**Associations of prenatal depressive symptoms with child DNA methylation and cortisol activity**

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# Abstract

Epigenetic DNA modifications in genes related to the hypothalamic–pituitary–adrenal (HPA) axis are discussed as an underlying mechanism for the association between prenatal depression and alterations in the child cortisol system. In a longitudinal study, DNA methylation changes related to maternal depressive symptoms in pregnancy were investigated in 167 children aged 6 to 9 years old. Further associations with the basal child HPA activity were examined. Children exposed to prenatal depressive symptoms exhibited lower bedtime cortisol (*p* = .003, ηp2= .07) and a steeper diurnal cortisol slope (*p* = .023, ηp2= .06). Effects for total cortisol release differed based on sex. Furthermore, prenatal depressive symptoms were associated with altered DNA methylation in *NR3C1*, *NR3C2* and *SLC6A4* at nominal significance level (*p* = .012-.040, ηp2  = .03-.04). In boys, prenatal depressive symptoms predicted bedtime cortisol, mediated by *NR3C2* methylation (indirect effect: -0.07, 95 % confidence interval: -0.16, -0.02). Results indicate sex-specific and -unspecific relations of prenatal depressive symptoms to both child basal HPA activity and DNA methylation, partially fitting a mediation model.

**Keywords**: pregnancy; prenatal depression; epigenetics; DNA methylation; cortisol

# Introduction

Depressive symptoms in the perinatal period are a frequent phenomenon, with an estimated prevalence between 6 % and 39 % depending on country and severity of symptoms (Field, 2011). Maternal depressive symptoms in pregnancy are regarded as risk factor for child development, manifesting in preterm delivery, delayed cognitive development or emotional and behavioral problems (Gentile, 2015). Furthermore, dysfunctions in the hypothalamic–pituitary–adrenal (HPA) axis have been observed in children exposed to prenatal depression, as elevated basal cortisol levels in newborns (Diego et al., 2004), lower cortisol levels in children (Laurent et al., 2013) as well as a reduced cortisol awakening response and a flattened diurnal cortisol slope in adolescents (O'Donnell et al., 2013). Studies investigating the association between prenatal depression and HPA reactivity also showed inconsistent results, with exposed infants exhibiting enhanced cortisol reactivity (Brennan et al., 2008), but exposed adolescents exhibiting a blunted stress response (Vedhara et al., 2012). Additionally, based on results from animal and human studies, effects of prenatal stress, depression or anxiety on offspring psychopathology and HPA axis regulation are found to be partially sex-specific (Glover & Hill, 2012).

According to the *Developmental Origins of Health and Disease*-hypothesis (DOHaD; Wadhwa, Buss, Entringer, & Swanson, 2009), environmental stimuli, especially in the pre- and postnatal period, can have long-lasting effects on the offspring’s development and health. It is hypothesized that fetal physiological processes adapt to the *in utero* environment as preparation for the anticipated postnatal environment. Prenatal exposure to depression or associated high cortisol levels in pregnancy might be interpreted as signal for an anticipated stressful life, resulting in altered metabolism and hormone sensitivity throughout the lifetime. The underlying biological mechanisms of these long-lasting effects remain unclear. However, epigenetic processes are discussed as promising [explanatory](http://www.dict.cc/englisch-deutsch/explanatory.html) [model](http://www.dict.cc/englisch-deutsch/model.html) (Wadhwa et al., 2009), especially modifications in the DNA methylation of cytosine-guanine-dinucleotides (CpGs) because of its role in regulating gene expression (Szyf & Bick, 2013). In the context of prenatal mood disorders, the epigenetic programming of the HPA axis is specifically discussed.

Key targets of the former studies investigating the epigenetic impact on the HPA axis were genes encoding receptors or other proteins that influence HPA axis functionality: The focus was on the glucocorticoid receptor (GR), which regulates the HPA axis together with the mineralocorticoid receptor (MR) by binding cortisol. Dysfunctions in the stress system are mainly associated with changes in the number or sensitivity of GR and MR, which are in turn related to altered cortisol levels and an impaired negative feedback of the HPA axis (e.g. Klok et al., 2011; Pariante, 2006). The FK506 binding protein 51 (FKBP5) has been shown to decrease GR-sensitivity to cortisol by reducing its cortisol-binding capacity (Jaaskelainen, Makkonen, & Palvimo, 2011), thus altering HPA activity. Moreover, the brain-derived neurotrophic factor (BDNF), which has a central role in neuronal development and plasticity, was regarded, because it was found to modulate the expression of corticotropin-releasing hormone (CRH) through interaction with glucocorticoids (Jeanneteau et al., 2012). The CRH receptor 1 (CRHR1) was examined since CRHR1 binding stimulates cortisol release and is therefore essential for initiating HPA axis responses to a stressor. Finally, the serotonin receptor was investigated due to the reciprocal interactions between the serotonergic system and the HPA axis. For example more serotonin is released in response to a stressor but higher cortisol levels are also a consequence of serotonin receptor binding (Lanfumey, Mongeau, Cohen-Salmon, & Hamon, 2008).

Epigenetic modifications related to prenatal depressive symptoms are present throughout development, with studies often differing in child age and in tissue used to extract DNA. Most findings for methylation changes refer to the GR encoding gene *NR3C1.* More maternal depressive symptoms during pregnancy were associated with higher *NR3C1* DNA methylation in newborns’ cord blood (Mansell et al., 2016; Oberlander et al., 2008), in saliva of 2-months and buccal cells of 14-months old infants (Braithwaite, Kundakovic, Ramchandani, Murphy, & Champagne, 2015; Murgatroyd, Quinn, Sharp, Pickles, & Hill, 2015). However, results for *NR3C1* methylation changes are not fully consistent and indicate sex-specific differences (Braithwaite et al., 2015) and a possible interaction with later maternal depression (Murgatroyd et al., 2015). Regarding the serotonin receptor encoding gene *SLC6A4*, more depressive symptoms were associated with a lower promoter methylation in newborns’ cord blood (Devlin, Brain, Austin, & Oberlander, 2010). For the BDNF-coding gene, higher prenatal depression scores were associated with reduced DNA methylation in DNA extracted from buccal cells in 2-months old infants (Braithwaite et al., 2015), but no differences were found in newborns’ cord blood (Devlin et al., 2010). Non, Binder, Kubzansky, and Michels (2014) reported a relation between prenatal depression and hypermethylation of *FKBP5* as well as hypomethylation of *CRHR1* in newborns. Despite the importance of MR for HPA axis functioning and findings of an altered expression of the MR-coding gene *NR3C2* in psychiatric disorders (ter Heegde, De Rijk, & Vinkers, 2015), studies investigating the effects of prenatal depression on *NR3C2* methylation in children are still missing.

In order to explain the underlying mechanisms, studying the functional consequences of DNA methylation changes that are related to prenatal depression is essential. Initial studies have reported associations of *NR3C1* and *SLC6A4* DNA methylation with internalizing and externalizing behavioral problems in children (e.g. Parade et al., 2016; Park et al., 2015). Other studies have looked at the association of DNA methylation and child cortisol. Higher *NR3C1* DNA methylation in DNA extracted from blood and saliva, respectively, was related to a delayed and slower cortisol recovery (van der Knaap, Oldehinkel, Verhulst, van Oort, & Riese, 2015) as well as increased morning cortisol levels in adolescents (Weder et al., 2014). Higher morning cortisol was additionally predicted by increased *FKBP5* methylation (Weder et al., 2014). For the first time, Oberlander et al. (2008) investigated the link of prenatal depression, DNA methylation and the functional outcome in terms of the cortisol reactivity. They found higher *NR3C1* methylation in cord blood, associated with prenatal depressive symptoms, predicting infants’ cortisol reactivity at 3-months of age.

The present study aimed to examine DNA methylation as a possible underlying mechanism for the association of prenatal depression and basal cortisol activity in primary school aged children. The longitudinal design enables investigating associations with prenatal depression, while controlling for postnatal and current maternal depression. Furthermore, the sample size allows comparing sex differences. The following hypotheses were tested in the current study: (1) Do children exposed to prenatal depressive symptoms show altered basal cortisol levels?; (2) Is the exposure to prenatal depression associated with DNA modifications in HPA related genes?; (3) Are DNA modifications that are related to prenatal depression also associated with altered basal cortisol levels?; (4) Do DNA modifications mediate the association of prenatal depressive symptoms and basal cortisol levels?

# Material and methods

* 1. *Study design*

Data were collected within the prospective, longitudinal Franconian Maternal Health Evaluation Studies (t1; FRAMES, Erlangen, Germany; Reulbach et al., 2009) and the follow-up Franconian Cognition and Emotion Studies (t2; FRANCES, Erlangen, Germany; Eichler, Grunitz, et al., 2016). From 2005 to 2007, pregnant women older than 18 years of age were asked during the third trimester of pregnancy to participate in FRAMES investigating perinatal maternal health. From 2012 to 2015, families were contacted again for participation in FRANCES in order to explore the effects of prenatal risk factors on child development in primary school age. Besides measuring the cognitive, language and motor development, cortisol samples as marker for the basal HPA activity and DNA samples for epigenetic analyses were collected at t2. Based on the aim of FRANCES, families with an existing prenatal risk were more intense recruited resulting in a risk-oversampling. The study was approved by the Local Ethics Committee of the Medical Faculty of the University of Erlangen-Nürnberg and conducted in accordance with the Declaration of Helsinki. All participants gave informed consent.

* 1. *Participants*

From the FRANCES cohort, 180 mother-child dyads with complete maternal depression data and child DNA samples were included. All children were from single pregnancies, enabling similar prenatal conditions, and had a Caucasian ethnicity in order to provide genetic homogeneity. In order to rule out possible medication effects, dyads were excluded when mothers reported antidepressive medication intake during pregnancy (*n* = 3). After quality control of DNA methylation data, additional children had to be excluded (*n* = 10), resulting in 167 mother-child dyads for the analyses.

At t2, children (82 boys, 85 girls) were between 6 and 9 years old (*M* = 7.6, *SD* = 0.6). The mothers were between 28 and 51 years old, with a mean age of 40.4 years (*SD* = 4.6). They were well-educated, with 53.9 % having completed university entrance qualifications, and most lived in a two parent household, either with the child’s father (86.2 %) or a new partner (3.6 %). Table 1 shows the sample characteristics.

*[Please insert Table 1 here.]*

* 1. *Measures*
		1. *Maternal depressive symptoms*

Maternal depressive symptoms were assessed with the Edinburgh Postnatal Depression Scale (EPDS; Cox, Holden, & Sagovsky, 1987) during the third trimester, two days postpartum and at t2 when the child was in primary school. The EPDS is a 10-item self-rating scale, assessing the severity and frequency of depressive symptoms on a 4-point Likert scale. The scale is validated for the prenatal and postnatal period. Raw-sum scores were calculated (range: 0-30), with a threshold ≥ 10 interpreted as exposure to prenatal depressive symptoms.

* + 1. *Further child and family characteristics*

Pregnancy characteristics, i.e. gestational age, birth weight, Apgar scores and maternal age at birth, were registered immediately after delivery. The Apgar scores represent the newborn’s physical condition at 1, 5 and 10 minutes after birth. They range from 1 to 10, with higher values representing a better adaption. The mean Apgar score (mean of the three time points) was used for analyses. Cigarette smoking and alcohol consumption during pregnancy were regarded as further prenatal risk factors. Prenatal cigarette smoking was assessed in self-report at t1. Smoking ≥ 1 cigarette per day was interpreted as exposed to prenatal smoking. Maternal alcohol consumption in pregnancy was assessed by the ethanol metabolite ethyl glucuronide (EtG) in the newborn’s meconium, described in Eichler, Grunitz, et al. (2016). The families’ socioeconomic status (SES) was calculated from maternal and paternal educational level and family income per month referring to Geißler (1994). The score ranged between 3 and 14, with higher values indicating higher SES. At t2, mothers completed the Strength and Difficulties Questionnaire (SDQ; Goodman, 2001). The subscales ‘Emotional Problems’ and ‘Conduct Problems’ were used as index for child’s psychopathology. Child intelligence was assessed with the standardized Intelligence and Development Scales (IDS; Grob, Meyer, & Hagmann-von Arx, 2009).

* + 1. *Salivary cortisol*

Mothers were instructed to collect five saliva samples at home using Salivette sampling devices (Sarstedt, Nümbrecht, Germany). The collection device and a daily protocol were given to each family to complete at home. In a single day, five samples (T1: at awakening, T2: 30 minutes after awakening, T3: 12 a.m., T4: at 5 p.m. and T5: at bedtime) were collected. Mothers were asked to document the child’s awakening time and sampling times as well as characteristics regarding the day of sampling: school day (yes/no), medication intake, diseases and special events. Saliva samples were stored at -20°C. Cortisol levels were analyzed with a photometric immunoassay (ELISA; IBL International, RE56211, Hamburg, Germany). Photometric measurements were conducted with the MultiskanTM GO microplate spectrophotometer (Thermo Fisher Scientific, Vantaa, Finland).

Due to the typical positive skew of cortisol data, natural logarithm transformation was employed to improve normal distribution. To represent the individual diurnal cortisol profile, five parameters were calculated out of the ln-transformed raw values. The first and last sample were used as waking cortisol respectively bedtime cortisol. The cortisol awakening response (CAR), indicating the typical cortisol increase during 30 to 45 minutes after awakening, was calculated as area under the curve with respect to increase (AUCI) from first to second sample (Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003). The diurnal cortisol slope represents the rate of cortisol decline from awakening to bedtime, calculated as a slope over all samples except the second sample. The total cortisol release throughout the day was computed as area under the curve with respect to ground (AUCG) including all samples (Pruessner et al., 2003).

For six children, salivary cortisol samples were missing, indicating a response rate for cortisol samples of 96.4 %. Children, who were administered corticosteroids (*n*= 7) or ketoconazole (*n* = 1) or reported Henoch-Schönlein purpura (*n* = 2), were excluded from the cortisol analyses. One child was excluded because the samples were collected on different days. Time frames were set for the first two samples in order to assess the sensitive cortisol reaction in the morning accurately. For the first assessment point, samples that were collected more than 15 minutes after awakening (*n* = 31) were excluded from analyses of waking cortisol, CAR and diurnal slope. If the second sample was collected less than 15 minutes or more than 45 minutes after awakening (*n* = 51), it was excluded from CAR calculation. Because of missing values for awakening time, resulting in unknown time frames for the morning, 17 children were further excluded from waking cortisol, CAR and diurnal slope analyses. Dyads with missing awakening times did not differ from other dyads in socioeconomic status, child or maternal psychopathology. According to the daily protocol, single samples that were associated with special day events (e.g. conflict about sample collection) were excluded in order to assess the basal stress activity, not stress reactivity. Outliers defined as values more than 3 standard deviations from group mean were removed. Depending on the parameter of interest, a minimum of *n* = 81 and a maximum of *n* = 145 subjects were included in the analyses. Table S1 represents the sample size as well as the descriptive statistics of the raw cortisol values, sampling times and cortisol parameters. Diurnal cortisol profiles are illustrated in Figure S1, separated into prenatal exposure to depressive symptoms and sex.

* + 1. *DNA methylation*

DNA samples were obtained from buccal cells with OmniSwab (Whatman®, Maidstone, UK). The OmniSwab pad was rubbed on the children’s inner cheek for 30 seconds, placed in a collection tube (Eppendorf Tubes®, Hamburg, Germany) and stored at +4°C. DNA was extracted with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer’s protocol. A total of 500 ng DNA for each sample was sent to the Helmholtz-Zentrum München (Germany), where genome-wide DNA methylation was analyzed with the Infinium Human Methylation 450K BeadChip (Illumina, San Diego, CA, USA) as described elsewhere (Zeilinger et al., 2013).

* + 1. *Epigenetic analyses*

Quality control and preprocessing of the methylation data were performed with R (version 3.2.2) and the R package *minfi* (Aryee et al., 2014), mostly according to the pipeline of Lehne et al. (2015). *Illumina* Background correction was applied to the raw intensity values. Raw intensity values were then normalized using *Quantile* (implemented in *minfi*) and subsequently converted to β values, as the proportion of DNA methylated at a single CpG site. Samples with a call rate less than 97 % were excluded (*n* = 10). Probes overlapping with single nucleotide polymorphisms or located on sex chromosomes were removed as well as probes with a detection *p*-value >.001, reflecting a low signal-detection rate from the background. Probes with a mean beta less than .01 or above .99, standard deviation less than .01 or defined as outliers with more than 4 standard deviations from mean were removed. Control Probe Adjustment (Lehne et al., 2015) was carried out to the normalized β values in order to adjust for technical bias. In addition to 23 control probe factors, which explained 95 % of the β value variance and represent technical biases as shown in Figure S2, children’s age at DNA sampling and birth parameters (gestational age, birth weight, maternal age at birth and mean Apgar score) were added to the regression model. To account for further biological variance, a PCA on the resulting regression residuals were performed and the first two factors were included as predictors in a final regression model. As presented in Figure S3, these factors were not associated with relevant psychosocial variables. The resulting residuals were then used as adjusted methylation values. Due to the different number of CpGs per gene on the BeadChip and the filtering procedure, the number of CpGs investigated varied dependent on gene (*BDNF*: 39 CpGs, *CRHR1*: 15 CpGs; *FKBP5*: 26 CpGs; *NR3C1*: 21 CpGs; *NR3C2*: 15 CpGs; *SLC6A4*: 10 CpGs).

* + 1. *Statistical analyses*

After preprocessing of the methylation data, adjusted methylation values were exported to SPSS (version 21, SPSS, Chicago, USA), where analyses were performed. The effect of prenatal depressive symptoms on child cortisol parameters and DNA methylation were tested with univariate analyses of covariance (ANCOVAs). Exposure to prenatal depressive symptoms (EPDSpre) and sex were entered as independent variables in each model, in order to check possible sex-specific or interaction effects. The cortisol parameters and the adjusted methylation values of each CpG were included as the dependent variables, respectively. Possible interaction effects were tested post-hoc with exposure-group specific ANCOVAs. To examine the functional relevance of differentially methylated CpGs for child cortisol, multiple regression models were applied. Adjusted methylation values were entered as the predictor and the cortisol parameters as the outcome in independent models. Therefore, only CpGs and cortisol parameters, which were associated with EPDSpre in previous analyses, were considered.

For significant associations, the mediation hypothesis was tested with the PROCESS macro for SPSS (version 2.16; Hayes, 2013). In independent models, EPDSpre was added as the regressor, adjusted methylation values as the mediator and cortisol parameters as the outcome variable. Bootstrapped 95 % confidence intervals (CI) based on 10.000 samples were used to interpret the indirect effects, with CIs not containing zero being interpreted as a mediation effect (A. P. Field, 2013).

Postnatal and current maternal depressive symptoms were consistently added as covariates in order to detect specific prenatal effects. Further covariates were added, if they were significantly associated with the dependent variable tested using Pearson correlation (r) or significantly different between the children non-exposed vs. exposed to prenatal depressive symptoms (independent t-test). Effect sizes for ANCOVA results were computed as partial η2 (ηp2), with the values ηp2< .06 interpreted as small, .06 ≤ ηp2< .14 as medium and ηp2 ≥.14 as large effects (Cohen, 1988). For all analyses, significance level was set to *p* = .05. In order to correct for multiple testing in the analyses considering the association of EPDSpre and DNA methylation, Bonferroni-adjustment for the number of tested CpGs per gene was applied.

# Results

* 1. *Covariates*

Children exposed to maternal depressive symptoms during pregnancy were older than non-exposed children at t2 (*p* < .001). Regarding maternal characteristics, depressive symptoms differed between groups not only during pregnancy (*p* < .001), but also for the postnatal (*p* < .001) and current period (*p* < .001). No other group differences in child, mother or pregnancy characteristics appeared, as shown in Table 1.

Regarding the cortisol analyses, significant associations between potential covariates and child cortisol differed between parameters, and were therefore specifically controlled in the analyses. Children’s antibiotic intake in the last 6 months before sample collection was significantly associated with waking cortisol (*p* = .032) as well as mean Apgar score with bedtime cortisol (*p* = .013) and the diurnal slope (*p* = .006). Child total cortisol release was higher on school days than on the weekend or during a school holiday (*p* < .001). Results are presented in Table S2. Additionally, parameter-relevant time frames were considered as covariates in order to control for time effects. Time between awakening and first sample was added as covariate for analyses with waking cortisol and CAR, time between first and last sample as covariate for analyses with bedtime cortisol, diurnal slope and total release.

* 1. *Prenatal depressive symptoms and child cortisol*

Analyses for bedtime cortisol revealed a significant main effect for EPDSpre as well as a significant interaction effect with child sex. Children who were exposed to depressive symptoms during pregnancy showed lower cortisol levels at bedtime. While boys and girls did not differ in the unexposed group, exposed boys tended to exhibit less cortisol than exposed girls at bedtime. A significant main effect of EPDSpre was also detected for the diurnal slope. Children of prenatal depressed mothers showed a steeper cortisol decline throughout the day. In addition, analyses revealed a significant interaction effect of EPDSpre and sex for the total cortisol release. In the exposed and non-exposed group, sex-differences were apparent, but marginally significant. Girls showed less total cortisol release than boys within the non-exposed group, but more total cortisol release than boys within the exposed group. No significant main effect of sex was found in either model, as well as no effects for waking cortisol and CAR. Results of the ANCOVAs are presented in Table 2, with significant effects illustrated in Figure 1. Table S3 shows the results of the conducted post-hoc ANCOVAs.

*[Please insert Table 2 here.]*

*[Please insert Figure 1 here.]*

* 1. *Prenatal depressive symptoms and DNA methylation of HPA related genes*

Subsequently reported results reached significance at uncorrected significance level (*p*= .05). For one CpG (cg07733851) of *NR3C1*, analysis revealed a significant main effect of EPDSpre. Children exposed to depressive symptoms in pregnancy showed higher methylation values. Interaction effects between EPDSpre and sex were observed for two further *NR3C1* CpGs (cg04111177, cg27107893). At both CpGs, no sex differences were observed in the non-exposed group, whereas boys exhibited higher methylation values than girls in the exposed group with medium effect sizes. Exposure to depressive symptoms in pregnancy was furthermore associated with lower methylation at one CpG (cg10288772) of *NR3C2*. For *SLC6A4*, modifications in DNA methylation at two CpGs were apparent. Exposed children showed higher methylation in cg18584905. For cg26741280, an interaction effect for EPDSpre and sex was identified. Again, only boys and girls exposed to prenatal depressive symptoms differed marginally in methylation values, whereas no sex-differences were observed in the non-exposed group.

No depression or sex effect on DNA methylation was found for *BDNF, CRHR1* or *FKBP5*. As presented in Table 3, reported differentially methylated CpGs in *NR3C1*, *NR3C2* and *SLC6A4* reached significance at *p*= .05, with effect sizes indicating small effects (ηp2= .03-.04). Significant main and interaction effects of EPDSpre or EPDSpre x sex are displayed in Figure 2. Results of the post-hoc analyses for significant interaction effects are shown in Table S3. Complete results of analyses testing modifications in DNA methylation at all CpGs of the candidate genes are shown in Table S4.

*[Please insert Table 3 here.]*

*[Please insert Figure 2 here.]*

* 1. *DNA methylation of HPA related genes and child cortisol*

Investigating the functional relevance of altered DNA methylation for basal HPA activity, multiple regression models were only calculated for those cortisol parameters and CpGs, which were significantly associated with EPDSpre in the preceding analyses. Only in boys, DNA methylation of one *NR3C2* CpG (cg10288772) significantly predicted bedtime cortisol (β = .30, *t*(66) = 2.64, *p* = .010), with higher methylation values resulting in higher bedtime values. Regarding all children and girls separately, DNA methylation of the HPA related genes did not predict either bedtime cortisol, diurnal slope or total cortisol release. Complete results are presented in Table S5.

* 1. *Prenatal depressive symptoms, DNA methylation of HPA related genes and basal cortisol in boys.*

Previous analyses indicated a specific association of prenatal depressive symptoms, DNA methylation in *NR3C2* and bedtime cortisol in boys. The hypothesis of DNA methylation as mediator between EPDSpre and cortisol was tested. Prenatal depressive symptoms significantly predicted boys’ bedtime cortisol. The bootstrapped confidence interval for the mediation effect did not include zero, indicating an existing mediation effect. Paths including bedtime cortisol were adjusted for postnatal and current depressive symptoms, time from first to last cortisol sample and mean Apgar score. The mediation effect was also apparent in an unadjusted model. Results of the unadjusted and adjusted model are presented in Figure 3.

*[Please insert Figure 3 here.]*

# Discussion

Investigating the association of prenatal depressive symptoms with child DNA methylation and basal HPA axis activity revealed small but clear effects. Results suggest that there is a sex-specific mediation effect of DNA methylation that explains, at least partially, basal HPA activity in primary school aged children.

* 1. *Prenatal depressive symptoms and child cortisol*

Children exposed to prenatal depressive symptoms showed altered basal cortisol levels. Whereas Laurent et al. (2013) reported decreased cortisol both at awakening and bedtime, the present study only found a downregulation of bedtime cortisol in exposed children. This might explain the additional finding for the diurnal cortisol slope, with exposed children displaying a steeper cortisol decline than non-exposed children. Exposed boys were more susceptible to the effects, exhibiting lower bedtime cortisol levels than exposed girls. Additionally, girls and boys showed different patterns of total release. Boys released more cortisol throughout the day than girls in the unexposed group, while the opposite was observed within the exposed group.

Other studies investigating the influence of peripartum maternal depression mainly reported higher basal cortisol levels – as found here in girls – or flattened cortisol slopes in exposed children, without reporting sex effects (e.g. Diego et al., 2004; O'Donnell et al., 2013). The diverging results might be explained by differences in the type and duration of maternal depressive symptoms as well as different child age ranges that were examined. As a consequence of different early life stress exposures, including maternal and paternal depression in the first year of life, Essex et al. (2011) reported profiles of hypo- and hypercortisolism as well as varying developmental profiles from 9 to 15 years of age. Likewise, Shirtcliff et al. (2012) detected developmental trajectories from prepubertal age to adolescence, e.g. decreased cortisol levels and flattened diurnal profiles in adolescents. They also found sex-specific effects, e.g. girls exhibiting more cortisol and steeper slopes than boys. Hence, it must be stated that the HPA-axis functionality during childhood and adolescence is not fully understood making a comparison of its function in studies performed at different developmental stages difficult. In further longitudinal studies relevant age- and sex-specific effects must be examined.

According to the DOHaD hypothesis, alterations in the HPA axis represent an adaption to the prenatal environment. The downregulated bedtime cortisol levels in exposed children, found in the present study, might be interpreted as an overcompensation of the HPA axis to the early stressful life circumstances. In expectation of a similar postnatal environment, the regulatory mechanisms of the HPA axis are increased, resulting in lower cortisol levels. It might be speculated that this overcompensation might result in a general blunted HPA axis and exhausted cortisol system over time, serving as a risk factor for mental health problems. Studies regarding the behavioral consequences of prenatal depression support this risk hypothesis of decreased cortisol levels, especially seen in boys. Prenatal depression was associated with more externalizing behavior and violence in children and adolescence, and for boys with a lower social-emotional competence (e.g. Eichler, Walz, et al., 2016; Korhonen, Luoma, Salmelin, & Tamminen, 2012). Low basal cortisol levels in turn predicted persistence and early-onset of aggressive behaviors in boys (McBurnett, Lahey, Rathouz, & Loeber, 2000). Despite the non-significant group difference in externalizing behavior in the present non-clinical sample, both the lower bedtime value and total cortisol release, especially in the exposed boys, might be interpreted as an underlying risk factor for these behavior problems in the long term. The increased total cortisol release found in exposed girls might be similarly interpreted as risk factor for later internalizing problems, which have already been associated with prenatal depression, especially in girls (e.g. El Marroun et al., 2014; Quarini et al., 2016).

* 1. *Prenatal depressive symptoms and DNA methylation of HPA related genes.*

For DNA methylation, modifications associated with prenatal depression were found for *NR3C1*, *NR3C2* and *SLC6A4*. With three CpGs, the GR-coding gene *NR3C1* was the most frequently influenced gene, corresponding to its central role in the previous methylation studies of early adversity. Whereas one CpG (cg07733851) was hypermethylated in exposed boys and girls, the others (cg04111177, cg27107893) were modified for each sex differently. Girls showed higher methylation values than boys after prenatal exposure, indicating sex-specific effects on DNA methylation. In Weder et al. (2014), the methylation of cg04111177 has already been associated with both early childhood maltreatment and morning cortisol in adolescents, but without investigating sex-differences. Indications for an impact of prenatal depression on the serotonergic system are given through methylation differences of two CpGs of the serotonin receptor coding gene *SLC6A4*. For one of these CpGs (cg18584905), exposed children showed consistently higher methylation, for the other (cg26741280) sex-specific modifications in the exposed group are apparent, with girls’ methylation being influenced stronger in form of a hypomethylation.

Methylation modifications at *NR3C1* and *SLC6A4* have already been associated with pre- and postnatal adversity, childhood trauma and risk for psychopathology, and are therefore discussed as biomarker for prenatal and early childhood adversity (Provenzi, Giorda, Beri, & Montirosso, 2016; Tyrka, Ridout, & Parade, 2016). Results presented here support this hypothesis, but with small effect size and not withstanding correction for multiple testing. Furthermore, the differentially methylated CpGs of both genes are not located in a promotor region, lowering the potential functional relevance for gene expression. This in turn is bolstered by the missing associations between the found altered CpGs at *NR3C1* and *SLC6A4* and child HPA activity.

The identified sex-specific relations of prenatal depression to both HPA axis activity and DNA methylation support the hypothesis of sex-specific intrauterine programming mechanisms (Bale, 2011; Glover & Hill, 2012). Sex-specific differences in the functionality of the placenta, which is suggested to be the primary source for the relation of maternal mood and fetal HPA axis development, might alter the *in utero* environment specifically, resulting in sex-specific consequences for HPA axis regulation.

* 1. *Prenatal depressive symptoms, DNA methylation of NR3C2 and bedtime cortisol*

For the MR-coding gene *NR3C2,* one CpG (cg10288772) was differentially methylated, with lower methylation values in exposed children. The modified CpG is located in the *NR3C2* promotor allowing the hypothesis that this CpG likely impacts gene expression. In further analyses, *NR3C2* methylation was found to be related to bedtime cortisol in boys and could be identified as sex-specific mediator of this association, suggesting a functional relevance of *NR3C2* for basal cortisol.

The MR is identified as an important regulator of the basal and stress-induced activity of the HPA axis (ter Heegde et al., 2015). MR binding of corticosteroids is thereby associated with an increased inhibition mechanism and, in turn, lower basal cortisol levels and a blunted stress response, respectively. The positive association of *NR3C2* methylation and bedtime cortisol levels, found in the present study, fits the typical expectation of DNA methylation associated with a downregulation of gene expression. In the case of *NR3C2*, lower methylation would result in an increased MR expression and a stronger feedback inhibition process with lower cortisol values as indeed observed. Summarizing the literature to MR, ter Heegde et al. (2015) interpreted increased MR expression or functionality as enhanced stress resilience.

The present study identified less *NR3C2* DNA methylation and a decreased bedtime cortisol level in boys prenatally exposed to depressive symptoms. Investigating the effects of a prenatal traumatic event on gene expression, Perroud et al. (2014) have reported higher MR mRNA levels in exposed children compared to non-exposed ones, which can be interpreted as analogous finding in terms of the proposed mechanism. More studies reporting modifications in *NR3C2* methylation or expression after exposure to early adversity in children are currently missing. According to ter Heegde et al. (2015), however, prenatal depressive symptoms accompanied by increased MR functionality would represent stress resilience and not, as discussed more often, a risk factor for HPA dysregulation and psychiatric disorders. Considering the fact that the association of prenatal depression, *NR3C2* DNA methylation and basal cortisol was found only in boys, it might be possible to integrate the findings into the risk hypothesis. As discussed previously, lower basal cortisol levels are considered to be a risk factor for externalizing behavior problems, which in turn are associated with prenatal depression in boys. Therefore, it is speculated that the altered *NR3C2* methylation following prenatal depression might represent one explanatory model for the higher risk for externalizing behavior in boys.

* 1. *Limitations*

It has to be mentioned again, that the identified associations were mainly small and, regarding the analyses of DNA methylation modifications, did not survive corrections for multiple testing. Knowing that methylation changes, especially in the gene promotor, are expected to influence gene expression, it must be recognized, that no CpG within the promoter region of *BDNF* or *SLC6A4* was included on the 450k BeadChip. Therefore, investigating the impact of quite important sites for gene regulation was not possible for these genes. Analyzing the association of differently methylated CpGs with variables such as the cortisol system, offers an opportunity for identifying their potential functional relevance. Nevertheless, gene expression data, which are necessary for validating this on the molecular level, were not available in the present study. Furthermore, DNA was extracted from buccal cells in the study. Because of the tissue-specificity of DNA methylation, conclusions from peripheral tissues upon brain processes and functioning should be drawn consciously.

An EPDS score of 10 or higher in the third trimester was interpreted as exposure to prenatal depressive symptoms. This score is used in several studies but indicates only a potential minor clinical depression. Besides the self-rating questionnaire, no other objective assessment was used. Furthermore, depressive symptoms were assessed just once in pregnancy. Regarding the diverging influences on child development dependent on the three trimesters was therefore not possible.

1. **Conclusions and future directions**

To the authors’ knowledge, this is the first study conducted in primary school aged children investigating the association between prenatal depression and basal child HPA activity and testing the mediation hypothesis of DNA methylation as underlying mechanism. Presented results give rise to DNA methylation, here found especially in *NR3C2*, as one underlying mechanism as well as sex-specific processes. Despite the known role of MR on HPA axis regulation, the current literature has mostly ignored DNA methylation and gene expression of *NR3C2* as marker of prenatal and early childhood adversity in comparison to *NR3C1*, *SLC6A4* or *BDNF*. Future studies should regard *NR3C2* as further relevant part of HPA axis regulation for validating the presented results. Looking at the sample sizes in several previous studies published in this topic, it is recommended to investigate effects in larger samples enabling the exploration of discussed sex-differences as well as the probable influence of ethnicity, medication or symptom severity. Additionally, expression data as a parameter of functional analyses of DNA methylation are necessary in order to validate the functional consequences of modifications in reported CpGs and to integrate the former studies, which examined DNA methylation and gene expression mainly separately. Comparative analyses of DNA methylation in peripheral tissue and the brain would further help with interpreting the findings of the studies currently available, which were mainly undertaken with DNA from blood. Understanding the mechanisms of prenatal depression effects on child HPA axis and psychopathology remains an important research field, with many open questions. This study emphasizes that one component should be the continued development of the research parameters.

# Conflicts of interest

None.

# Contributors

Valeska Stonawski, Stefan Frey, Yulia Golub, Nicolas Rohleder, Jennifer Kriebel, Hartmut Heinrich and Anna Eichler analyzed the data and / or interpreted the results. Tamme W. Goecke, Johannes Kornhuber, Peter A. Fasching and Matthias W. Beckmann initiated and designed the FRAMES project. Tamme W. Goecke and Peter A. Fasching supervised the clinical data acquisition in FRAMES. Oliver Kratz, Gunther H. Moll, Hartmut Heinrich and Anna Eichler were responsible for the study design of FRANCES. Valeska Stonawski and Anna Eichler supervised the clinical data acquisition in FRANCES. Valeska Stonawski drafted the initial manuscript. All authors reviewed the manuscript and have approved the final manuscript

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**Tables and Figures**

## Table 1. *Demographic and pregnancy characteristics of the sample*

|  |  |  |  |
| --- | --- | --- | --- |
|  | Total sample | Prenatal depressive symptomsa | Non-exposed vs. exposed |
|  | *(n* = 167) | Non-exposed*(n* = 117) | Exposed*(n* = 50) | *t*(165) / χ2(1) | *p* |
| Maternal characteristicsb |
| Age (years) | 40.44 (4.62) | 40.73 (4.30) | 39.76 (5.29) | 1.24 | .216 |
| SES  | 11.2 (2.17) | 11.37 (2.08) | 10.88 (2.37) |  1.33 | .185 |
| EPDS current | 6.43 (4.78) | 5.12 (4.04) | 9.50 (5.02) | 0.46c\*\* | < .001 |
| Child characteristicsb |
| Age (years) | 7.6 (0.6) | 7.5 (0.6) | 7.9 (0.6) | 4.62\*\* | < .001 |
| Sex |  |  |  |  |  |
| Boys | 82 (49.1) | 59 (50.4) | 23 (46.0) | 0.28 | .617 |
| Girls | 85 (50.9) | 58 (49.6) | 27 (54.0) |  |  |
| Psychopathology (SDQ) |  |  |  |  |  |
| Emotional problems | 1.84 (1.76) | 1.76 (1.64) | 2.02 (2.03) | 0.87 | .385 |
| Conduct problems | 1.91 (1.68) | 1.82 (1.48) | 2.12 (2.08) | 0.92c | .359 |
| IQ (IDS) | 104.81 (10.25) | 105.20 (10.52) | 103.92 (9.62) | 0.74 | .463 |
| Pregnancy characteristics |
| Gestational age (weeks) | 39.3 (1.5) | 39.3 (1.48) | 39.3 (1.63) | 0.32 | .751 |
| Birth weight (g) | 3441.3 (491.4) | 3434.7 (474.7) | 3456 (533.2) | 0.27 | .791 |
| Apgar | 9.43 (0.6) | 9.47 (0.56) | 9.35 (0.66) | 1.08c | .284 |
| Maternal age at birth (years) | 32.8 (4.71) | 33.2 (4.36) | 32.0 (5.41) | 1.40c | .165 |
| EPDS pre | 6.69 (5.07) | 3.93 (2.63) | 13.16 (3.13) | 19.58\*\* | < .001 |
| EPDS post | 4.42 (4.74) | 3.52 (3.96) | 6.52 (5.69) | 3.39c\*\* | < .001 |
| Alcohol consumption pre |  |  |  |  |  |
| Yes | 34 (20.4%) | 27 (23.1%) | 7 (14%) | 0.36 | 1.00 |
| No | 113 (67.7%) | 88 (75.2%) | 25 (50%) |  |  |
| Cigarette smoking pre |  |  |  |  |  |
| Yes | 19 (11.4%) | 16 (13.7%) | 3 (6%) | 2.05 | .190 |
| No | 148 (88.6%) | 101 (86.3%) | 47 (94%) |  |  |

*Note*. Continuous variables are expressed as mean (*SD*) and tested with independent t-tests, categorial variables are expressed as *n* (%) and tested with chi-squared tests. *t*-scores are displayed as absolute values. EPDS = Edinburgh Postnatal Depression Scale (Cox et al., 1987). SES = socioeconomic family status. IDS = Intelligence and Development Scales (Grob et al., 2009). SDQ = Strength and Difficulties Questionnaire (Goodman, 2001). Pre = prenatal, post = postnatal. aPrenatal EPDS score < 10 interpreted as non-exposed, ≥ 10 as exposed. bat time of cortisol and DNA sampling. c*df* adjusted for unequal variances based on Levene. \**p* < .05, \*\**p* < .01.

## Table 2. *Effects of exposure to prenatal depressive symptoms and sex on diurnal cortisol parameters: results of ANCOVAs*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  |  | ME EPDSpre | ME sex | IA EPDSpre x sex |
|  | *n* | *F* | *p* | ηp2 | *F* | *p* | ηp2 | *F* | *p* | ηp2 |
| Waking Cortisola,b | 99 | 1.92 | .169 | .02 | 0.10 | .756 | .00 | 1.19 | .278 | .01 |
| Bedtime Cortisolc,d | 145 | 9.47\*\* | .003 | .07 | 0.80 | .372 | .01 | 4.35\* | .039 | .03 |
| CARa | 81 | 1.29 | .260 | .02 | 0.00 | .951 | .00 | 0.54 | .465 | .01 |
| Diurnal Slopec,d | 99 | 5.36\* | .023 | .06 | 0.18 | .670 | .00 | 1.94 | .167 | .02 |
| Total releasec,e | 145 | 0.03 | .859 | .00 | 0.23 | .632 | .00 | 5.62\* | .019 | .04 |

*Note*. Models were adjusted for maternal postnatal and current depressive symptoms as well as specific covariates for the cortisol parameters (atime between awakening and first sample, bAntibiotic intake in the 6 months before sample collection, ctime between first and last sample, dmean Apgar score, eschool day: yes/no). ME = main effect; IA = interaction effect. EPDS = Edinburgh Postnatal Depression Scale (Cox et al., 1987). EPDSpre = exposure to prenatal depressive symptoms (EPDS score ≥ 10) or no exposure (< 10). CAR = cortisol awakening response. Total release = total cortisol release throughout day. \**p* < .05, \*\**p* < .01.

## Table 3. *Effects of exposure to prenatal depressive symptoms and sex on DNA methylation: results of ANCOVAs*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  |  | ME EPDSpre | ME sex | IA EPDSpre x sex |
| Gene | CpG | *n* | *F* | *p* | ηp2 | *F* | *p* | ηp2 | *F* | *p* | ηp2 |
| *NR3C1* | cg04111177 | 167 | 1.11 | .294 | .01 | 1.04 | .308 | .01 | 4.73\* | .031 | .03 |
| *NR3C1* | cg07733851 | 167 | 4.68\* | .032 | .03 | 0.07 | .793 | .00 | 0.22 | .644 | .00 |
| *NR3C1* | cg27107893 | 144 | 0.12 | .731 | .00 | 0.82 | .368 | .01 | 4.74\* | .031 | .03 |
| *NR3C2* | cg10288772 | 166 | 6.51\* | .012 | .04 | 0.26 | .611 | .00 | 1.01 | .318 | .01 |
| *SLC6A4* | cg18584905 | 166 | 5.19\* | .024 | .03 | 0.07 | .797 | .00 | 0.21 | .649 | .00 |
| *SLC6A4* | cg26741280 | 167 | 3.11 | .080 | .02 | 0.77 | .381 | .01 | 4.28\* | .040 | .03 |

*Note*. Maternal postnatal and current depressive symptoms were included as covariates. ME = main effect; IA = interaction effect. EPDS = Edinburgh Postnatal Depression Scale (Cox et al., 1987). EPDSpre = exposure to prenatal depressive symptoms (EPDS score ≥ 10) or no exposure (< 10). \**p* < .05, \*\**p* < .01, not Bonferroni adjusted.

## Figure 1



***Figure 1****.* Effects of exposure to prenatal depressive symptoms and sex on diurnal cortisol parameters. Parameters were calculated based on ln-transformed raw values. Means are adjusted for maternal postnatal and current depressive symptoms as well as specific covariates for the cortisol parameters (atime between first and last sample, bmean Apgar score, cschool day: yes/no). Error bars represent standard mean error. EPDS = Edinburgh Postnatal Depression Scale (Cox et al., 1987). EPDSpre = exposure to prenatal depressive symptoms (EPDS score ≥ 10) or no exposure (< 10). Interaction effects were tested post-hoc with ANCOVAs comparing boys vs. girls in the non-exposed and exposed group separately. *+p* < .10, \**p* < .05, \*\**p*< .01.

## Figure 2



***Figure 2*.**Effects of exposure to prenatal depressive symptoms and sex on DNA methylation at CpGs of *NR3C1*, *NR3C2* and *SLC6A4*. DNA methylation is displayed as z-standardized adjusted methylation value. Error bars represent standard mean errors. EPDS = Edinburgh Postnatal Depression Scale (Cox et al., 1987). EPDSpre = exposure to prenatal depressive symptoms (EPDS score ≥ 10) or no exposure (< 10). Interaction effects were tested post-hoc with ANCOVAs comparing boys vs. girls in the non-exposed and exposed group separately. *+p* < .10, \**p* < .05, not Bonferroni-adjusted.

## Figure 3

**A**

DNA methylation cg10288772 (*NR3C2*)

Bedtime cortisol

*b* =- 0.005\*\*,
*p* = .006

*b* = 11.92+,
*p* = .055

Direct effect*: b* = -0.18\*, *p* = .041

Indirect effect: *b* = -0.06, 95% CI [-0.14; -0.01], *p* = .123

EPDSpre

**B**a

DNA methylation cg10288772 (*NR3C2*)

Bedtime cortisol

*b* =- 0.005\*\*,
*p* = .006

*b* = 14.37\*,
*p* = .021

Direct effect*: b* = -0.27\*\*, *p* = .003

Indirect effect: *b* = -0.07+, 95% CI [-0.16; -0.02], *p* = .079

EPDSpre

***Figure 3*.** Unadjusted (A) and adjusted (B) model of exposure to prenatal depressive symptoms as predictor of bedtime cortisol in boys, mediated by *NR3C2* DNA methylation (*n* = 70). Results of testing the mediation hypothesis by PROCESS (Hayes, 2013), with exposure to prenatal depression as independent variable, boys’ bedtime cortisol as outcome variable and cg10288772 (*NR3C2*) DNA methylation as mediator. EPDS = Edinburgh Postnatal Depression Scale (Cox et al., 1987). EPDSpre = exposure to prenatal depressive symptoms (EPDS score ≥ 10) or not (< 10). aPostnatal and current depressive symptoms, time between first and last sample and mean Apgar score were included as covariates for paths including bedtime cortisol. +*p* < .10, \**p* < .05, \*\**p* < .01.