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Epigenome-wide associations between observed maternal sensitivity and offspring DNA methylation: a population-based prospective study in children

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Abstract

Background. Experimental work in animals has shown that DNA methylation (DNAm), an epigenetic mechanism regulating gene expression, is influenced by typical variation in maternal care. While emerging research in humans supports a similar association, studies to date have been limited to candidate gene and cross-sectional approaches, with a focus on extreme deviations in the caregiving environment.

Methods. Here, we explored the prospective association between typical variation in maternal sensitivity and offspring epigenome-wide DNAm, in a population-based cohort of children ($N = 235$). Maternal sensitivity was observed when children were 3- and 4-years-old. DNAm, quantified with the Infinium 450 K array, was extracted at age 6 (whole blood). The influence of methylation quantitative trait loci (mQTLs), DNAm at birth (cord blood), and confounders (socioeconomic status, maternal psychopathology) was considered in follow-up analyses.

Results. Genome-wide significant associations between maternal sensitivity and offspring DNAm were observed at 13 regions ($p < 1.06 \times 10^{-07}$), but not at single sites. Follow-up analyses indicated that associations at these regions were in part related to genetic factors, confounders, and baseline DNAm levels at birth, as evidenced by the presence of mQTLs at five regions and estimate attenuations. Robust associations with maternal sensitivity were found at four regions, annotated to *ZBTB22*, *TAPBP*, *ZBTB12*, and *DOCK4*.

Conclusions. These findings provide novel leads into the relationship between typical variation in maternal caregiving and offspring DNAm in humans, highlighting robust regions of associations, previously implicated in psychological and developmental problems, immune functioning, and stress responses.

Introduction

Parental sensitivity, i.e. the responsiveness to children's signals and communications, is an important predictor of developmental outcomes across the behavioral, emotional, and cognitive domains (Kok et al., 2013; Madigan et al., 2019; Thomas, Letourneau, Campbell, Tomfohr-Madsen, & Giesbrecht, 2017). Low sensitivity of primary caregivers – typically mothers – has been associated with a host of negative outcomes, including higher risk for child psychopathology (Haltigan, Roisman, & Fraley, 2013; Kimbrel, Nelson-Gray, & Mitchell, 2007), externalizing and internalizing problems (Kok et al., 2013; Rijlaarsdam et al., 2014), and lower cognitive abilities (Bernier, Carlson, Deschênes, & Matte-Gagné, 2012). This influence can be long-lasting, as shown by prospective human studies (Raby, Roisman, Fraley, & Simpson, 2015; Stams, Juffer, & van IJzendoorn, 2002) and experimental

work in animals (Meaney, 2001). Yet, the molecular mechanisms underlying the enduring effects of maternal care on neurodevelopmental and behavioral outcomes in humans remain unclear.

Previous studies have provided initial support for DNA methylation (DNAm) – an epigenetic modification regulating gene expression – as a mechanism of interest for these processes (Mulder, Rijlaarsdam, & Van IJzendoorn, 2017; Szyf, 2013; Weaver et al., 2004). DNAm involves the addition of a methyl group to DNA base pairs, primarily to the 5-carbon of cytosine nucleotides, resulting in 5-methylcytosine. DNAm is sensitive to both environmental and genetic influences (Ladd-Acosta & Fallin, 2016; Smith et al., 2014; Weaver et al., 2004), with the latter being evidenced by changes in the methylome due to DNA variation, named methylation quantitative trait loci (mQTLs) (Gaunt et al., 2016). Further, DNAm plays an essential role in healthy development and functioning by modulating the programming of wider biological systems (e.g. neural and immune functioning) and by coordinating key cellular processes (e.g. tissue differentiation) (Carey, 2012). DNAm might thus represent a mechanism by which genetic and environmental factors, including the early caregiving environment, jointly and/or independently predict developmental outcomes (Ladd-Acosta & Fallin, 2016).

Most evidence of maternal care effects on DNAm comes from animal models. In a seminal study by Weaver et al. (2004), low levels of maternal care in the first week of life – operationalized as the frequency of licking/grooming and arched-back nursing behaviors – altered hippocampal DNAm patterns in offspring at the glucocorticoid receptor (*gr*, also known as *nr3c1*), a key regulator of stress response (Geer et al., 2010). Importantly, these epigenetic changes were long-lasting, but could be reversed via cross-fostering or chemical interventions, leading to a normalization of physiological and behavioral responses to stress (Weaver et al., 2004, 2005). These findings generated widespread interest, as they indicated (i) a causal role of maternal care on offspring's epigenetic dysregulation and downstream phenotypes, independent of genetic liability, and (ii) the possibility of influencing developmental trajectories through environmental interventions, mediated by DNAm. Since this initial work, other studies have replicated the effects of maternal care on *gr* methylation in rodents (Turecki & Meaney, 2016) and extended findings to demonstrate DNAm changes in other tissues and genes (Beery, McEwen, MacIsaac, Francis, & Kobor, 2016; Blaze et al., 2017; Doherty, Forster, & Roth, 2016) [e.g. brain-derived neurotrophic factor (*bdnf*) and oxytocin receptor (*oxtr*)] as well as in other species such as rhesus macaques (Provençal et al., 2012).

Although rodents and primates widely differ from humans in their caregiving, a number of similarities in maternal–infant relationships have been observed across mammalian species (Feldman, 2016; Knop, Joëls, & van der Veen, 2017). Parallels at the sensory, hormonal, behavioral, and brain circuit levels have been noted (Feldman, 2016; Glynn & Baram, 2019; Knop et al., 2017), including the touch-based behavior characterizing rodents, primates, and humans in the early caregiving and the involvement of the limbic network in maternal–infant relationships (Feldman, 2016). Guided by the animal literature, a growing number of studies have sought to determine the extent to which different forms of caregiving and adversities affect DNAm in humans.

Human studies have focused on different forms of adversities (Daskalakis & Yehuda, 2014) including poly-victimization (Marzi et al., 2018), and on extreme deviations in the early caregiving environment, such as maltreatment (Cecil et al., 2016; Gouin et al., 2017; Mehta et al., 2013; Stenz et al., 2016; Weder et al., 2014), institutionalization (Naumova et al., 2012), and maternal psychopathology (Oberlander et al., 2008). Generally, literature focusing on the caregiving environment has provided preliminary support in line with animal findings, identifying, for example, similar increases in *GR* methylation in both *postmortem* hippocampal tissue and peripheral tissues from individuals exposed to childhood maltreatment or early-life stress (Turecki & Meaney, 2016). Studies also indicate that epigenetic patterns associated with the caregiving environment extend beyond *GR*, implicating other genes related to, among other processes, neurodevelopment and stress, such as *OXTR* and *BDNF*. Moreover, by leveraging epigenome-wide DNAm, novel genes were identified (e.g. *KCNQ2*, *miR124-3*) in relation to maltreatment and child abuse in individuals with post-traumatic stress disorder (Mehta et al., 2013), borderline personality disorder (Stenz et al., 2016), and depression (Weder et al., 2014).

While these results are promising and suggest a role of the caregiving environment in the human methylome, the current evidence in humans is limited in a number of key ways. First, since research has mostly focused on extreme deviations in the caregiving environment in selected samples, little is known about how typical variation in maternal sensitivity associates with offspring DNAm in the general population. Second, while studies on extreme deviations in maternal care have leveraged epigenome-wide approaches, the literature on normative variation in maternal care has solely focused on candidate genes. This has impeded the identification of novel DNAm loci associated with maternal sensitivity, which might instead be detected with a hypothesis-free approach by performing an epigenome-wide association study (EWAS). Third, studies have typically relied on cross-sectional designs, in which the early caregiving environment is measured retrospectively via the use of questionnaires, raising doubts about the directionality of observed associations and about the validity of measurements, which may be prone to recall bias (Baldwin, Reuben, Newbury, & Danese, 2019; Reuben et al., 2016). Moreover, previous studies rarely investigated whether the identified associations may be confounded by genetic background shared between parents and offspring. The examination of the relationship between maternal care and DNAm might indeed capture intergenerational genetic transmission. Lastly, the influence on offspring DNAm of factors preceding postnatal maternal care, including the prenatal environment, remains unexplored.

To address these gaps, we firstly examined how typical variation in observed maternal sensitivity prospectively associates with epigenome-wide DNAm patterns in a general population of children. Secondly, with a series of follow-up analyses, we explored the extent to which associations reflected genetic influences as well as confounding by 'baseline' DNAm levels at birth, which precede exposure to postnatal maternal care and might constitute a biological indicator of the prenatal environment as well as of genetic effects on the methylome.

Materials and methods

Participants

The present research was embedded in the Generation R Study, a prospective population-based cohort study from fetal life onwards in Rotterdam, The Netherlands (Kooijman et al., 2016) (online Supplementary Information 1). Ethical approval was obtained

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from the Medical Ethics Committee of Erasmus MC, University Medical Center Rotterdam. For the purposes of this study, children within the Generation R Study with data on maternal sensitivity (at 3 and/or 4 years) and DNAm (at 6 years) were selected ($N = 235$). Since 5 sibling-pairs were present, we later excluded one sibling per pair ($N = 230$) to ensure genetic relatedness did not impact results.

Participant characteristics are shown in online Supplementary Table S1. Participants with data on both maternal sensitivity and DNAm (age 6) differed from participants invited to the age 6 assessment in gestational age at birth [$M_{subsample} = 40.3$ weeks (S.D. = 1.4), $M_{fullsample} = 39.8$ (S.D. = 1.9), $t = 5.6$, $p = 6.50 \times 10^{-08}$], but not other covariates.

Measures

Maternal sensitivity

Maternal sensitivity was assessed at ages 3 and 4 years through observations of mother–child interactions during teaching tasks too complex for the age of the child. These involved (i) building a tower and (ii) completing an etch-a-sketch drawing. Mother–child interactions were recorded and subsequently coded, according to the revised Erickson seven-point rating scales (Egeland, Erickson, Clemenhagen-Moon, Hiester, & Korfmacher, 1990), based on two interdependent subscales: intrusiveness (IN) and supportive presence (SP), which together form the maternal sensitivity construct. Inter-coder reliability amounted to 0.81 at age 3 and 0.84 at age 4 (Kok et al., 2015).

Eight measures of maternal sensitivity (i.e. IN and SP scales × two tasks × two time-points) were available. IN scores were reversed, and both IN and SP scores were standardized. An overall maternal sensitivity score was calculated, for participants with data at age 3 and/or 4, by averaging such standardized measures (Cents et al., 2014). This was done in line with previous literature (Kok et al., 2015), due to the stability of the maternal sensitivity scores between age 3 and 4 years (Kok et al., 2013), the temporality of these assessments, which both precede DNAm at age 6, and to maximize our sample size. Cronbach's α reliability of the obtained measure was acceptable (Cronbach's $\alpha = 0.70$) (Cortina, 1993).

DNA methylation

DNAm in whole blood at age 6 was used for our epigenome-wide analyses. This was selected due to it being the closest DNAm assessment after maternal sensitivity observations (age 3 and 4 years), and to test the prospective association of maternal sensitivity with DNAm. Based on previous studies in animals, which found maternal care to have long-lasting influences on the methylome (Weaver et al., 2004), we expected for maternal care effects to endure in early childhood.

To obtain DNAm data, DNA extraction and bisulfite conversion via the EZ-96 DNA Methylation kit (Shallow) (Zymo Research Corporation, Irvine, California, USA) were performed, and samples were processed with the Illumina Infinium HumanMethylation450 BeadChip (Infinium 450 K), which measures 485 577 CpGs. The incorporating control probe adjustment and reduction of global correlation pipeline (Lehne et al., 2015) was employed for the preparation and normalization of the data using R. Firstly, the *minfi* package (Aryee et al., 2014) in R was used to read the idat files. Probes that had a detection p value above background (based on the sum of methylated and unmethylated intensity values) $\geq 1 \times 10^{-16}$ were set to missing per array. Next, the intensity values were stratified by autosomal and non-autosomal probes and quantile normalized for each of the six probe-type categories separately: type II red/green, type I methylated red/green, and type I unmethylated red/green. For each probe, DNAm levels were indexed by β values (i.e. the ratio of methylated signal divided by the sum of the methylated and unmethylated signal [M/(M + U + 100)]). Quality control procedures were additionally performed (e.g. check for sex mismatch). Only arrays with a call rate above 95% per sample were considered for additional processing. DNAm data were winsorized (>3 S.D.) to reduce the influence of potential outliers. In total, we obtained information on 457 872 autosomal sites in 493 6-year-olds.

We additionally used DNAm data collected at birth in cord blood for a follow-up analysis. This was subject to the same pipeline as the DNAm data at age 6 and was also measured based on the Infinium 450 K BeadChip. Only CpGs identified as significant or within DNAm significant regions were selected for these analyses.

Covariates

All analyses were adjusted for a key set of covariates guided by previous literature (Birney, Smith, & Greally, 2016; Breton et al., 2009; Liang & Cookson, 2014; Rakyan, Down, Balding, & Beck, 2011), including batch effects (plate number), sex, gestational age at birth, maternal smoking during pregnancy (never smoked, smoked until pregnancy known, continued during pregnancy), and estimated cell-type proportions (Houseman et al., 2012) (online Supplementary Information 1). We additionally adjusted for two sets of covariates: (i) maternal education (highest level completed) as a proxy for socioeconomic status, and postnatal maternal psychopathology (Brief Symptom Inventory), and (iii) DNAm levels at birth (cord blood tissue), together with respective cell-type and batch effect adjustments (online Supplementary Information 1).

Statistical analyses

Analyses were performed in R (version 4.0.0) and are described in-depth in online Supplementary Information 1. A *probe-level EWAS* (multiple linear regression models) was run with the CpGassoc R package (Barfield, Kilaru, Smith, & Conneely, 2012), to test for associations of maternal sensitivity with each DNAm site individually (Bonferroni epigenome-wide significance threshold: $p < 1.09 \times 10^{-07}$). To account for potential bias and inflation, the *BACON* R package (van Iterson, van Zwet, & Heijmans, 2017) was used.

Moreover, to capture correlations across CpGs, reduce data dimensionality, and attenuate the multiple testing burden, a *regional-level EWAS* was performed by using the R package DMRff (Suderman et al., 2018). This estimates correlations across nominally-significant probes within a 500 bp window (default setting) and combines the EWAS summary statistics of such neighboring CpGs to identify differentially methylated regions while accounting for multiple testing with a Bonferroni procedure in both gene regions and sub-regions (Suderman et al., 2018).

A *candidate gene look-up* was also performed to maximize comparability with previously reported DNAm–maternal care associations. To date, DNAm levels of four genes have been associated with maternal care in humans (Bosmans, Young, & Hankin, 2018; Conradt et al., 2016; Provenzi et al., 2017; Unternaehrer et al., 2015), by at least one study: *GR*, *BDNF*, the

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serotonin receptor (*SLC6A4*), and *OXTR*. We looked-up the EWAS results for probes located within these genes, as annotated in the HumanMethylation450 v1.2 Manifest File. Following previous studies (Cecil et al., 2017; Marzi et al., 2018), gene-level Bonferroni correction was used as a significance threshold (i.e. $p < 0.05$/number of annotated probes).

To identify enriched biological pathways, we performed an in-house *gene ontology* (*GO*) *analysis* (Cecil et al., 2017, 2018; Hannon et al., 2016) on sites with $p < 0.001$ in the probe-level EWAS, in line with previous literature (Cecil et al., 2017, 2018; Mulder et al., 2020; Roberts et al., 2019). We performed p value adjustments based on default procedures (Hannon et al., 2016). Enriched pathways were confirmed by an independent GO approach from the missMethyl R package (Phipson, Maksimovic, & Oshlack, 2016) ($p < 0.05$).

Finally, a series of *follow-up analyses* were run. Firstly, the influence of genetic factors on our top hits (i.e. Bonferroni-significant sites or sites within Bonferroni-significant DNAm regions) was assessed by drawing on an *mQTL database* (Gaunt et al., 2016) (www.mqtldb.org). We examined whether hits were associated with known mQTLs during childhood, based on the results from a genome-wide complex trait conditional analysis. Secondly, we explored the robustness of top hits to *additional adjustments* for (i) postnatal maternal education and maternal psychopathology ($N = 223$) and (ii) pre-exposure DNAm ($N = 226$). The latter was done to account for the effect of DNAm at birth on DNAm at age 6 and to capture potential pre-existing influences (e.g. intrauterine exposures) on DNAm in childhood. Spearman correlations between DNAm at birth and age 6 were also calculated, per CpG. Thirdly, based on a list of our CpG hits, the in-house *GO analysis* and missMethyl validation were run, with the same procedures as the main GO analysis specified above. Finally, to understand the relevance of our findings to the brain, which is linked to the caregiving environment (Kok et al., 2015; Weaver et al., 2004), we looked-up *brain–blood concordance* values for our top hits using the BECon online tool (https://redgar598.shinyapps.io/BECon/) (Edgar, Jones, Meaney, Turecki, & Kobor, 2017).

Results

Probe-level EWAS

Maternal sensitivity was not associated with any single CpGs at age 6, after genome-wide correction ($p < 1.09 \times 10^{-07}$) (Fig. 1, online Supplementary Table S2). BACON analysis revealed a normal λ ($\lambda = 1.00$), minimal bias (Bayesian estimate of bias = −0.002), and deflation in the test results – indicative of low power (Bayesian inflation factor = 0.925) (online Supplementary Fig. S1). Following BACON correction for deflation, one intergenic CpG reached genome-wide significance: cg25628898 (estimate = −0.008; S.E. = 0.002; $p = 1.03 \times 10^{-07}$) (online Supplementary Table S2).

Regional-level EWAS

With a regional-level EWAS, we identified 13 DNAm regions associated with maternal sensitivity ($p < 1.09 \times 10^{-07}$; $\alpha = 0.05$) (Table 1, Fig. 2, online Supplementary Table S3), spanning 143 CpGs. The top three DNAm regions coincided with the *ANKMY1*, *RNF39*, and *ZBTB22* and *TAPBP* genes (Table 1). The largest estimates were shown at regions encompassing *COLEC11* and *DOCK4*. None of the CpGs within our significant regions was related to prenatal maternal smoking, based on previous research in neonates and children (Joubert et al., 2016; Rzehak *et al.*, 2016), suggesting adjustments in the EWAS accounted for its confounding role. When siblings ($N = 230$) were excluded, all but one region (annotated to *RNF5P1*, *RNF5*, *AGPAT1*) remained significantly associated with maternal sensitivity.

Candidate gene look-up

The candidate gene look-up showed that, of the four selected genes (*NR3C1*, *BDNF*, *SLC6A4*, *OXTR*), which included 14–74 sites, no CpG met Bonferroni-adjusted gene-wide significance in association with maternal sensitivity (Table 2, online Supplementary Table S4). Only three sites reached nominal significance ($p < 0.05$).

Gene ontology

The in-house GO analysis, based on sites with $p < 0.001$ in the probe-level EWAS, revealed enrichment for 148 pathways. Yet, this threshold might have been overinclusive. Thirty-nine of the 148 pathways were confirmed by the missMethyl GO method ($p < 0.05$) (online Supplementary Table S5). Both methods indicated enrichment for, among others, calcium ion channels functioning, phosphorylation, and tissue and cell polarity.

Follow-up analyses

Firstly, an mQTL search revealed that five of the 13 significant DNAm regions contained at least one CpG associated with one or more known SNPs (Table 3, online Supplementary Table S6). Eight regions, including *ZBTB22/TAPBP* (one of our top regions), did not present any mQTLs. Of the 143 sites within the 13 significant regions, 22% ($n = 31$) associated with one or more known SNPs. All associations were in *cis*.

Secondly, after additional adjustments for socioeconomic status and maternal psychopathology, associations attenuated at seven regions (median: −1%, range: −44% to 13%). Regions which did not decrease in effect were *TAPBP*, *RNF39*, two non-annotated regions, *ANKMY1*, and *ALOX12P2* (online Supplementary Table S7). When adjusting for pre-exposure DNAm levels (online Supplementary Table S8), associations attenuated at 10 regions (median: −45%, range: −97% to 17%), with *RNF39* being the most affected. Regions whose estimates did not decrease were *ZBTB12*, *FBXO44/FBXO2*, and a non-annotated region (chromosome 7). The median correlation between each CpG DNAm level at birth and age 6 was of $\rho = 0.43$ (range: 0.11–0.86) (online Supplementary Table S9).

Thirdly, in a follow-up GO analysis, based on the sites within the significant DNAm regions ($n = 143$), enrichment was found at 63 pathways (in-house method). Of these, 33 were validated by missMethyl ($p < 0.05$). Both methods indicated enrichment for, among others, several lipoprotein processes (e.g. particle remodeling), and peptide binding (online Supplementary Table S10).

Lastly, of the 13 significant DNAm regions, six contained half or more sites with greater than average blood–brain tissue concordance (Edgar et al., 2017) in at least one brain tissue (for BA7 $r > |0.36|$, for BA10 $r > |0.40|$, for BA20 $r > |0.33|$), for a total of 67 sites (online Supplementary Table S11) (not empirically tested).

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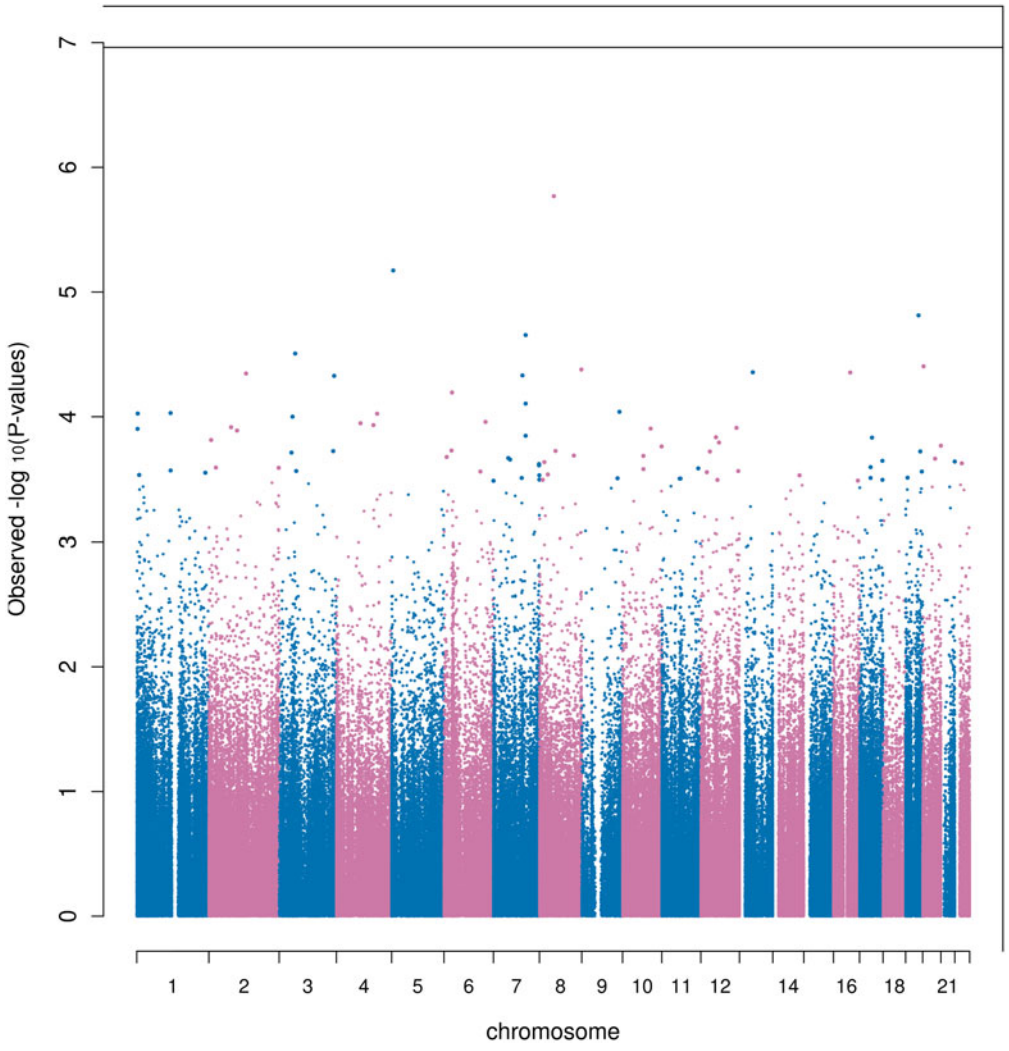

Fig. 1. Manhattan plot of CpG sites associated with maternal sensitivity. *Note.* The Manhattan plot displays the log *p* values for each site tested in association with maternal sensitivity in the EWAS, across autosomal chromosomes. No genome-wide significant association was observed ($p < 1.06 \times 10^{-07}$).

Discussion

This is the first epigenome-wide study investigating the prospective association between typical variation in maternal sensitivity (observed) and offspring DNAm, in a general population of children. Genome-wide significant associations were observed at 13 DNAm regions, four of which did not contain mQTLs and were minimally affected by adjustments for postnatal confounders and by pre-exposure DNAm levels, thus showing robustness in associations.

Summary of key findings

Our first aim was to examine the prospective relationship between maternal sensitivity and child DNAm using complementary approaches. Firstly, no individual CpG was identified in the *probe-level EWAS* after genome-wide correction. This might indicate that associations at site-level are subtle and challenging to identify, especially considering this study assessed typical variation in maternal care as opposed to extreme deviations (e.g. abuse). The high multiple testing correction burden that probe-level EWASs entail may also impede the detection of single sites of small effect, which could be uncovered with larger samples. For instance, with our sample ($N = 235$) and model (multiple linear regression, 10 predictors), 80% power, and a genome-wide threshold, only moderate estimates (as small as 0.27) could be detected.

When employing a *regional approach*, which can detect weaker but more widespread signals by accounting for correlations across CpGs, 13 DNAm regions were significantly associated with maternal sensitivity ($p < 1.06 \times 10^{-07}$, $\alpha = 0.05$). These findings support the presence of offspring methylomic signatures of maternal care, which may be best uncovered through hypothesis-free approaches with methods capturing the correlational patterns of DNAm. Yet, replication of these findings is needed, and the possibility of false-positive findings should not be excluded. Notably, when considering a more stringent significance threshold ($p < 2.18 \times 10^{-09}$; $\alpha = 0.001$), as suggested to reduce false-positive rates (Colquhoun, 2014), most of the regions (77%, $N = 10$) remained significantly related to maternal sensitivity.

Further, we failed to detect an association between maternal sensitivity and DNAm variation at *candidate genes* previously identified by studies of maternal care in humans (Bosmans et al., 2018; Conradt et al., 2016; Provenzi et al., 2017; Unternaehrer et al., 2015). Inconsistencies may reflect several

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Table 1. DNA methylation regions significantly associated with maternal sensitivity from the regional-level EWAS

DNAm region location	Annotated gene(s)	*N* CpGs included	Estimate	Standard error	Raw *p* value	Bonferroni Adj. *p* value
chr2: 241458886–241460002	*ANKMY1*	8	0.365	0.043	1.17×10^{-17}	5.61×10^{-12}
chr6: 30039027–30039600	*RNF39*	22	−0.227	0.028	5.03×10^{-16}	2.42×10^{-10}
chr6: 33282879–33283184	*ZBTB22*; *TAPBP*	17	−0.215	0.027	1.83×10^{-15}	8.77×10^{-10}
chr2: 21266727–21267334	*APOB*	10	−0.302	0.040	2.83×10^{-14}	1.36×10^{-08}
chr2: 3642629–3642867	*COLEC11*	6	−0.875	0.135	9.80×10^{-11}	4.71×10^{-05}
chr17: 6797034–6797771	*ALOX12P2*	6	−0.571	0.088	1.00×10^{-10}	4.80×10^{-05}
chr7: 111368367–111368847	*DOCK4*	4	−0.822	0.127	1.02×10^{-10}	4.90×10^{-05}
chr6: 32145383–32146595	*RNF5P1*; *RNF5*; *AGPAT1**	27	0.047	0.007	3.55×10^{-10}	0.000171
chr7: 158749953–158751591	*Non-annotated region*	8	0.558	0.090	4.80×10^{-10}	0.000231
chr6: 33280149–33280436	*TAPBP*	9	−0.282	0.046	8.89×10^{-10}	0.000427
chr6: 31867757–31868169	*ZBTB12*	19	−0.100	0.018	2.35×10^{-08}	0.011285
chr4: 147164778–147165097	*Non-annotated*	4	0.427	0.077	2.53×10^{-08}	0.012128
chr1: 11714218–11714254	*FBXO44*; *FBXO2*	3	−0.439	0.081	5.82×10^{-08}	0.027955

DNAm region location: genomic location of the DNA methylation region (chromosome, start position, and end position); Annotated gene(s): gene(s) annotated to the CpGs within the DNA methylation region; N CpGs included: number of CpGs included in the DNA methylation region; Estimate: estimate for the association of maternal sensitivity with DNA methylation at a region; Standard error: standard error for the association of maternal sensitivity with DNAm at a region; Raw *p* value: unadjusted *p* value for the association of maternal sensitivity with DNA methylation at a region; Bonferroni adj. *p* value: *p* value adjusted for multiple testing with Bonferroni correction.
*This region was not genome-wide significant when siblings were excluded from the sample.

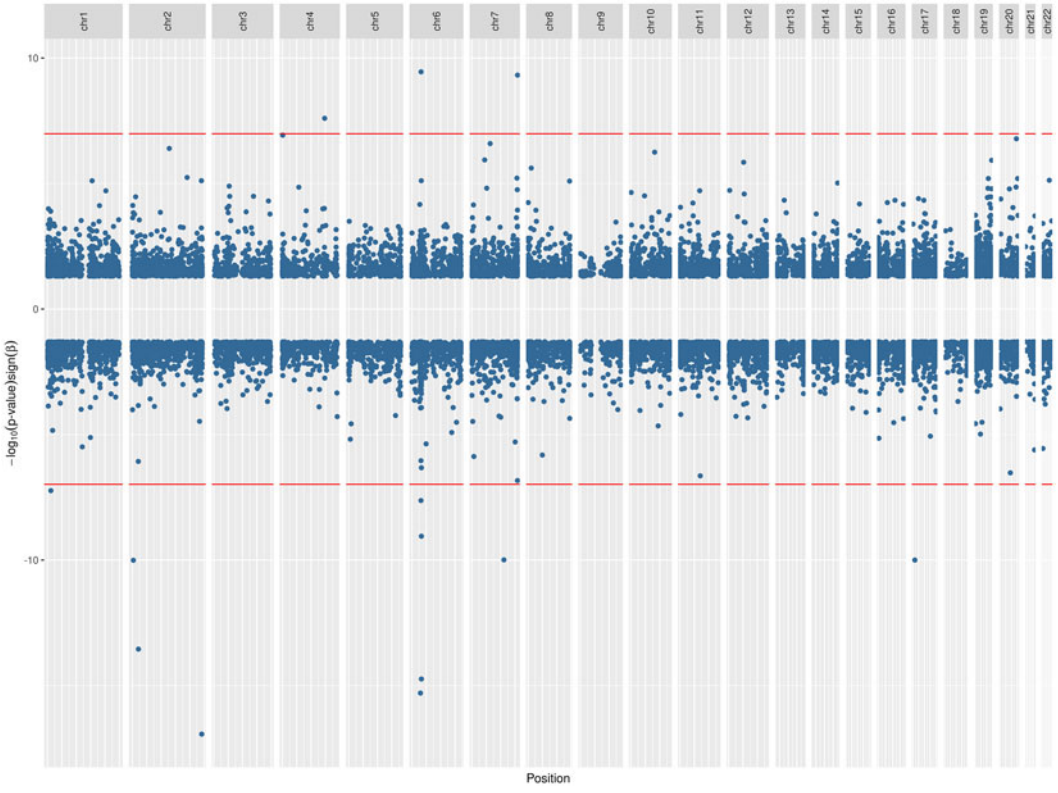

Fig. 2. Miami plot of DNA methylation regions associated with maternal sensitivity. *Note.* The Miami plot displays the log *p* values and estimates direction for each DNA methylation region tested in association with maternal sensitivity, across autosomal chromosomes. Thirteen regions were Bonferroni significant, three of which showed a positive relation with maternal sensitivity and 10 a negative one.

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Table 2. The association between maternal sensitivity and DNA methylation: candidate gene look-up

Gene	Chr	*N* probes	Gene-level sign.	Nominal sign.	Estimate range	% Positive associations	% Negative associations
NR3C1	5	40	No	Yes (cg17342132)	−0.004 to 0.006	65%	35%
BDNF	11	74	No	Yes (cg26840770)	−0.010 to 0.005	50%	50%
SLC6A4	17	14	No	Yes (cg06841846)	−0.004 to 0.005	29%	71%
OXTR	3	18	No	No	−0.006 to 0.006	56%	44%

Gene: candidate gene; Chr: chromosome; *N* probes: number of probes annotated to the gene (based on the Infinium 450 K); Gene-level sign.: gene-level Bonferroni significance in any of the probes annotated to the candidate gene ($p < 0.05$/number of annotated probes); Nominal sign.: nominal significance in any of the probes annotated to the candidate gene ($p < 0.05$); Estimate range: range of estimates for the probes annotated to the candidate genes; % Positive associations: percentage of probes with a positive association with maternal sensitivity; % Negative associations: percentage of probes with a negative association with maternal sensitivity.

Table 3. mQTLs within the statistically significant DNA methylation regions

DNAm region location	Annotated gene(s)	*N* CpGs included	*N* mQTL associations	*N* CpGs with mQTLs	% CpGs with mQTLs
chr2: 241458886–241460002	*ANKMY1*	8	16	7	88%
chr6: 30039027–30039600	*RNF39*	22	0	0	0%
chr6: 33282879–33283184	*ZBTB22*; *TAPBP*	17	0	0	0%
chr2: 21266727–21267334	*APOB*	10	19	10	100%
chr2: 3642629–3642867	*COLEC11*	6	6	6	100%
chr17: 6797034–6797771	*ALOX12P2*	6	0	0	0%
chr7: 111368367–111368847	*DOCK4*	4	0	0	0%
chr6: 32145383–32146595	*RNF5P1*; *RNF5*; *AGPAT1*	27	0	0	0%
chr7: 158749953–158751591	*Non-annotated region*	8	5	5	63%
chr6: 33280149–33280436	*TAPBP*	9	0	0	0%
chr6: 31867757–31868169	*ZBTB12*	19	0	0	0%
chr4: 147164778–147165097	*Non-annotated region*	4	0	0	0%
chr1: 11714218–11714254	*FBXO44*; *FBXO2*	3	3	3	100%
Total		**143**	**49**	**31**	**22%**

DNAm region location: genomic location of the DNA methylation region (chromosome, start position, and end position); Annotated gene(s): gene(s) annotated to the DNA methylation region; *N* CpGs included: number of CpGs included in the DNA methylation region; *N* mQTL associations: number of SNPs–DNA methylation associations at a region; *N* CpGs with mQTLs: number of CpGs presenting one or more mQTL(s) at a region; % CpGs with mQTLs: percentage of CpGs presenting one or more mQTL(s) at a region.

factors, including differences in sample characteristics (e.g. psychiatric *v.* population-based samples), maternal care assessments (retrospective *v.* prospective reports), and analysis (e.g. gene regions covered by pyrosequencing *v.* Infinium 450 K). Lastly, candidate gene studies may be particularly vulnerable to false positives, as shown in the genetic field (Sullivan, 2007).

As a second aim, we explored whether identified maternal sensitivity–DNAm associations may be influenced by genetic factors, based on mQTL mapping. Twenty-two percent of the sites in our significant regions were linked to known SNPs. This suggests that associations for those sites may be in part confounded by genetic factors and corroborates previous research highlighting DNAm responsiveness to both external exposures and genetic variation (Ladd-Acosta & Fallin, 2016). However, the presence of mQTLs alone does not preclude environmental effects. Indeed, recent studies have found that interindividual variability in DNAm is primarily explained by gene–environment combinations (additive and interactive effects) (Czamara et al., 2019; Teh et al., 2014). Moreover, mQTLs were identified based on a publicly available database, as our sample was underpowered to directly test for genetic confounding. Future studies employing genetically-sensitive designs could more precisely quantify the effect of maternal sensitivity on DNAm by directly modeling genetic influences.

When exploring the robustness of findings to additional adjustments, we observed attenuations at half of the regions, after controlling for socioeconomic status and maternal psychopathology. When considering pre-exposure DNAm levels, estimates attenuated at most regions. Although neonatal methylomic patterns were measured in cord blood at birth and not in peripheral blood (used at age 6), which may lead to additional differences, these findings indicate that associations partly reflected pre-existing DNAm levels. This was clearly exemplified by *RNF39*, a region strongly associated with sensitivity, robust to postnatal confounders, and genetic influences. After adjustments, its estimate reduced by 97%, showing that associations did not result from postnatal caregiving, as they were already present at baseline (birth). These findings cast doubts on previous studies of caregiving which did not consider pre-exposure DNAm levels, and raise questions on the directionality of associations between maternal care and DNAm, as well as on the

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potential role of other factors affecting child DNAm (at birth and in childhood) and maternal sensitivity (e.g. shared genetics, maternal distress).

Here, we highlight four 'high-confidence' associations with maternal caregiving, which were not linked to any mQTLs, and were most robust to adjustments for confounders and pre-exposure DNAm levels. These spanned (i) ZBTB22/*TAPBP*, (ii) *ZBTB12*, (iii) *DOCK4*, and (iv) a non-annotated region in chromosome 4. All four genes are protein-coding (Geer et al., 2010). *DOCK4* is implicated in neuronal processes, such as neuronal migration, and dendritic arborization (Shi, 2013) and its DNAm region presented higher than average blood–BA10 concordance in this study. *ZBTB22* and *ZBTB12* are involved in transcriptional regulation and nuclear chromatin localization (Agapite et al., 2020). These two genes, together with *TAPBP*, are within the major histocompatibility complex (MHC). While these associations should be carefully interpreted as the MHC is characterized by extensive linkage disequilibrium (Price et al., 2008), this genomic region plays an important role in immune functioning and has been implicated in neuronal plasticity (Shatz, 2009; Sobue et al., 2018). *TAPBP* specifically is involved in MHC class I protein complex assembly, gene expression regulation, and immunodeficiency (Agapite et al., 2020). In this study, enrichment for MHC class I protein assembly and peptide binding was found for maternal sensitivity, potentially suggesting that such exposure might enact on *TAPBP*-related functions via DNAm.

Generally, our high-confidence genes have been previously associated with psychological and developmental problems, inflammation, and stress responses. Molecular changes were shown at *TAPBP* for major depressive disorder and suicide (Murphy et al., 2017), *TAPBP* and *DOCK4* for schizophrenia (Alkelai et al., 2012; Lee, Kim, & Song, 2013; Zhang et al., 2020), *ZBTB22* for intellectual disability (Agapite et al., 2020) and psychopathologies following hypercortisolism (Glad et al., 2017), and *DOCK4* for autism and dyslexia (Liang et al., 2014; Maestrini et al., 2010). Enrichment for pathways including Dock4 has been repeatedly associated with stress-related responses in mice (Lee et al., 2005; Lisowski et al., 2011; Papale, Madrid, Li, & Alisch, 2017), while *ZBTB12* DNAm is related to markers of inflammation (e.g. white blood cell counts) (Noro et al., 2019).

Limitations and suggestions for future research

Our findings should be interpreted in light of several limitations. Firstly, identified associations may have been influenced by additional parental factors that we could not control for in the present study, either because this information was not available (e.g. parental temperament, parental genotype) or due to the low number of cases (e.g. maternal medication and substance use in pregnancy). Nevertheless, we did control for the most important maternal confounders (smoking during pregnancy, socioeconomic status, psychopathology). Secondly, if unmeasured changes in maternal sensitivity and covariates occurred during the 2–3-year time-lag between our exposure and outcome, noise would be introduced in the identified associations. A prospective design, as opposed to a cross-sectional one, remains however preferable due to the possibility to better understand the directionality of associations. Nonetheless, repeated postnatal measurements of both DNAm and maternal sensitivity would be ideal to longitudinally examine how associations change over time and disentangle directionality. Thirdly, we did not have information on whether the mothers included in this study were primary or secondary caregivers (at 4 years only). Yet, within Generation R, most mothers are primary caregivers (White et al., 2018). Additionally, while the use of the Infinium 450 K provided novel insights into the genes affected by maternal sensitivity, future research should employ, when possible, the EPIC 850 K array due to its wider and more diverse genomic coverage (Illumina, 2020). Lastly, our investigation solely focused on the association of maternal sensitivity with the child methylome. Related molecular signatures, such as transcription changes and epigenetic clocks, could be examined in future research to better understand the biological consequences of maternal care.

In conclusion, this exploratory population-based study suggests a prospective association of typical variation in maternal sensitivity with epigenome-wide DNAm in children. We highlight four DNAm regions that showed the strongest associations with maternal sensitivity as well as limited evidence of genetic and pre-exposure influences, and which should thus be prioritized in future confirmatory research. These results permit further delineation of the relationship between DNAm and maternal care in humans and warrant corroboration by future research with large, longitudinal, and genetically-sensitive studies.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0033291720004353

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Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

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