when compared with the main models (*r* > .92, *p* < .001).

Dose
Among 157 sustained smoking mothers, the third-trimester dose ranged between <1 and >20 cigarettes/d (Supplementary Table 1). Smoking ≥5 cigarettes (*N* = 76) was associated with newborn DNA methylation levels at seven CpGs in the main model (smallest FDR *p*-value = .001), when compared with smoking <5 cigarettes (*N* = 81): cg05575921 (*AHRR*), cg11641006 (*ARL4C*), cg07339236 (*ATP9A*), cg01621764 (*ACTL9*), cg04180046 (*MYO1G*), cg12803068 (*MYO1G*), and cg22377963 (*BCL7C*) (Supplementary Table 3d; Supplementary Figure 3). The trend analysis among 1260 newborns (1103 with nonsmoking mother) showed evidence toward

Table 1. Characteristics of the study population

	Maternal smoking: timing					Paternal smoking: any smoking[1]		
	Total mothers (*N* = 1261)	Never smoking mother (*N* = 708)	Former smoking mother (*N* = 251)	Early quitting mother (*N* = 125)	Sustained smoking mother (*N* = 177)	Total fathers (*N* = 938)	Nonsmoking father (*N* = 656)	Smoking father (*N* = 282)
Maternal age (y)	31.7 (4.2)	32.0 (3.9)	31.9 (3.9)	31.5 (4.2)	30.3 (5.5)	31.9 (3.9)	32.1 (3.8)	31.5 (4.0)
Maternal BMI (kg/m²)	23.2 (3.8)	23.3 (3.9)	23.5 (3.9)	22.5 (2.9)	23.2 (4.1)	23.4 (3.9)	23.2 (3.8)	23.7 (4.1)
Maternal education[2]								
Low	441 (35.0)	205 (29.0)	84 (33.5)	40 (32.0)	112 (63.3)	282 (30.1)	186 (28.4)	96 (34.0)
High	820 (65.0)	503 (71.0)	167 (66.5)	85 (68.0)	65 (36.7)	656 (69.9)	470 (72.6)	186 (66.0)
Parity								
Nulliparous	764 (60.6)	414 (58.5)	166 (66.1)	88 (70.4)	96 (54.2)	570 (60.8)	382 (58.2)	188 (66.7)
Multiparous	497 (39.4)	294 (41.5)	85 (33.9)	37 (29.6)	81 (45.8)	368 (39.2)	274 (41.8)	94 (33.3)
Newborn sex								
Male	640 (50.8)	345 (48.7)	130 (51.8)	61 (48.8)	104 (58.8)	467 (49.8)	317 (48.3)	150 (53.2)
Female	621 (49.2)	363 (51.3)	121 (48.2)	64 (51.2)	73 (41.2)	471 (50.2)	339 (51.7)	132 (46.8)
Birth weight SDS[3]	0.10 (0.99)	0.16 (0.95)	0.17 (0.97)	0.18 (0.97)	−0.33(1.06)	0.17 (0.96)	0.20 (0.95)	0.09 (0.95)
Paternal age (y)						34.2 (4.9)	34.4 (4.9)	33.6 (4.8)
Paternal BMI (kg/m²)						25.2 (3.1)	25.1 (3.1)	25.2 (3.1)

Table displays characteristics of participants included in the main models of the maternal smoking timing analysis and the paternal smoking analysis. For characteristics of participants included in the dose analysis, see Supplementary Table 1. Values are mean (standard deviation) or *N* (percentage). (1) Analysis of paternal smoking among newborns with never or former smoking mothers only. For results of the sensitivity analysis among all newborns, see Supplementary Table 3e. (2) Highest completed education, categorized into lower (secondary education or less) versus higher (more than secondary education). (3) Birthweight corrected for sex and gestational age at birth.[14] Missing data: maternal smoking timing (*N* = 135), paternal smoking (*N* = 458), maternal BMI (*N* = 205), maternal education (*N* = 20), parity (*N* = 2), birthweight (*N* = 1), paternal age (*N* = 18), and paternal BMI (*N* = 51). BMI = body mass index; SDS = standard deviation score.

Table 2. Associations of sustained maternal smoking during pregnancy with newborn DNA methylation, when compared with never smoking

CpG	Mapped gene	Nearest gene (within 10 Mb)	Effect estimate (beta)	Standard error	*p*-value	*p*-value (FDR)
cg05575921	*AHRR*	*AHRR*	−0.069	0.004	3.57E-71	2.11E-67
cg18316974	*GFI1*	*GFI1*	−0.059	0.004	8.69E-37	2.57E-33
cg04180046	*MYO1G*	*MYO1G*	0.051	0.004	1.09E-29	2.15E-26
cg12803068	*MYO1G*	*MYO1G*	0.068	0.006	4.18E-28	6.18E-25
cg09935388	*GFI1*	*GFI1*	−0.083	0.008	1.21E-26	1.43E-23
cg14179389	*GFI1*	*GFI1*	−0.064	0.007	5.85E-22	5.77E-19
cg06338710	*GFI1*	*GFI1*	−0.044	0.005	2.07E-21	1.75E-18
cg25949550	*CNTNAP2*	*CNTNAP2*	−0.023	0.002	3.41E-21	2.52E-18
cg22132788	*MYO1G*	*MYO1G*	0.039	0.004	1.45E-19	9.52E-17
cg12876356	*GFI1*	*GFI1*	−0.085	0.010	7.68E-18	4.54E-15
cg18146737	*GFI1*	*GFI1*	−0.098	0.012	5.35E-16	2.87E-13
cg23067299	*AHRR*	*AHRR*	0.033	0.004	1.26E-15	6.21E-13
cg19089201	*MYO1G*	*MYO1G*	0.028	0.004	2.97E-15	1.35E-12
cg21161138	*AHRR*	*AHRR*	−0.022	0.003	9.93E-14	4.19E-11
cg11902777	*AHRR*	*AHRR*	−0.015	0.002	5.28E-13	2.08E-10
cg09662411	*GFI1*	*GFI1*	−0.050	0.007	7.81E-12	2.89E-09
cg04598670	—	*AUTS2*	−0.031	0.005	1.14E-11	3.96E-09
cg08606254	*AHRR*	*AHRR*	0.022	0.003	4.62E-11	1.52E-08
cg22549041	*CYP1A1*	*CYP1A1*	0.055	0.008	8.46E-11	2.63E-08
cg14817490	*AHRR*	*AHRR*	−0.025	0.004	9.16E-11	2.71E-08
cg18703066	—	—	−0.011	0.002	3.15E-10	8.87E-08

Shown are all CpGs with a FDR-corrected *p*-value of <1E-07 in the main model. Full results are shown in Supplementary Table 3a.

a dose effect at 1167 CpGs (smallest FDR *p* for trend 1.85E-87 for cg05575921 [*AHRR*]).

Paternal Smoking

Among 938 newborns with a never smoking or former smoking mother, 282 had a smoking father. Paternal smoking during pregnancy was not associated with DNA methylation in the main model (smallest FDR *p*-value = .54; Supplementary Table 3e). A sensitivity analysis among 1221 newborns (483 with smoking father), adjusting for maternal smoking status, showed similar results (Supplementary Table 3e).

Discussion

These analyses suggest that newborn DNA methylation levels at CpGs associated with sustained maternal smoking are not associated with former maternal smoking or quitting smoking early in pregnancy. We observed some evidence of dose effects among sustained smoking mothers during the third trimester. Paternal smoking was not associated with newborn DNA methylation at these CpGs.

Timing

Fetal changes in DNA methylation might underlie the known associations of maternal smoking during pregnancy with adverse health outcomes in the children.[1,2] A meta-analysis reported associations of sustained maternal smoking during pregnancy with over 6000 CpGs.[5] Here, sustained maternal smoking during pregnancy was associated with newborn DNA methylation at 24% of the 5915 evaluated CpGs. Among these was the consistently reported cg05575921 (*AHRR*).[5,7–9] Aryl-Hydrocarbon Receptor Repressor is a transcription factor, part of the aryl hydrocarbon receptor signaling pathway, involved in the detoxification of toxins such as dioxin and polyaromatic hydrocarbons, both substances of cigarette smoke.[8] The fact that we found no associations for 76% of the evaluated

CpGs most likely reflects the smaller sample size in this study when compared with the meta-analysis.[5]

Neither maternal former smoking nor quitting smoking in early pregnancy was associated with newborn DNA methylation at these CpGs. As we only analyzed CpGs known to be associated with sustained smoking, we could not detect CpGs specifically associated with former smoking or early quitting, but not with sustained smoking. Our results are largely in line with previous studies.[6–10] Four of these studies observed associations of sustained smoking with newborn DNA methylation.[6–8,10] Of the two studies that included analyses of early quitting,[6,10] one did not observe any associations of early quitting with DNA methylation.[10] The other study reported a single CpG, cg01290904 (*EVC*) specifically associated with early quitting in a Japanese population.[6] This CpG was not among the 5915 CpGs analyzed in our study, but it was found to be associated with adult former smoking in a large EWAS meta-analysis.[19]

Our analyses suggest that quitting smoking earlier in pregnancy may still generate benefit in terms of associations with newborn DNA methylation at CpGs associated with sustained smoking. Future studies should explore whether this results from a lower cumulative dose or less exposure during a critical period in pregnancy. Whether smoking earlier in pregnancy is associated with DNA methylation at other CpGs not studied here remains to be elucidated.

Dose

Previous studies showed associations of the number of cigarettes smoked by pregnant women with fetal growth and birth weight, suggesting a dose–response association.[1,2] Four EWAS examined and found dose effects for some CpGs.[7–10] We observed evidence for dose–response effects in late pregnancy at seven CpGs and a trend toward dose-dependent associations at 1167 CpGs. Except for cg01621764 (*ACTL9*) and cg22377963 (*BCL7C*), the dose-associated CpGs in our study have been examined in relation

to maternal smoking dose previously.[7–9] Of these, cg05575921 (*AHRR*), cg07339236 (*ATP9A*), cg04180046 (*MYO1G*), and cg12803068 (*MYO1G*) showed a tendency toward dose-dependent associations in at least two studies.[7–9]

Paternal Smoking

Like maternal smoking, paternal smoking has been associated with adverse child health outcomes.[2,3,20] If these associations are mediated through DNA methylation, this could either reflect transmission through effects on the sperm epigenome, or in utero exposure to secondhand smoke.[10] In line with a previous study, focusing on a small number of CpGs, we observed no associations of paternal smoking with newborn DNA methylation at CpGs associated with sustained maternal smoking, independent of the mother's smoking status.[10] A recent full EWAS did report evidence for associations of paternal smoking with differential DNA methylation in offspring at older ages, after adjustment for maternal smoking.[21] This indicates that there may be CpG sites at which DNA methylation is specifically related to paternal smoking.

Strengths and Limitations

This study was embedded in a large population-based prospective cohort study, and the sample size for DNA methylation was relatively large. However, this study may still not be adequately powered to find small effects. Therefore, our results should be considered as hypothesis generating. Because of power, we only included CpGs previously identified in relation to sustained maternal smoking. Consequently, we may have failed to detect CpGs specifically associated with maternal smoking earlier in pregnancy or with paternal smoking. The Generation R Study provided 13% of the meta-analysis sample size, so has contributed to the discovery of the CpGs of interest.[5] As DNA methylation is tissue specific, the relevance of findings in blood in relation to other tissues needs further study. For example, maternal smoking during pregnancy has been associated with childhood asthma.[1] To better understand the role of DNA methylation in this, analyses of DNA methylation in lung tissue, which may be more relevant for respiratory phenotypes, should be performed. This study included relatively few heavy smokers, most smokers consumed low doses and variation in dose was limited. Furthermore, there may be measurement error in dose, as women may not have accurately reported small differences in daily smoking dose. These factors may have led to underestimation of any dose–response effects.

Conclusion

Our results indicate that CpGs robustly associated with sustained maternal smoking are not associated with maternal smoking earlier in pregnancy or with paternal smoking and that the association with sustained maternal smoking shows dose effects at some CpGs. Further research is needed to study the relevance of DNA methylation for clinical outcomes.

Supplementary Material

A Contributorship Form detailing each author's specific involvement with this content, as well as any supplementary data, are available online at https://academic.oup.com/ntr.

Declaration of Interests

None declared.

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