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Epigenetic Programming by Maternal Behavior in the Human Infant

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Abstract

Objective: We sought to determine if variations in maternal care alter DNA methylation in term, healthy five- month-old infants. This work was based on landmark studies in animal models demonstrating that nurturing care by dams would alter their newborn's stress responses through epigenetic mechanisms. We used breastfeeding as a proxy for animal maternal behavior. We hypothesized alterations in DNA methylation of the glucocorticoid receptor gene and less hypothalamic stress response in infants of mothers who breastfed their infants versus infants of mothers who did not breastfeed.

Methods: A cohort study of term, healthy infants and their mothers who did (n=21) or did not (n=21) breastfeed for the first 5 months was utilized in this analysis. Cortisol stress reactivity was measured in infant saliva using a mother-infant interaction procedure, and DNA methylation of an important regulatory region of the glucocorticoid receptor gene. Changes in DNA methylation of this gene in the human were compared to homologous regions of the rat gene. DNA samples were

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Barry Lester: Dr. Lester conceptualized and designed the study, analyzed and interpreted data, drafted the article and revised critically for important intellectual content and approved the final manuscript as submitted.

Elisabeth Conradt: Dr. Conradt conceptualized and designed the study, analyzed and interpreted data, drafted the article and revised critically for important intellectual content and approved the final manuscript as submitted.

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Carmen Marsit: Dr. Marsit performed the laboratory studies, drafted the article and revised critically for important intellectual content and approved the final manuscript as submitted.

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prepared from cheek swabs and subjected to quantitative analysis of extent of methylation using sensitive sequencing techniques.

Results: Breastfeeding decreased DNA methylation of the glucocorticoid receptor promoter and decreased cortisol reactivity in 5-month-old infants. Decreased DNA methylation occurred in the promoter region involved in regulation of the hypothalamic-pituitary-adrenal (HPA) and immune system responses.

Conclusions: Maternal care in humans may impact HPA stress response through behavioral programming and manifest as offspring epigenetic change. These results explain, in part, some of the positive effects observed in children with breastfeeding.

Table of Contents Summary:

A human replication of rodent work relates maternal care to epigenetic variation and reduced infant stress with implications for the positive effects of breastfeeding.

Keywords

Epigenetics; Genetics

INTRODUCTION

Epigenetics is the study of how environmental influences affect gene expression. Now classic animal studies demonstrate that variations in maternal care can have profound effects on early development, stress regulation and behavior.[1–5] Dams with high nurturing activity (licking and grooming, (LG) and arched-back nursing, (ABN)) have pups that display less anxiety, less fear conditioning and less hypothalamic stress responsivity than pups reared by dams that are less-nurturing.[2] Amazingly, if the subsequent litters born to dams that display these different behaviors are switched at birth, the altered stress responsivity and behavioral conditioning has been shown to be dependent on maternal behavior. Even more extraordinary is the demonstration that these effects are preserved across subsequent generations. The mechanism for these remarkable environmental effects on behavior and stress responsivity is mediated by epigenetics. Alterations in DNA methylation of the glucocorticoid receptor promoter modifies hypothalamic pituitary expression of this important gene and stress responsivity. Further, mechanistic exploration of these effects on DNA methylation have been shown to be mediated by alterations in the binding site for important transcription factors that regulate expression of this gene. These effects have also been shown to be reversible following pharmacologic modulation of chromatin (chromosome materials) remodeling. Thus, the role of maternal behavior in behavioral conditioning during early life in newborn animals is widely accepted and demonstrated. In humans, children exposed to physical maltreatment,[6] infants of depressed mothers,[7,8] adolescents whose mothers were exposed to intimate partner violence during pregnancy[9] and adults with a history of child abuse[10,11] have increased DNA methylation of the human glucocorticoid receptor, the homolog of the rat glucocorticoid receptor.[12] While these studies did not examine HPA stress reactivity or parenting behavior, they suggest that similar molecular mechanisms in rodents and humans could modulate risk for psychopathology. We

wondered whether any of these fundamental mechanisms, mediated through epigenetic effects, were demonstrable in human infants.

METHODS

We used breastfeeding as a proxy for high nurturing activity in the rodent to determine if human maternal care in the form of breastfeeding would elicit similar epigenetic programming to the rodent. Therefore, we hypothesized that infants of mothers who provide high levels of breastfeeding would have less DNA methylation of the glucocorticoid receptor promoter region than infants of mothers who provide low levels of breastfeeding. We further hypothesized that less DNA methylation of the glucocorticoid receptor in this region would be associated with decreased HPA response to stress measured by cortisol reactivity in these infants. Informed consent was obtained from all of the mothers. Mothers and their infants born at Women and Infants Hospital of Rhode Island were enrolled as part of the larger Rhode Island Child Health Study,[13] and then recruited for this follow-up examination during the period, June 2013 through July 2014. Inclusion criteria included uncomplicated delivery of a singleton, viable, term infant (>37 weeks). Exclusion criteria included maternal age <18 or >40 years, life-threatening complications in mother or child, and congenital or chromosomal abnormality of the infant. Mothers were administered a self-report questionnaire consisting of three questions to measure the extent of breastfeeding since the birth of their infant. Mothers were asked: 1) if they ever breastfed their baby (yes/no); 2) the proportion of feedings that are currently breast versus bottle (mothers indicated % of feedings from breast vs. % of feedings from bottle) and 3) the age of the infant when she stopped breastfeeding. Mothers who breastfed their infants continuously over the first 5 months were in the high breastfeeding group (n=21). Mothers who did not breast their infant at all or who stopped breastfeeding by 3 weeks were in the low breastfeeding group (n=21).

Cortisol stress reactivity was elicited from the infant using a mother-infant interaction procedure which utilizes a “still-face” episode during which the mother is non-responsive to her infant[14] (Fig. 1, frame 3). The “still-face” is a stressor and has been reliably shown to elicit cortisol reactivity.[15] Specifically, the procedure begins with the mother and infant sitting across from each other and playing face-to-face as they normally would at home for 2 minutes (Fig. 1, frames 1–2). This is followed by a “still-face” episode (Fig. 1, frame 3) during which time the mother is instructed to maintain a blank “poker” face and not to smile and interact with her infant for 2 minutes. Here, the infant shows a negative response to the mothers “still face.” (Fig. 1, frame 4). After the 2 minute “still-face” the mother and infant again play together as they normally would for 2 minutes, just as they might during the first “play” episode.

Infant saliva samples were collected before (pre-stress) and 30 minutes after (post–stress) the “still-face” procedure using a small sponge that was swabbed in the infant’s mouth until it became saturated with saliva. The swab was then placed into a storage vial and frozen until analyzed. If infants ate or drank 30 minutes prior to the collection of the saliva sample, their mouths were first swabbed with a wet paper towel. The saliva samples were sent to Salimetrics LLC, (Tempe, Arizona), and cortisol was measured by Enzyme Immunoassay in

µg/dL units. The cortisol values were log transformed (Ln). Cortisol reactivity was computed as a difference score (post-stress µg/dL–pre-stress µg/dL).

An additional set of buccal swabs was also collected from each infant using the Oragene Discover for assisted collection system, and from those genomic DNA was extracted using the prepIT kit (DNA Genotek, Ontario, Canada). The DNA was subjected to bisulfite modification using the EZ DNA methylation Kit (Zymo Research, Irvine, CA), and bisulfite pyrosequencing was performed on PCR products amplified from bisulfite modified DNA as described previously.[16] The primers for amplification of the human glucocorticoid receptor promoter were Forward: 5′-TTT TTT TTT TGA AGT TTT TTT A-3′ and Reverse: 5′-Biotin-CCC CCA ACT CCC CAA AAA-3′. Two primers were used to sequence the amplification product in the 1F region, sequence 1: GAG TGG GTT TGG AGT-3' and sequence 2: 5'-AGA AAA GAA TTG GAG AAA TT-3'. Percent DNA methylation at each cytosine guanine (CpG) site was quantified using the Pyro Q-CpG software, version 1.0.11 (Qiagen).

Statistical analysis

Mean percent of methylation was compared between the high and low breastfeeding groups at each CpG site using one-way ANOVA. Following statistically significant group differences, bivariate correlations were used to relate DNA methylation to cortisol reactivity at CpG sites 7, 10, 12 and 13. We controlled for False Discovery among the 13 tests relating DNA methylation of the 13 CpG sites on the glucocorticoid receptor gene and breastfeeding status using the Benjamini and Hochberg procedure.[17] Instead of a corrected p-value, a q-value is obtained to determine the percent of findings that could be a false discovery. As is standard in the epigenetic literature, we chose a q-value of .10. In the results we present both the p-value and q-values.

RESULTS

Participants included 42 mothers and their 5-month-old infants, 21 who breastfed exclusively for the first four months (high levels of breastfeeding) and 21 mothers who did not breastfeed for the first four months (low levels of breastfeeding). The mothers were of middle socioeconomic status (SES), with a median income of $50,000-$79,000. They ranged in age from 21 – 38 (M = 29.64 years, SD = 4.53 years) and 91% had at least a high school education. The infants were born full-term and healthy.

Infants of mothers in the high breastfeeding group showed less methylation with low false discovery rates at CpG sites 7 (p = .05, q = .03), 10 (p = .02, q = .008), 12 (p = .02, q = .02), and 13 (p = .04, q = .02) in the glucocorticoid receptor gene exon 1F promoter region than infants of mothers in the low breastfeeding group (Fig. 2). Lower DNA methylation at CpG sites 10 (r=. 41, p<. 05, Fig. 3) and 12 (r=. 35, p < .05, Fig. 4) was associated with decreased cortisol reactivity.

DISCUSSION

We report, to our knowledge, the first translational study recapitulating the effects of maternal care in rodents by demonstrating that in the human infant, maternal breastfeeding impacts the infant's epigenome and is associated with altered stress responsivity. Specifically, in rodents, increased maternal nurturing behaviors led to decreased methylation of the glucocorticoid receptor promoter region in the rat hippocampus. This effect was mirrored in our population. Infants that experienced increased breastfeeding demonstrated decreased methylation in the homologous region of the human gene. Also, in parallel to observations in rodents, decreased methylation of this gene in the human infant was associated with decreased cortisol stress reactivity. In rodent pups, and presumably in human infants, this decreased methylation of the glucocorticoid receptor gene is associated with enhanced glucocorticoid receptor expression facilitating regulation of the HPA stress reactivity system. Our findings indicate that variations in early human maternal care alter the epigenome of the infant, which, in turn, alters HPA stress reactivity. These are unique results that demonstrate that the functional consequences of behavioral programming, due to maternal care, may be through an altered epigenetic state in the human infant.

Our findings in human infants differ only modestly from the observations in animals. The effects of breastfeeding on DNA methylation in human infants were in homologous regions of the glucocorticoid receptor gene of the rodent. This suggests that the effects of maternal behavior on DNA methylation of the offspring and the functional consequences of DNA methylation are conserved between rodents and human.

Mechanistic studies have been possible in animal models. In the rodent, less nurturing maternal behavior is associated with increased methylation at a CpG site for a nerve growth factor binding site important for healthy brain development and other biological functions.[18,19] Methylation of this binding site reduces nerve growth factor occupancy, leading to reduced glucocorticoid receptor expression and subsequent disruption of the HPA system.[1] We[8] and others[7] have observed that methylation of the human glucocorticoid receptor promoter region, including the nerve growth factor binding site is associated with maternal mood disorders during pregnancy and infant behavior in term infants and aberrant neurobehavior in preterm infants.[20] We found altered methylation at CpG sites 7, 10, 12 and 13. This region of the human glucocorticoid receptor gene promoter is involved in the regulation of the HPA system. CpG sites 7 and 12 are binding sites for the specificity protein transcription factor and epigenetic variation of these binding sites can impact binding and function of this transcription factor.[21] The specificity protein transcription factor is activated for a number of cellular processes including cell differentiation, cell growth, apoptosis, response to DNA damage, chromatin remodeling and, interestingly, immune responses. With respect to the HPA axis, the specificity protein is a mediator of steroid hormone nuclear signaling. We would expect the increased DNA methylation to have effects on chromatin, leading to decreased specificity protein binding, reduced rate of transcription, interference with cellular processes and disruption of the HPA axis. This argument is supported by our finding that increased DNA methylation is associated with increased cortisol stress reactivity.

We used breastfeeding as a proxy for a high level of maternal nurturing behavior in the rodent to replicate the epigenetic effects of maternal care on the glucocorticoid receptor gene promoter region. That the specificity protein binding factor is involved in immune responses could suggest epigenetic associations related to the positive effects of breastfeeding. Breastfeeding and the accompanying tactile stimulation have effects comparable to a high degree of maternal nurturing in young animals. Higher levels of maternal care in both rodent and humans decrease DNA methylation in analogous regions of the glucocorticoid receptor promoter that results in decreased reactivity of the HPA system response to stress. As in the rodent, behavioral programming by the mother affects the epigenome of the infant. There are undoubtedly multiple pathways for behavioral programming within species. We would expect additional pathways for behavioral programming that are conserved between rodents and humans to be identified, including pathways that alter, for example, serotonin activity.[22]

Strengths and Limitations

Our study had some limitations. For obvious reasons, we could not measure DNA methylation in hippocampal cells or conduct the kind of cross-fostering, intergenerational transmission or pharmacologic studies that can be conducted in animal models. Although we recognize that there may be differences in DNA methylation at specific CpG sites between hippocampal tissues and the cells available from a buccal swab, we note that buccal cells come from the same primordial lineage, and that findings are similar from buccal and brain specimens used in studies of psychiatric traits.[23] We also highlight the comparability of our findings in human buccal cells to animal work using brain cells, suggesting that they could be measuring similar processes. We did not study gene expression and we used salivary cortisol, a peripheral glucocorticoid. Although DNA methylation is correlated with gene expression in human epigenetic studies[24,25] and salivary cortisol is a well-established measure of HPA stress reactivity[26] correlated with serum cortisol, our findings would be strengthened by using these measures and they certainly should be considered in future studies in this area.

Rodents are the most frequently used species in both experimental and translational studies. The risks involved in the translation of rodent to human findings are well recognized. Although determining the exact equivalence in development between these species is difficult, we can say that in terms of age, the rodent at 6 postnatal days, the age used in the rodent work,[1] roughly corresponds to the human infant at 5 months,[27] the age of the infants that we studied. This underscores the translational importance of our study.

Despite these limitations, we have identified a conserved pathway of maternal behavioral programming in humans associated with the altered epigenetic state of a gene that affects HPA stress reactivity, an important functional consequence. Dysregulation of the HPA axis has been implicated in the etiology of a range of psychological disorders.[28–32] Interestingly, such disruptions are also related to responsivity of immune systems.[33] Understanding how variations in maternal care alter the infant epigenome through behavioral programming and modification of HPA stress reactivity provides insights into molecular mechanisms that could confer risk or protection for psychopathology. It may be too early, but we can also

speculate that future translational work could lead to the development of interventions that increase maternal care and reprogram the HPA system.

CONCLUSION

In summary, we have presented translational evidence that, as in developing animals, variation in maternal care in humans may impact the epigenome of the offspring through behavioral programming, which, in turn, could alter HPA stress reactivity, and may, in part, provide a mechanistic link for the positive impacts of breastfeeding on infant development.

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Abbreviations:

(DNA)	Deoxyribonucleic acid
(LG)	licking and grooming
(ABN)	arched-back nursing
(HPA)	hypothalamic- pituitary-adrenal
(CpG)	cytosine guanine site

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What's Known on This Subject:

Variations in early maternal care in the rodent, including nursing, affect epigenetic changes (DNA methylation) in the glucocorticoid receptor gene of offspring. We report similar findings in human mothers and their infants.

What This Study Adds:

This partial replication of rodent work shows that maternal care alters the human infant epigenome through behavioral programming, altering HPA stress reactivity. In addition, we show that behavioral programming could explain, in part, the protective effects of breastfeeding.

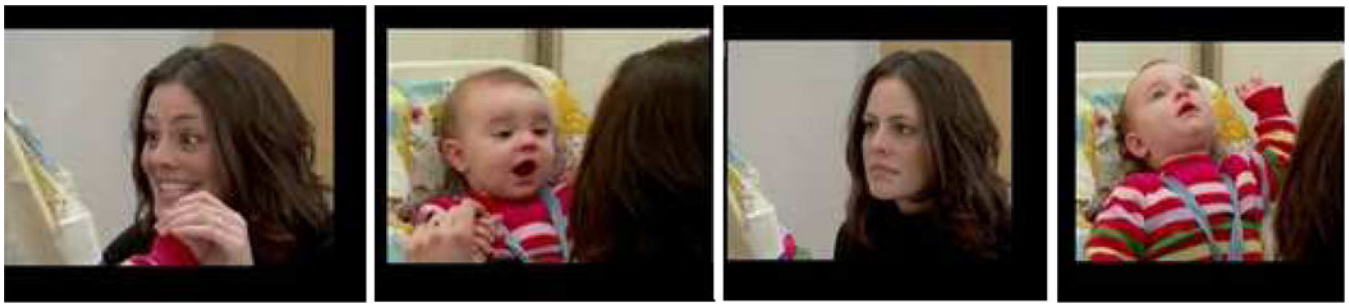

Fig. 1. The Face-to-Face Still-Face Paradigm: A mild stress paradigm.

Frames 1–2 show a playful mother-infant play sequence. In frames 3–4 the mother maintains a blank “still-face” and does not smile or interact with her infant.

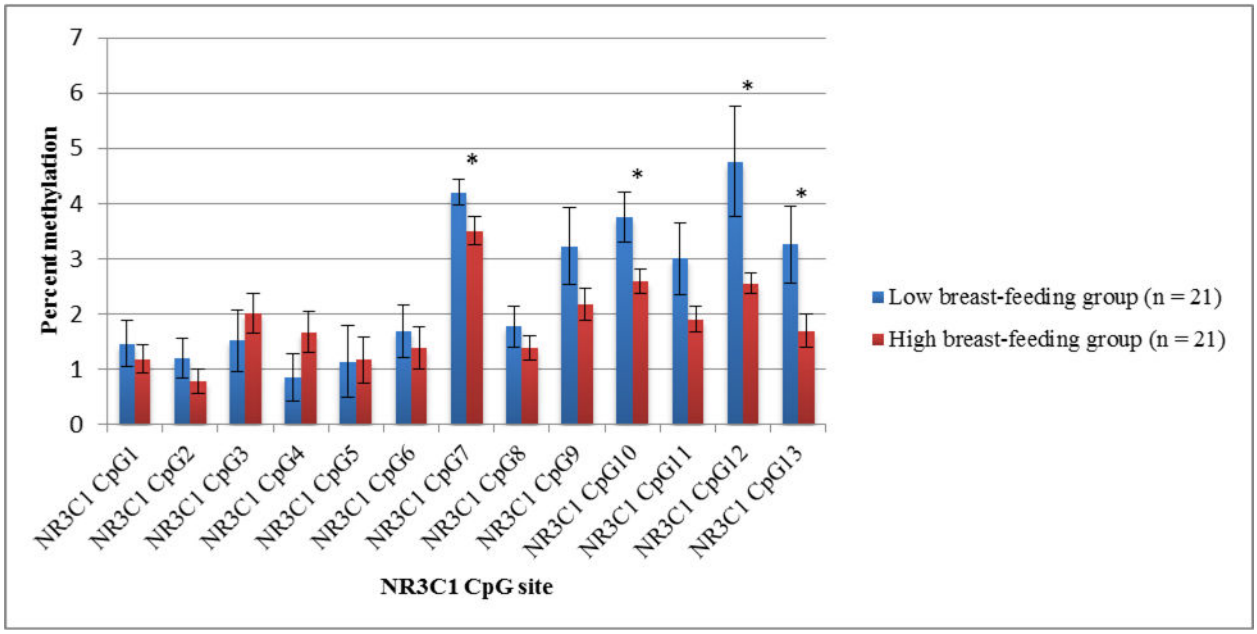

Fig. 2. Differences in the extent of DNA methylation at CpG sites across the glucocorticoid receptor gene (NR3C1) in buccal cells derived DNA from infants experiencing low (blue bars) or high (red bars) breastfeeding behaviors.

Bar heights represent mean methylation in all subjects within the group and error bars, the standard error of the mean. *False discovery rate, $q<0.05$.

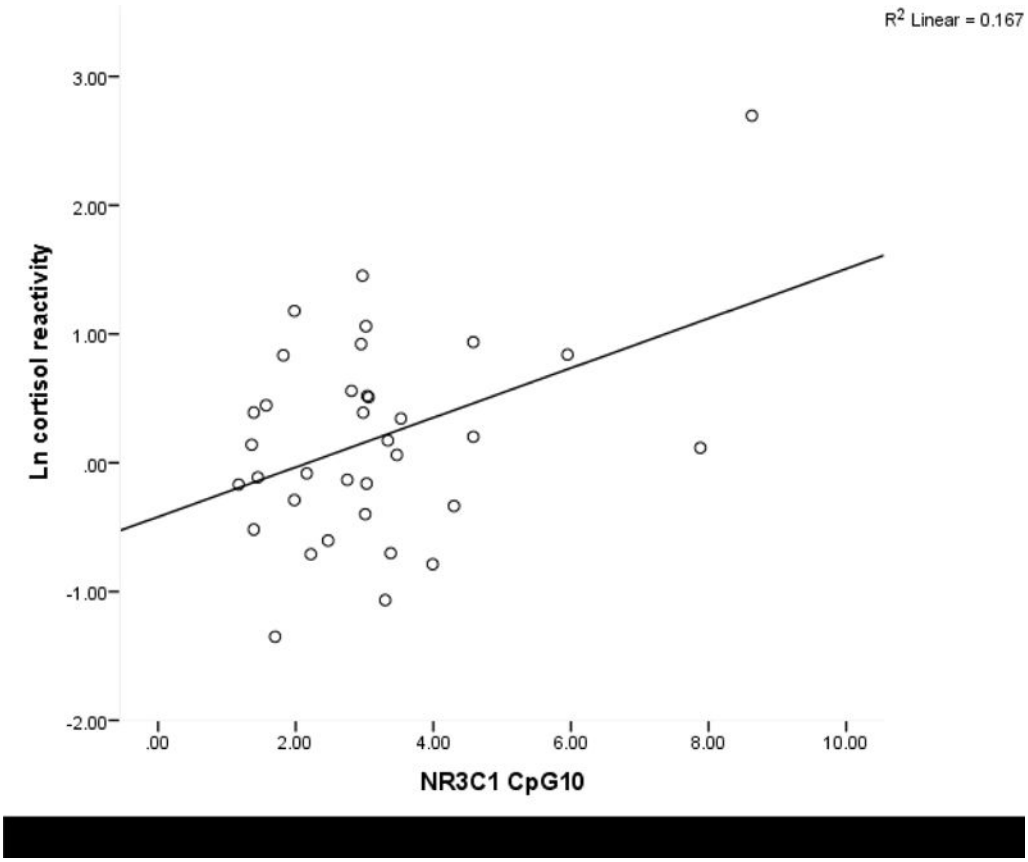

Fig. 3. Scatterplot of the correlation between amount of DNA methylation of the glucocorticoid receptor gene (NR3C1) at CpG site 10 and cortisol reactivity.

Cortisol was measured from saliva collected before and after a mother infant interaction still-face stress procedure. Cortisol reactivity was computed as a difference score (post stress μg/dL – prestress μg/dL) from the natural log transformation (Ln) shown on the Y-axis. Mean percent of DNA methylation of the glucocorticoid receptor gene (NR3C1) at CpG site 10 is shown on the X-axis. The line in the figure is the linear regression that was statistically significant r= .41, p<.05) and the circles are the individual subjects.

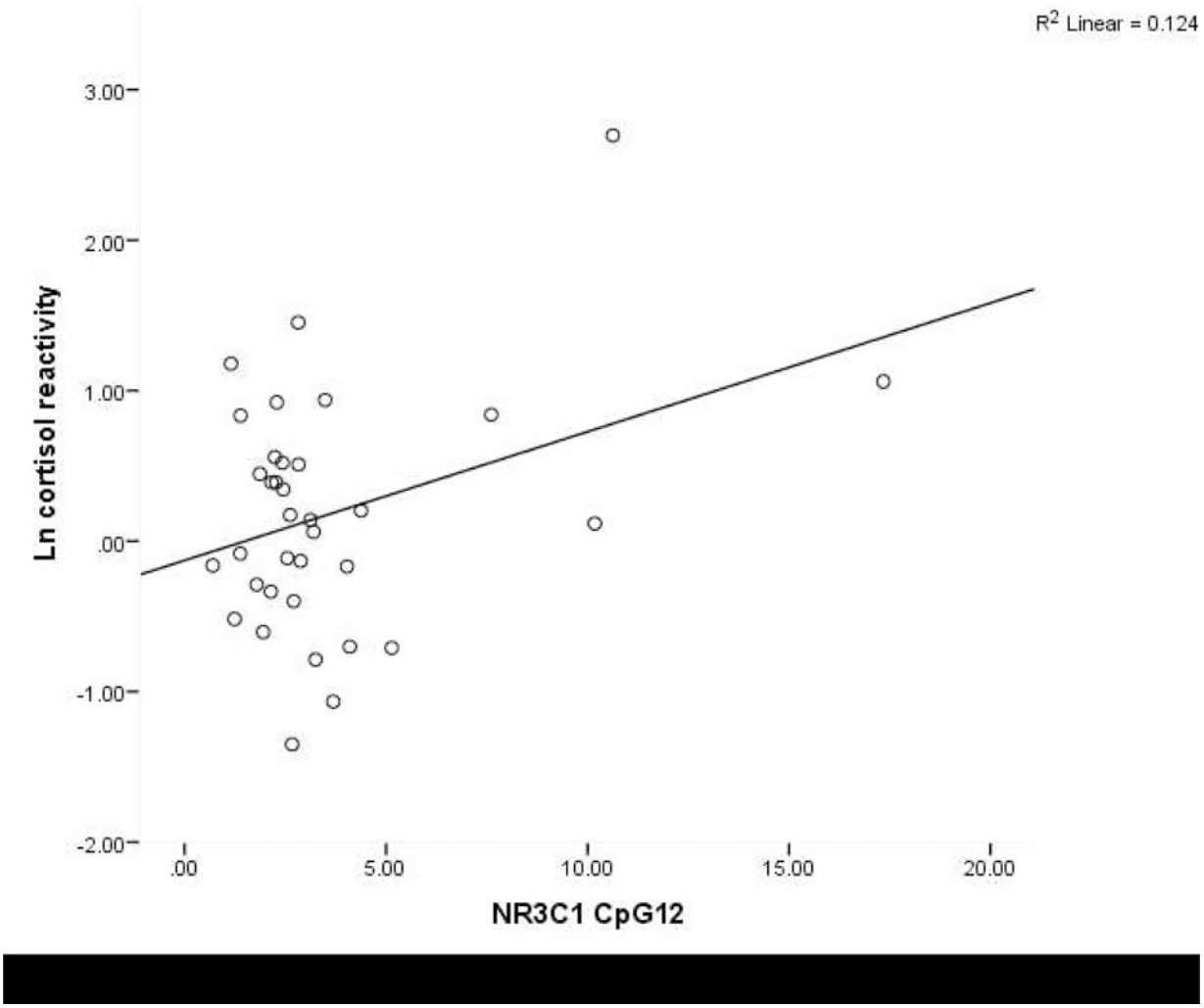

Fig. 4. Scatterplot of the correlation between amount of DNA methylation of the glucocorticoid receptor gene (NR3C1) at CpG site 12 and cortisol reactivity.

Cortisol was measured from saliva collected before and after a mother infant interaction still-face stress procedure. Cortisol reactivity was computed as a difference score (post stress μg/dL – prestress μg/dL) from the natural log transformation (Ln) shown on the Y-axis. Mean percent of DNA methylation of glucocorticoid receptor gene (NR3C1) at CpG site 12 is shown on the X-axis. The line in the figure is the linear regression that was statistically significant r= .35, p<.05) and the circles are the individual subjects.

Table 1.

Patient Demographic Characteristics

	Mean/percentage		
	Low/no breastfeeding group (n=21)	**Breastfeeding group (n=21)**	***p***
Age			
Maternal (years)	29.05	31.52	*ns*
Gestational (weeks)	39.25	39.38	*ns*
Infant (weeks)	18.76	19.14	*ns*
Pregnancy complications	0	0	*ns*
Birth weight (grams)	3389.75	3564.48	*ns*
Infant sex (female)	47.6%	42.9%	*ns*
Household income			*ns*
$0–24,999	14.3%	9.5%	
$25,000–49,999	40%	23.9%	
>$50,000	45.7%	66.6%	
Married or living with partner	60%	81%	*ns*
Ethnicity			*ns*
European American	47.6%	76.2%	
African American	23.8%	4.8%	
Hispanic	14.3%	9.5%	
Asian	4.8%	0%	
Other	9.5%	9.5%	