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RESEARCH ARTICLE

Maternal distress, DNA methylation, and fetal programing of stress physiology in Brazilian mother–infant pairs

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Abstract

Maternal prenatal psychosocial stress is associated with adverse hypothalamic–pituitary–adrenal axis (HPAA) function among infants. Although the biological mechanisms influencing this process remain unknown, altered DNA methylation is considered to be one potential mechanism. We investigated associations between maternal prenatal psychological distress, infant salivary DNA methylation, and stress physiology at 12 months. Mother's distress was measured via depression and anxiety in early and late pregnancy in a cohort of 80 pregnant adolescents. Maternal hair cortisol was collected during pregnancy. Saliva samples were collected from infants at 12 months to quantify DNA methylation of three stress-related genes (*FKBP5*, *NR3C1*, *OXTR*) ($n = 62$) and diurnal cortisol ($n = 29$). Multivariable linear regression was used to test for associations between prenatal psychological distress, and infant DNA methylation and cortisol. Hair cortisol concentrations in late pregnancy were negatively associated with two sites of *FKBP5* (site 1: $B = -22.33$, $p = .003$; site 2: $B = -15.60$, $p = .012$). Infants of mothers with elevated anxiety symptoms in late pregnancy had lower levels of *OXTR2* CpG2 methylation ($B = -2.17$, $p = .03$) and higher evening salivary cortisol ($B = 0.41$, $p = .03$). Furthermore, *OXTR2* methylation was inversely associated with evening cortisol ($B = -0.14$, p-value $\leq .001$). Our results are, to our knowledge, the first evidence that the methylation of the oxytocin receptor may contribute to the regulation of HPAA during infancy.

KEYWORDS
anxiety, cortisol, depression, DNA methylation, glucocorticoid receptor, oxytocin receptor, pregnancy

1 | INTRODUCTION

Maternal psychosocial distress during pregnancy is associated with a wide array of adverse developmental outcomes in offspring. A substantial body of prenatal psychosocial stress literature has described associations with adverse birth (Grote et al., 2010; Straub et al., 2012), psychological (Pearson et al., 2013; Van den Bergh et al., 2008), behavioral (O'Connor et al., 2002), and physical health outcomes (Beydoun & Saftlas, 2008). Although these effects are thought to be a result of fetal programing effects, the biological mechanisms underlying prenatal stress and infant development remain poorly understood (Conradt et al., 2018; Dunkel Schetter, 2010). To date, research on fetal programing mechanisms has been conducted almost exclusively in high-income countries, with socioeconomically and ethnically homogenous samples. This limitation of the literature hampers the generalizability of results of extant research to populations frequently exposed to chronic and severe adversity (Bush et al., 2017). This study focused on adolescent mothers in the western region of São Paulo, Brazil. This region is characterized by high rates of urban violence, poverty, and adverse living conditions (Ferri et al., 2007; Jacobi, 1994; Ribeiro et al., 2013). We investigated potential mechanisms of fetal programing by examining associations between maternal psychological distress, infant DNA methylation of three genes (*NR3C1*, *FKBP5*, and *OXTR*), and infant stress physiology indexed by diurnal cortisol rhythms.

1.1 | Mechanisms of fetal programing

Prenatal exposure to glucocorticoids (GCs) has been proposed as one of the primary mechanisms of fetal programing of pre- and postnatal developmental trajectories and a driver of epigenetic change across the life course (Seckl & Meaney, 2004; Zannas & Chrousos, 2017). Cortisol levels in pregnancy may be sensitive to maternal psychological distress: Elevated salivary levels are associated with prenatal depression (O'Connor et al., 2014) and anxiety (Pluess et al., 2010). Elevated prenatal cortisol during gestation has been associated with a variety of postnatal neurodevelopmental consequences (Davis et al., 2007; Davis & Sandman, 2010) and altered infant hypothalamic–pituitary–adrenal axis (HPAA) activity (Davis et al., 2011; Gutteling et al., 2005; Irwin et al., 2021; Van den Bergh et al., 2008) for infants living in the United States and the Netherlands.

Epigenetic modifications, such as DNA methylation, are thought to be mediators in the fetal programing of infant stress physiology in response to maternal prenatal stress (Turecki & Meaney, 2016). Despite the hypothesized role of cortisol as one of the primary mechanisms of fetal programing, few studies have examined DNA methylation in relation to circulating GC levels during pregnancy. One such study by Hompes et al. (2013) found that maternal diurnal cortisol levels in the second trimester were associated with cord blood methylation of several *NR3C1* sites. Another study that quantified placental methylation levels of *NR3C1*, *FKBP5*, and *HSD11B2* (the gene coding for 11B-HSD2) found no significant associations with salivary cortisol levels (Monk et al., 2016). More research is needed to investigate the methylation of these genes in response to pregnancy GCs and their involvement in regulating postnatal HPAA physiology in infancy.

1.2 | HPAA gene methylation and fetal programing

The GR gene, *NR3C1*, has been a focus of many studies of prenatal stress and methylation due to its role in regulating the HPAA through a negative feedback loop in response to GC release (Palma-Gudiel, Córdova-Palomera, Eixarch, et al., 2015; Palma-Gudiel, Córdova-Palomera, Leza, et al., 2015). Elevated methylation of *NR3C1* has been associated with a decreased expression of GRs in the rat hippocampus, which results in a prolonged increase in circulating GRs due to a weakening of the negative feedback loop (Weaver et al., 2004). In humans, maternal anxiety and depression (Braitwaite et al., 2015; Conradt et al., 2013; Hompes et al., 2013; Monk et al., 2016; Oberlander et al., 2008; Stonawski et al., 2018), intimate partner violence (Radtke et al., 2011), and war-related stress (Mulligan et al., 2012; Perroud et al., 2014; Rodney and Mulligan, 2014; Kertes et al., 2016) have been associated with methylation of *NR3C1*. A meta-analysis of studies examining associations between prenatal stress and *NR3C1* methylation supported the role of methylation in this process (Palma-Gudiel, Córdova-Palomera, Eixarch, et al., 2015).

Despite this historical focus on *NR3C1*, it is likely that other genes are involved in regulating the complex biological pathway of the HPAA. One other gene that has received attention is FK506 binding protein 5 (*FKBP5*), coding for the co-chaperone of the GR that is involved in the termination of the HPAA response via regulation of GR sensitivity (Binder, 2009). Even fewer studies have examined patterns of *FKBP5* methylation in response to prenatal stress. For example, Monk et al. (2016) in a study of American women documented a positive association between maternal perceived stress, increased placental *FKBP5* methylation, and lower fetal coupling, a measure of fetal neurobehavior. Similarly, Kertes et al. (2016) found positive associations between war stress and *FKBP5* methylation, though it was not associated with their primary outcome measure, birth weight in a sample of women and infants from the Democratic Republic of Congo. Another study found associations between higher levels of placental *FKBP5* methylation with altered neurobehavioral outcomes in neonates (Paquette et al., 2014). Although the methylation of these genes has been shown to vary in response to prenatal stress, it is unclear how this process is affected by proposed mediators, such as pregnancy cortisol levels.

1.3 | OXTR methylation and fetal programing

The neuropeptide oxytocin (OXT) plays a key role in regulating human social and emotional behaviors, including prosocial behaviors such as attachment and bonding (Lee et al., 2009; Levine et al., 2007). Although the oxytocin system has received considerable attention for its roles in social behavior, childbirth, lactation, and maternal–infant bonding, it has recently been proposed to be involved in pre- and postnatal transmission pathways of maternal stress (Toepfer et al., 2017). This

argument is supported by evidence that suggests that stress exposure is associated with alterations to functioning of the OXT system, as well as studies that suggest that OXT plays a role in the development of stress-related disorders such as depression and anxiety (McQuaid et al., 2014; Neumann & Slattery, 2016). Additionally, OXT may modulate the activity of the HPAA and the immune system, which have been proposed to affect fetal development (Cardoso et al., 2014; Wang et al., 2015). A meta-analysis showed that intranasal OXT administration can attenuate HPAA reactivity in response to a laboratory stressor that stimulates the HPAA (Cardoso et al., 2014). Early life stress has also been shown to impact OXT signaling in childhood and altered cerebral spinal fluid and plasma concentrations of OXT in adulthood (Fries et al., 2005; Heim et al., 2009; Opacka-Juffry & Mohiyeddini, 2012). This co-occurrence of abnormal OXT and HPAA signaling suggests that disruption to the bidirectional associations between these systems may shape trajectories of stress-related disorders later in life (Toepfer et al., 2017). In fact, the stress modulating effects of OXT may be reversed in individuals exposed to early life stress, as intranasal OXT has been shown to increase cortisol reactivity in such individuals (Grimm et al., 2014). Although OXT levels have been more extensively studied, novel research has begun to implicate the oxytocin receptor (OXTR) in this process.

Studies of *OXTR* methylation have investigated the associations between prenatal stress and methylation levels and suggest that DNA methylation of *OXTR* may be one pathway from early life experiences to adverse socio-behavioral outcomes later in life (Kraaijenvanger et al., 2019). Epigenetic regulation of *OXTR* may also serve as a mechanism by which the OXT system helps regulate allostasis (Danoff et al., 2021). Cecil et al. (2014) found that maternal prenatal stress, such as violence exposure, was associated with an increased methylation of a CpG island of *OXTR* at birth in a subsample of the Avon Longitudinal Study of Parents and Children. Reporting conflicting associations, Unternaehrer et al. (2016) reported maternal prenatal depression, life stress, and pregnancy cortisol levels were associated with lower *OXTR* cord blood methylation of Swiss infants. However, another study of cord blood methylation reported null findings between prenatal stressors and *OXTR* methylation at birth (Rijlaarsdam et al., 2017). Null associations have also been reported for associations between prenatal maternal depression and *OXTR* methylation of placental tissue, as well as salivary DNA collected from children (Galbally et al., 2018; King et al., 2017).

1.4 | Methylation and infant cortisol

Although such studies have documented associations between prenatal stress and infant methylation, only a few have examined associations between methylation and infant stress physiology. To our knowledge, only three studies have investigated the relationship between *NR3C1* methylation and infant HPAA function, both focused on reactivity in response to an age-appropriate laboratory-based stressor. Specifically, Conradt et al. (2016) found no association between *NR3C1* methylation and basal cortisol level, but that greater methylation was associated with lower posttest cortisol levels in a cohort of American women and their infants. The other study, also conducted with an American sample, found that increased *NR3C1* methylation was associated with greater cortisol response to the stressor (Oberlander et al., 2008). Barha et al. (2019) reported no association between the methylation of *NR3C1* and urinary or salivary cortisol of Canadian infants. No studies, to our knowledge, have investigated associations between *FKBP5* methylation and infant HPAA function in infancy.

Despite hypothesized interactions with the HPAA (Entringer et al., 2015), no studies have investigated relationships between prenatal stress and offspring *OXTR* methylation and HPAA activity.

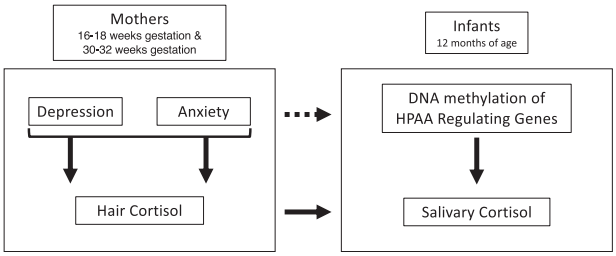

FIGURE 1 Conceptual schema of relationships between study variables. We hypothesized that mothers' prenatal distress would be associated with hair cortisol levels during pregnancy and that both distress and cortisol would be associated with programing of infant DNA methylation and hypothalamic–pituitary–adrenal axis regulation. Furthermore, we hypothesize that infant DNA methylation would also play a role in regulation of infant stress physiology. Such associations could be fetal programing effects that occur through fetal programing by maternal stress physiology (solid arrows) or indirect effects through another unmeasured pathway (dashed arrow).

1.5 | Our study

This study investigated maternal prenatal psychological distress and pregnancy cortisol levels as predictors of infant salivary DNA methylation of *NR3C1*, *FKBP5*, and *OXTR* at 12 months postnatal and diurnal cortisol levels (Figure 1). We used two measures of maternal prenatal psychological distress, evaluated as symptoms of depression and anxiety. We quantified maternal hair cortisol levels at two timepoints during pregnancy, 8–16 and 30 weeks, as a measure of maternal HPAA activity. We investigated associations between the methylation of *NR3C*, *FKBP5*, and *OXTR* and infant salivary cortisol levels at 12 months in order to investigate potential functional consequences of prenatal stress-induced changes of methylation of these genes. The aims of this study were to investigate associations between:

1. Maternal psychological distress and maternal hair cortisol in early and late pregnancy.
2. Maternal prenatal psychological distress and hair cortisol concentration and infant salivary DNA methylation of three genes related to HPAA regulation—the GC receptor (*NR3C1*) and its co-chaperone (*FKBP5*), and the *OXTR* and salivary cortisol levels.

3. DNA methylation of genes related to HPAA function and infant salivary cortisol levels.

We hypothesized that greater maternal prenatal psychological distress and increased cortisol during pregnancy would be associated with an altered methylation of infant's stress genes and diurnal HPAA rhythm of infants. We did not test a formal mediation model due to our modest sample size.

2 | METHODS

2.1 | Participants

Eighty pregnant adolescents between 14 and 19 years of age were recruited and completed a baseline interview (T1) during the first trimester of their pregnancy (8–16 weeks), as participants in a randomized controlled trial to test the efficacy of Primeiros Laços, a home visiting program (HVP) to support positive parenting skills for adolescent mothers living in a poor urban area of São Paulo, Brazil (NCT02807818). A second interview (T2) was conducted at 30 weeks of gestation. Inclusion and exclusion criteria were low socioeconomic status youth aged living in the western region of São Paulo, pregnant for the first time, and between 8 and 16 weeks of pregnancy at recruitment (Fatori et al., 2021). The analyses presented here were conducted on the entire sample. Written informed consent was obtained from all participants and from a parent or guardian if the participant was <18 years old. The study was approved by the ethical review boards at the University of São Paulo and the São Paulo Municipal Health Department. Additional approval for analyses of saliva samples was received from the institutional review board at Yale University.

2.2 | Maternal depression

Maternal depressive symptoms were measured using a Portuguese-language version of the Beck Depression Inventory (BDI) (Beck et al., 1961; Gomes-Oliveira et al., 2012). The BDI was administered at enrollment at 8–16 weeks, again at 30 weeks of gestation, and at the postnatal interview. BDI scores were dichotomized in order to conserve statistical power in our analyses. Following the BDI manual, we used cutoffs of 0–13, reflecting minimal depressive symptoms, and 14 to the maximum reported score, to group those with more severe depressive symptoms (Beck et al., 1996).

2.3 | Maternal anxiety

Maternal anxiety symptoms were measured using a Portuguese-language version of the Beck Anxiety Inventory (BAI) (Beck et al., 1961; Quintão et al., 2013). The BAI was administered at the same weeks of gestation as the BDI. We also dichotomized the BAI scores. Following the BAI manual, we used cutoffs of 0–7, reflecting zero to few anxiety symptoms, and 8 to the maximum reported score, representing more severe anxiety symptoms (Beck & Steer, 1993).

2.4 | Maternal hair cortisol

We collected two hair samples from participants during pregnancy. Hair was cut as close as possible to the scalp at the vertex posterior of the head, upon enrollment between 8 and 16 weeks of gestation, and again at follow-up at 30 weeks of gestation. Hair samples were cut into 3 cm segments to measure the average of the previous 3 months of cortisol production prior to the sampling date, using an estimated rate of 1 cm/month of hair growth (Russell et al., 2012; Stalder & Kirschbaum, 2012). Hair samples were processed and assayed at a commercial research lab, the Laboratório Especializado em Análises Científicas (LEAC), in São Paulo, following a previous extraction protocol used in our studies (Liu et al., 2017, 2020). First, 50 mg hair samples were washed twice with 40 ml of water, followed by two washes with 40 ml of isopropanol on a plate rotator. After the washes, the samples were cut into pieces using surgical scissors and were added to scintillation vials. HPLC methanol was added to the vials at a concentration of 100 μl/mg of hair. Samples were sonicated for 30 min and then incubated at 50°C for 24 h. After the incubation, samples were centrifuged for 30 min at 3000 rpm and the supernatant was aliquoted into glass tubes. The supernatant was evaporated under a stream of nitrogen. Samples were reconstituted in phosphate-buffered saline and vortexed. Commercially available salivary enzyme-linked immunosorbent assays were used to quantify hair cortisol extract concentrations (Diasource, New York, New York, USA, product number KAPDB290). Sufficient hair samples were collected from 62 participants at baseline and follow-up. At baseline, 18 participants declined to participate in hair sample collection. Nine participants were lost to follow-up at the 30-week interview; another nine declined the hair sample collection. Following previous work published by our research group (Liu et al., 2017), we excluded hair cortisol concentrations >2 standard deviations (SD) above the mean, resulting in available data for 54 participants at recruitment and 58 at follow-up.

2.5 | Infant salivary cortisol

Infants establish a typical diurnal cortisol rhythm by 3–4 months of age (Price et al., 1983; Santiago et al., 1996). This allowed us to quantify waking and bedtime cortisol, as well as decline across the day. Mothers were asked to collect saliva samples from infants at 12 months of age using Salimetrics saliva collection swabs and tubes (College Station, Pennsylvania, USA), on two consecutive days at home within 30 min of waking and prior to bedtime. Complete saliva samples for cortisol analyses were not available for all infants. At least 1 saliva sample was collected from 45 infants. Mothers completed the saliva collection protocol for 37 infants, although collection times were unreported for 8 of them. Two morning samples were available from 37 infants and 2 evening samples from 34 infants. Some mothers failed to report

collection times (11 for infants with complete morning samples and 9 with complete evening samples). We calculated the average sample collection time for morning and evening samples and assigned these to infants missing collection time data.

We used commercially available enzyme immunoassay kits (Arbor Assays, Ann Arbor, Michigan, USA) and performed all assays at the Institute of Psychiatry at the University of São Paulo Medical School (IPq-FMUSP). In order to reduce batch effects, we ran all samples from each individual on the same assay plates. We then averaged cortisol concentrations for both the two morning and two evening samples to calculate the average am and pm values used in this analysis. Salivary cortisol values were not normally distributed before or after log-transforming the data. We excluded outliers >2 SDs above the mean, which left 26 infants with completed morning sample collection and 30 with completed evening samples and collection information. After an exclusion of these outliers, the cortisol values were normally distributed after a log transformation.

2.6 | Pyrosequencing

Salivary DNA samples were collected from infants at 12 months of age during the 12-month interview and assessment visit using Oragene DNA OG-575 Kits (DNA Genotek Inc, Ottawa, Ontario, Canada). Samples for pyrosequencing analyses were successfully collected from 66 of the infants. Sufficient DNA was extracted for pyrosequencing from 62 infants. DNA extraction was performed using the QIAamp DNA Blood Mini Kit (Qiagen Inc, Hilden, Mettmann, Germany) according to the manufacturer's protocol. Genomic DNA (~300 ng) was then bisulfite-converted using the EZ DNA Methylation Kit (Zymo Research, Irvine, California, USA).

Bisulfite-specific polymerase chain reaction (PCR) amplification was performed using the PyroMark PCR Kit (Qiagen) and PyroMark Q24 system (Qiagen), according to the manufacturer's instructions. The PCR conditions for the *FKBP5* and *NR3C1* genes were 15 min at 95°C, 45 cycles at 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s, and a final extension of 72°C for 10 min. The conditions for *OXTR1* and *OXTR2* sequences were 15 min at 95°C, 42 cycles at 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s with a final extension of 72°C for 5 min.

Pyrosequencing was performed using four primers to measure the methylation levels of one target sequence each of *NR3C1* and *FKBP5* and two target sequences of the *OXTR* gene (*OXTR1*—catalog number PM00016821 and *OXTR2*—catalog number PM00016828, Qiagen). Sequences located on *NR3C1* and *FKBP5* genes were selected based on sites of interest from other studies in the literature (Braithwaite et al., 2015; Kertes et al., 2016; Monk et al., 2016). For the regions of interest of *NR3C1* and *FKBP5* genes, primers were designed using PyroMark Assay Design SW 2.0 software (Qiagen) (Table S1). The *NR3C1* primer included 5 CpG sites, including the exon 1_F CpG site 36, which was the most commonly replicated CpG site in a meta-analysis of studies of prenatal stress and methylation of this gene (Palma-Gudiel, Córdova-Palomera, Eixarch, et al., 2015). The *OXTR* sites were selected as they had been used in previous studies conducted by our research group (Cappi et al., 2016).

Samples were randomly distributed on plates in the same position for each assay. A pooled sample was included to evaluate bias between plates. Methylated and unmethylated bisulfite-converted human control DNA (EpiTect PCR control DNA, Qiagen) were diluted to create a 5-point standard curve (0%, 25%, 50%, 75%, and 100%). The software included at least one control dispensation to ensure adequate signal over background noise and verify the efficiency of bisulfite conversion. The CpG methylation percentages provided by pyrosequencing analysis were calculated as the ratio of C to C+T as implemented in the PyroMark Q24 2.0.7 software (Qiagen). Percent methylation for each sample was quantified using the 5-point standard curve, from a cubic polynomial regression or hyperbolic regression, according to the best fit model (Moskalev et al., 2011).

2.7 | Statistical analyses

The data were first examined for normality and the presence of outliers. Hair and salivary cortisol concentrations were log transformed. Following previous work (Cappi et al., 2016), *NR3C1*, *FKBP5*, and *OXTR* CpG sites were first analyzed individually. Statistical analyses were only run for participants with complete cortisol and methylation data. We chose not to impute data given the modest sample size. We first used *t*-tests and Spearman's correlation to identify bivariate associations between maternal variables and infant DNA methylation and cortisol (Tables S2 and S3). We then used multivariable regression models to assess significant associations for associations above $p \leq .1$ in bivariate correlations for each aim, controlling for theoretically relevant covariates. Intervention group was included as a covariate in all models. Other covariates, including any maternal medication use other than vitamins or prescribed supplements and infant sex, were included in the models as previous studies have documented their associations with our outcomes of interest (Martin et al., 2019; Ostlund et al., 2016; Vidal et al., 2013). Standard significance testing thresholds of $p < .05$ were used for all multivariable models and predictors of interest. Statistical analyses were conducted in the R statistical programing language and environment, version 4.2.1.

3 | RESULTS

Sample characteristics for mothers and infants are presented in Table 1. Complete data were available for $n = 62$ infants with pyrosequencing data, $n = 26$ for infants with morning cortisol levels, and $n = 30$ for infants with evening cortisol levels and sample collection times. Methylation levels in our sample were low in *NR3C1* and *OXTR1* and high in *FKPB5* gene regions after normalizing against the 5-point standard curve. Only two sites from the *OXTR1* region had sufficiently high methylation levels for analysis; CpG 1 and 4 as sites 2 and 3 were below the detection limit of the standard curve.

TABLE 1 Participant descriptive, mother–infant pairs in São Paulo, Brazil

Maternal characteristics	
Age, M (SD)	16.87 (1.46)
Pre-pregnancy BMI, M (SD)	22.36 (4.29)
Baseline BDI (% depressed)	39.70
Baseline BAI (% anxious)	28.60
30-week BDI (% depressed)	65.10
30-week BAI (% anxious)	60.70
Baseline hair cortisol, M (SD)	46.4 (24.6)
30-week hair cortisol, M (SD)	29.21 (27.91)
Infant characteristics	
Male offspring	55%
Birth weight, M (SD)	3126.45 (2071.15)
Gestational age, M (SD)	38.57 (2.77)
Infant AM cortisol, M (SD), $n = 26$	2025.74 (2071.15)
Infant PM cortisol, M (SD), $n = 30$	1125.73 (1241.24)
Mean FKBP5 % methylation, M (SD), $n = 62$	63.77 (10.31)
CpG 1	43.08 (12.49)
CpG 2	84.46 (8.83)
Mean NR3C1 % methylation, M (SD), $N = 62$	1.3 (1.91)
CpG 1	1.38 (1.96)
CpG 2	1.67 (2.28)
CpG 3	1.63 (2.05)
CpG 4	0.80 (1.79)
CpG 5	1.01 (2.01)
Mean OXTR1 % methylation (CpG 1 and 4), M (SD), $N = 62$	0.55 (0.43)
CpG 1	0.43 (0.54)
CpG 4	0.66 (0.54)
Mean OXTR2 % methylation, M (SD), $N = 62$	4.14 (2.43)
CpG 1	4.81 (3.17)
CpG 2	4.94 (2.92)
CpG 3	3.64 (2.19)
CpG 4	5.21 (2.88)
CpG 5	2.11 (1.76)

BDI, Beck Depression Inventory; BAI, Beck Anxiety Inventory; *FKBP5*, FKBP51 gene; *NR3C1*, glucocorticoid receptor gene; *OXTR1* and *OXTR2*, oxytocin receptor gene region; SD, standard deviation.

3.1 | Prenatal stress and hair cortisol concentration

We first ran bivariate *t*-tests to identify associations between maternal mental health and hair cortisol concentrations at baseline and 30 weeks of pregnancy. Scores below the BDI cutoff of 14 points were not associated with hair cortisol at baseline ($t(23.1) = 0.034$, p-value = .973) or at 30 weeks ($t(12.4) = 0.436$, p-value = .670). A score on the BAI below the cutoff of 7 points at baseline was associated with lower baseline hair cortisol ($t(14.6) = -2.352$, p-value = .033). Scores at 30 weeks were not associated with hair cortisol measured ($t(28.5) = -0.033$, p-value = .839). Baseline anxiety was associated with hair cortisol after controlling for covariates, including intervention group, fetal sex, and medication use at interview ($B = 0.450$, p-value = .013, Adj. $R^2 = .205$).

3.2 | Prenatal stress and infant salivary biomarkers

We first ran bivariate correlation analysis and *t*-tests to identify relevant associations between maternal stressors, hair cortisol, and infant DNA methylation and cortisol levels. Results of Spearman's rank correlation analyses are presented in Table S2. Significant negative correlations were detected between maternal baseline cortisol levels and *NR3C1* CpG 1 ($r_s = -.469$, p-value = .009), 2 ($r_s = -.55$, p-value = .002), 3 ($r_s = -.612$, p-value ≤ .001), and 4 ($r_s = -.493$, p-value = .006). We also observed a significant negative correlation between 30-week hair cortisol and infant DNA methylation at *FKBP5 CpG 1* ($r_s = -.492$, p-value = .005) and *2* ($r_s = -.463$, p-value = .009). We did not detect any statistically significant correlations between maternal hair cortisol and infant *OXTR* methylation or diurnal cortisol levels at 12 months of age (p-value > .05). *OXTR2* CpG 1 ($r_s = -.386$, p-value = .037), 2 ($r_s = -.417$, p-value = .027), 3 ($r_s = -.412$, p-value = .029), and 4 ($r_s = -.492$, p-value = .042) were negatively correlated with infant evening cortisol.

Results of *t*-tests using clinical cutoffs of the depression and anxiety scale scores are presented in Table S3. Two noteworthy results included associations between maternal depression and infant *NR3C1* methylation and maternal anxiety and infant *OXTR* methylation. At 30 weeks, infants of depressed mothers had higher *NR3C1* methylation at CpG 1 ($t(54.4) = 2.202$, p-value = .032) and 2 ($t(57.2) = 2.043$, p-value = .046). Maternal depression at baseline and 30 weeks were not associated with methylation at any of the *OXTR* sites or with infant cortisol. Infants of non-anxious mothers at T1 had higher methylation at *OXTR2* CpG 3 ($t(36.7 = 2.27$, p-value = .03). Infants of non-anxious mothers at 30 weeks had higher methylation levels at *OXTR2* CpG 2 ($t(37.9) = 2.27$, p-value = .03) and CpG 3 ($t(39.9 = 2.19$, p-value = .03). Infants whose mothers scored below the anxiety cutoff at 30 weeks had lower evening cortisol ($t(27) = -2.58$, p-value = .02).

3.2.1 | Maternal cortisol and infant DNA methylation

We then ran multivariable tests to adjust for relevant covariates. The associations between baseline hair cortisol values and *NR3C1* methylation at CpG 1, 2, 3, and 4 were no longer significant after the addition of covariates (p-values > .05). Hair cortisol at 30 weeks was significantly associated with FKBP5 methylation at CpG 1 ($B = -17.041$, p-value ≤ .001, Adj. $R^2 = .220$, Table 2) and 2 ($B = -10.461$, p-value = .007, Adj. $R^2 = .093$; Table 2).

TABLE 2 Maternal hair cortisol at 30 weeks and infant FKBP5 DNA methylation ($n = 50$)

	FKBP5 CpG 1			*FKBP5* CpG 2		
	Estimate	Standard error	*p*-Value	Estimate	Standard error	*p*-Value
Intervention group	−1.890	3.354	.576	−0.660	2.630	.803
Medication use	6.313	3.624	.088	1.718	2.841	.548
Infant sex	3.308	3.278	.318	1.213	2.571	.639
30-week hair cortisol	−17.041	4.762	.001	−10.461	3.734	.007
Adjusted R^2	.220			.093		

TABLE 3 Multivariate regression of baseline maternal anxiety and infant DNA methylation of the oxytocin receptor region 2 (OXTR2) CpG3 methylation ($n = 61$)

	Prenatal model			Postnatal model		
	Estimate	Standard error	*p*-Value	Estimate	Standard error	*p*-Value
Intervention group	−0.182	0.553	.744	−0.080	0.598	.894
Medication use	−0.222	0.605	.715	−0.179	0.634	.779
Infant sex	0.270	0.554	.629	0.361	0.595	.547
Baseline anxiety	−1.323	0.571	.024	−1.456	0.659	.032
Postnatal anxiety				0.571	0.726	.435
Adjusted R^2	.036			.010		

3.2.2 | Maternal depression and infant DNA methylation

The associations between depression at 30 weeks and *NR3C1* methylation at CpG site 1 remained significant after the addition of covariates ($B = -1.201$, p-value = .037, Adj. $R^2 = .081$). However, the addition of postnatal depression into the model attenuated this association (Table S4). Methylation at CpG site 2 was no longer significant after the addition of covariates (p-value > .05).

3.2.3 | Maternal anxiety and infant salivary biomarkers

Associations between maternal anxiety and *OXTR* methylation were present at 30 weeks, but not at baseline after adjusting for covariates (Tables 3 and 4). Baseline anxiety was significantly associated with *OXTR2* CpG 3 after adjusting for covariates ($B = -1.323$, p-value = .024, Adj. $R^2 = .036$; Table 3). The association between 30-week anxiety and *OXTR2* methylation remained significant for CpG site 2 ($B = -1.762$, p-value = .021, Adj. $R^2 = .064$; Table 3) and site 3 ($B = -1.276$, p-value = .029, Adj. $R^2 = .029$; Table 4), suggesting that infants of anxious mothers had lower salivary OXTR2 methylation than infants of non-anxious mothers. We then added postnatal anxiety into the models to check for confounding effects of the postnatal environment. The addition of postnatal maternal anxiety did not affect the results, with the exception of attenuating the relationship between 30-week anxiety and *OXTR2* site 3 methylation (Tables 3 and 4).

When mothers had severe anxiety symptoms, their infants had elevated cortisol at 12 months (Figure 2). Maternal anxiety at 30 weeks was positively associated with infant evening cortisol levels at 12 months ($B = 0.368$, p-value = .007, Adj. $R^2 = .321$, Table 5, Figure 2), after adjusting for intervention group, maternal medication use, infant sex, and time of cortisol collection. We then added postnatal anxiety into the models to check for confounding effects of the postnatal environment. The addition of postnatal maternal anxiety to the model did not affect the results (Table 5).

3.3 | Infant methylation and cortisol

When infants had lower *OXTR2 methylation* levels, they also had higher evening cortisol levels (Figure 3). Infant evening cortisol levels were negatively correlated with *OXTR2* CpG sites 1 ($r_s = -.396$, p-value = .037), 2 ($r_s = -.417$, p-value = .027), 3 ($r_s = -.412$, p-value = .029), and 4 ($r_s = -.386$, p-value = .042; Table S2). After controlling for intervention group, maternal medication use, infant sex, and time of cortisol collection, *OXTR2* methylation at CpG sites 1 ($B = -0.063$, p-value = .008, Adj. $R^2 = .246$), 2 ($B = -0.82$, p-value = .002, Adj. $R^2 = .327$), 3 ($B = -0.093$; p-value = .011, Adj. $R^2 = .230$), and 4 ($B = -0.081$, p-value = .001, Adj. $R^2 = .338$) were negatively related to infant bedtime cortisol levels (Table 6, Figure 3).

4 | DISCUSSION

We investigated associations between maternal prenatal psychological distress and epigenetic fetal programing of stress-associated genes and diurnal HPAA function in a sample of Brazilian adolescents. We

TABLE 4 Multivariate regression of 30-week maternal anxiety and infant DNA methylation of oxytocin receptor region 2 (OXTR2) methylation ($n = 59$)

	CpG site 2					
	Prenatal model			Postnatal model		
	Estimate	Standard error	*p*-Value	Estimate	Standard error	*p*-Value
Intervention group	−0.744	0.735	.316	−0.692	0.811	.398
Medication use	−0.573	0.807	.481	−0.519	0.861	.550
Infant sex	0.543	0.737	.465	0.599	0.813	.465
30-week anxiety	−1.762	0.742	.021	−1.773	0.881	.049
Postnatal anxiety				0.179	1.005	.859
Adjusted R^2	.064			.022		
	CpG site 3					
	Prenatal model			Postnatal model		
	Estimate	Standard error	*p*-Value	Estimate	Standard error	*p*-Value
Intervention group	−0.245	0.562	.665	−0.165	0.615	.790
Medication use	−0.382	0.617	.538	−0.392	0.653	.552
Infant sex	0.109	0.563	.848	0.098	0.617	.875
30-week anxiety	−1.276	0.567	.029	−1.269	0.668	.063
Postnatal anxiety				0.319	0.762	.677
Adjusted R^2	.029			.015		

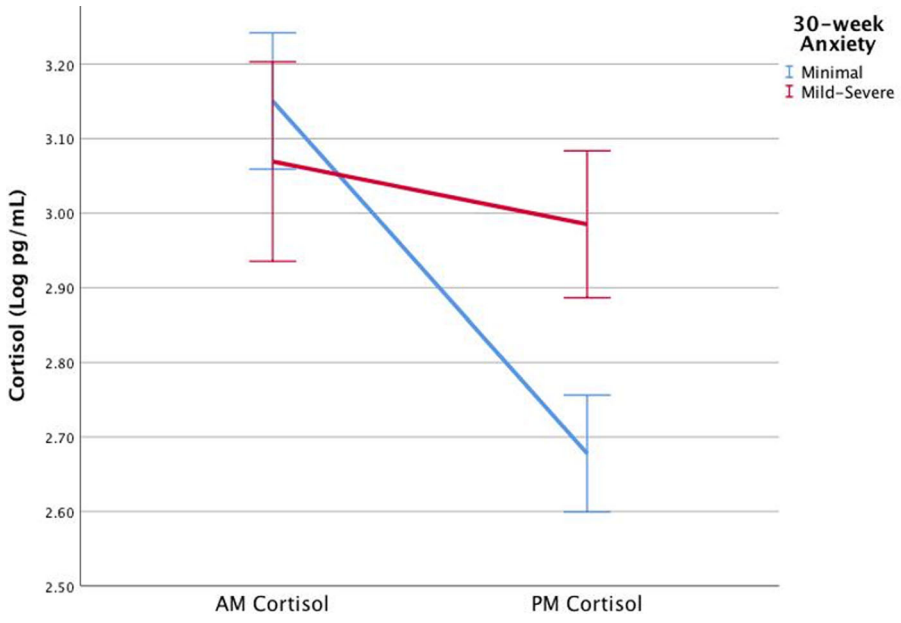

FIGURE 2 Associations between 30-week maternal anxiety and infant diurnal cortisol

examined maternal psychological distress, indexed by symptoms of anxiety and depression, and hair cortisol levels during pregnancy as predictors of infant methylation and postnatal HPAA function. We found three main findings. First, maternal hair cortisol levels in early pregnancy were negatively associated with the DNA methylation of several CpG sites of the GC receptor gene, *NR3C1*, whereas late pregnancy cortisol levels were significantly negatively associated with *FKBP5 sites*. Second, we also found that maternal anxiety was associated with lower levels of *OXTR* methylation and higher evening salivary cortisol levels of infants at 12 months of age. Third, *OXTR* methylation was also associated with elevated infant evening cortisol levels. The results of our multivariable adjusted models suggest that the methylation of the OXTR gene may be involved in the fetal programing of fetal HPAA physiology and contribute to postnatal HPAA function. This study contributes to extant literature by specifically investigating the relationship between OXTR methylation and infant HPAA function.

TABLE 5 Multivariate regression of maternal anxiety and infant salivary evening cortisol ($n = 30$)

	Prenatal model			Postnatal model		
	Estimate	Standard error	*p*-Value	Estimate	Standard error	*p*-Value
Intervention group	−0.256	0.122	.046	−0.222	0.125	.087
Medication use	0.174	0.165	.302	0.219	0.167	.201
Infant sex	−0.093	0.128	.472	−0.103	0.131	.440
Collection time	−0.047	0.072	.520	−0.016	0.075	.834
30-week anxiety	0.368	0.125	.007	0.437	0.133	.003
Postnatal anxiety				−0.189	0.157	.240
Adjusted R^2	.231			.251		

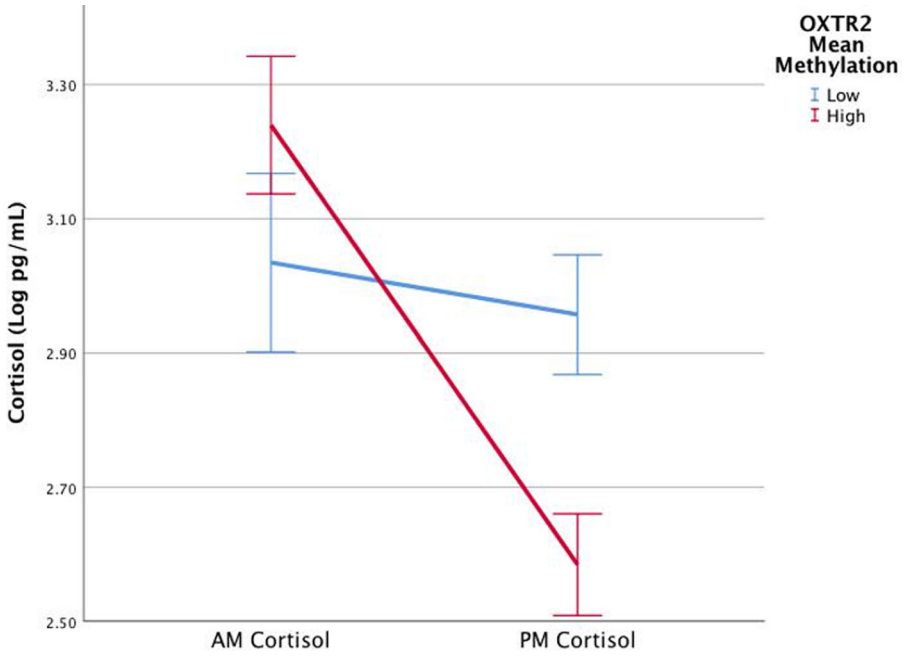

FIGURE 3 Associations between infant *OXTR2* methylation and infant diurnal cortisol

Few studies have examined associations between cortisol concentrations during pregnancy and psychological distress despite its hypothesized role as a biological mechanism of fetal programing. We found some evidence that maternal mood in pregnancy, particularly anxiety early in gestation, was associated with maternal hair cortisol concentration. However, such results should be interpreted with caution given our modest sample size. Other studies suggest that trajectories of prenatal depressive symptoms may more strongly associate with hair cortisol than symptoms at single assessments (Mustonen et al., 2019). Trajectories of cortisol concentrations from pre- to postpartum may better map on to maternal distress (King et al., 2022). Preconception mental health may also play a role in shaping hair cortisol concentrations in pregnancy (Orta et al., 2019). However, others have reported negative or mixed results. Several studies found that chronic stress, anxiety, or depression were not associated with hair cortisol levels in the last trimester of pregnancy (Braig et al., 2016; Lobmaier et al., 2020), and a large study of serum cortisol reported that levels in pregnancy are more strongly influenced by biological and lifestyle factors (Bleker et al., 2017). Other studies report that hair cortisone/cortisol ratio, as an index of 11B-HSD type 2 activity, correlates with maternal distress and both markers should be considered markers of physiological stress in pregnant women (Scharlau et al., 2018). More studies are needed to investigate how prenatal maternal mental health is related to hair cortisol levels across pregnancy, particularly in diverse geographical and social contexts. Additional work is needed to examine associations with other possible pathways of fetal programings, such as maternal immune activation, the autonomic nervous system, and oxidative stress (Minakova & Warner, 2018; Monk et al., 2003; Thompson & Al-Hasan, 2012).

We found that pregnancy hair cortisol levels in pregnancy may be associated with postnatal methylation of infant stress regulatory, particularly *FKBP5*, *suggesting that this gene may be sensitive to glucocorticoid*

TABLE 6 Multivariate regression of oxytocin receptor region 2 (OXTR2) methylation and infant salivary evening cortisol ($n = 30$)

	OXTR2 CpG 1			OXTR2 CpG 2			OXTR2 CpG 3			OXTR2 CpG 4		
	Estimate	Standard error	*p*-Value	Estimate	Standard error	*p*-Value	Estimate	Standard error	*p*-Value	Estimate	Standard error	*p*-Value
Intervention group	−0.374	0.127	.007	−0.425	0.123	.002	−0.340	0.125	.012	−0.363	0.117	.005
Medication use	0.097	0.158	.545	0.090	0.149	.553	0.172	0.162	.297	0.148	0.149	.330
Infant sex	−0.122	0.126	.343	−0.103	0.120	.396	−0.148	0.128	.259	−0.094	0.119	.437
Collection time	−0.081	0.072	.269	−0.070	0.066	.302	−0.047	0.070	.512	−0.028	0.064	.670
OXTR2 methylation	−0.063	0.022	.008	−0.082	0.024	.002	−0.093	0.034	.011	−0.081	0.023	.001
Adjusted R^2	.246			.327			.230			.338		

levels during pregnancy. However, we did not find any significant associations between maternal pregnancy hair cortisol levels and infant DNA methylation of *OTXR* or with cortisol levels at 12 months. This suggests that maternal circulating cortisol levels may not be one of the primary mechanisms by which prenatal stress alters the developmental trajectory of the HPAA. Despite the hypothesized role that cortisol is thought to play a role in the patterning of DNA methylation in humans, there remains limited evidence to support this (Zannas & Chrousos, 2017). Studies examining associations between pregnancy cortisol and cord blood methylation have found positive (Hompes et al., 2013) and negative associations with *NR3C1* (Braithwaite et al., 2015). The only other study to investigate pregnancy cortisol levels and *OXTR* methylation found that diurnal cortisol levels in the second trimester were associated with lower cord blood *OXTR* methylation in a sample of 39 Swiss women and their infants (Unternaehrer et al., 2016). These conflicting results may be due to heterogeneity of sample type or timing of collection for both cortisol and methylation analyses (Mill & Heijmans, 2013; Zijlmans et al., 2015). For example, hair cortisol is thought to reflect an integrated assessment of long-term cortisol production that is less sensitive than salivary cortisol to daily variability (Rippe et al., 2016). The significance of the association between prenatal cortisol and *FKBP5* methylation requires further investigation as we did not find associations between the methylation of this gene and infant postnatal cortisol. However, this does not preclude its role in other developmental processes.

Contrary to other studies, we found no significant effect of maternal mental health on infant FKBP*5* or *NR3C1* methylation. Maternal prenatal depression has been consistently associated with *NR3C1* methylation in infancy (Palma-Gudiel, Córdova-Palomera, Eixarch, et al., 2015). Although less frequently investigated, *FKBP5* cord blood and placental methylation have also been associated with maternal mental health and war stress (Kertes et al., 2016; Monk et al., 2016). It is possible that such effects reverse within the first year of life. Another potential explanation is that given the heterogeneity of tissue types commonly used in epigenetic research such effects are not detectable in salivary DNA methylation. Parenting skills developed over the course of the HVP "Primeiros Laços" may also have improved postnatal care, reversing potential differences across the first year of life in response to nurturing parenting. Several studies have shown that maternal sensitivity and responsiveness buffer the effect of prenatal depression and are associated with lower *NR3C1* promoter methylation of infants (Conradt et al., 2016, 2019; Lester et al., 2018). In our models, we found no differences in salivary DNA methylation levels between intervention and control groups in response to the intervention. However, another study of this cohort found that infants in the intervention group exhibited differences in cord blood epigenome-wide DNA methylation and less DNA methylation age acceleration than infants in the control group (Euclydes et al., 2022). A more direct measure of maternal care and larger sample size may contribute to the replication of the findings described above.

To our knowledge, this is one of the only studies to have tested for associations between pregnancy cortisol levels and an infant's DNA methylation and diurnal cortisol levels. Although we found no

associations between FKBP5 or NR3C1 methylation levels and infant cortisol, other studies have investigated associations between methylation and cortisol reactivity to a stressor. For example, several studies by Conradt et al. (2015, 2016) found that a factor based on the DNA methylation of several NR3C1 CpG sites was positively correlated with cortisol reactivity at 4 months in response to an age-appropriate stressor. Oberlander et al. (2008) also showed that NR3C1 methylation was associated with infant cortisol reactivity at 3 months of age. It is possible that the DNA methylation of these genes may be related to reactivity, but not diurnal cortisol production, or that other dimensions of the HPAA are involved. One study of postconception urinary cortisol levels and postnatal HPAA function found several sites that were associated with pregnancy cortisol levels and others that were associated with HPAA activity in childhood, though no significant sites were related to both (Barha et al., 2019). It is also possible that differences in cortisol regulation change during the first year of life and are not detectable in late infancy. Our results suggest that the methylation of the OXTR gene is sensitive to maternal anxious mood, but not with depressed mood. We did not find evidence of an association between maternal depressive symptoms and infant OXTR methylation. This is in contrast to findings reported by the only other study to investigate this association in a site-specific manner (Unternaehrer et al., 2016). However, the reported associations between methylation and depression may not persist into childhood (Galbally et al., 2018; King et al., 2017). More work is needed to investigate the role of maternal prenatal depression in the patterning of methylation of the OXTR gene of infants.

In our study, higher maternal anxiety symptoms in pregnancy and lower OXTR gene methylation predicted greater evening cortisol levels of infants at 12 months of age. These results are broadly in-line with studies that have shown that endogenous oxytocin and oxytocin administered intranasal may attenuate the cortisol response to social stressors (Heinrichs et al., 2003; Pierrehumbert et al., 2010). However, it is unclear if these associations are a compensatory response to buffer individuals from the development of stress-related disorders, or if the buffering effects of the oxytocin system are diminished in response to prenatal stress. More work is needed to disentangle the relationship between oxytocin and cortisol in this context.

Maternal anxiety may serve as an intrauterine signal that alters fetal developmental trajectories in ways that anticipate a stressful postnatal environment and epigenetic mechanisms may be one of the primary mechanisms involved in this process (Entringer et al., 2015; Wadhwa et al., 2009). Lower *OXTR* methylation, if associated with increased gene expression, may serve as an anticipatory and "adaptive" response to such maternal signals as oxytocin is believed to have a downregulatory effect on the stress response (Neumann, 2002). Such a phenotype may be advantageous in a stressful postnatal environment. It is also possible that upregulated OXTR expression, suggested by decreased *OXTR* methylation, is a compensatory mechanism induced in response to high levels of HPAA activity. This is supported by evidence suggesting that the downregulating effects of oxytocin on cortisol are diminished or reversed in individuals with severe life stress (Grimm et al., 2014; Meinlschmidt & Heim, 2007). It is clear that dysregulation of the oxytocin system is involved in the intergenerational transmission of prenatal stress (Toepfer et al., 2017). This may occur through the dysregulation of the oxytocin and HPAA systems.

Some of the associations in our study should be considered in light of the unique characteristics of our participant population. Pregnant adolescents have to balance multiple energetic demands, including the growth and development of their own tissues, but also that of their developing infant. Changes in HPAA function may mediate changes in energy allocation strategies and energy devoted to pregnancy or maternal growth (Rowlands et al., 2021). For example, maternal growth occurs in about 50% of adolescent pregnancies but may come at the cost of fetal growth (Scholl et al., 1990). On the other hand, investment in fetal and infant growth may hinder the growth and nutritional status of adolescent mothers (Casanueva et al., 2006; Rah et al., 2008). Thus, energetic trade-offs that occur in adolescent pregnancy may exert some programing effects on infants and have potential ramifications for mothers' development.

This study has several strengths. First, we included multiple measures of psychological and physiological measures of maternal prenatal stress and investigated the methylation of multiple genes hypothesized to regulate the HPAA. Second, we found several associations that were consistent with other studies, including the associations between lower methylation of the OXTR gene in response to maternal stress and the negative associations between *OXTR* methylation and infant evening cortisol. This study also has four main limitations. The analysis is based on a modest sample size, particularly for the infant salivary cortisol analyses (26 complete samples for morning cortisol and 30 for evening cortisol). Although small sample sizes of 50–100 participants are common for epigenetic studies in field settings, they limit our ability to detect small to moderate effect sizes. This likely explains some of the inconsistencies between our results and associations reported in other studies, such as those between maternal pregnancy cortisol levels and infant OXTR gene methylation (Unternaehrer et al., 2016). However, some inconsistencies may also be due to the use of different tissues for cortisol and methylation analyses. The second limitation is the use of salivary DNA. Methylation estimates from peripheral tissues may not accurately reflect methylation patterns in the brain (Armstrong et al., 2014; Thompson et al., 2013), although some studies suggest that salivary DNA methylation may better reflect patterns of methylation in the brain, especially in infants and children (Smith et al., 2015). A third limitation is the use of self-report measures of anxiety and depression. Clinical assessments may correlate more strongly with stress biomarker levels than self-report measure scores (O'Connor et al., 2014). The fourth limitation is that we did not quantify gene expression. Future studies should examine the functional relevance of the methylation of target regions by analyzing gene expression data in relation to methylation data. They should also include assessments of infant and child development in order to investigate the relationships between methylation, infant stress physiology, and postnatal health and developmental outcomes.

5 | CONCLUSION

This study of a cohort of adolescent mothers and their infants in São Paulo, Brazil found that maternal prenatal cortisol was related to methylation of *FKBP5* and anxiety was related to DNA methylation of the OXTR and to diurnal variation of salivary cortisol of their infants at 12 months of age. Additionally, methylation of the OXTR predicted infant evening cortisol levels. Our results are, to our knowledge, the first evidence that methylation of the OXTR may contribute to the regulation of HPAA regulation and cortisol production during infancy. These findings suggest that the oxytocin system may play a role in the intergenerational transmission of prenatal stress, particularly in relation to maternal anxiety. Such results highlight the need to consider other biological pathways in research investigating the mechanisms involved in the intergenerational transmission of prenatal stress, as well as interactions between such systems. However, we did not detect significant associations between maternal mood and hair cortisol levels in pregnancy (with the exception of anxiety and cortisol in early pregnancy), suggesting that maternal cortisol may not be the primary biological mechanism involved in the fetal programing of stress regulation. Future studies should continue to examine other biological pathways, such as the immune system, as well as the functional consequences of methylation of these CpG sites on downstream traits, including gene expression and developmental outcomes.

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CONFLICT OF INTEREST

The authors have no conflict of interests to declare.

DATA AVAILABILITY STATEMENT

Data are shared upon reasonable request to the corresponding or senior authors.

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