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# DNA methylation of *NR3c1* in infancy: Associations between maternal caregiving and infant sex

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## ABSTRACT

Caregivers play a critical role in scaffolding infant stress reactivity and regulation, but the mechanisms by which this scaffolding occurs is unclear. Animal models strongly suggest that epigenetic processes, such as DNA methylation, are sensitive to caregiving behaviors and, in turn, offspring stress reactivity. We examined the direct effects of caregiving behaviors on DNA methylation in infants and infant stress reactivity. Infants and mothers ($N = 128$) were assessed during a free play when infants were 5 months old. Maternal responsiveness and appropriate touch were coded. and infant buccal epithelial cells were sampled to assess for DNA methylation of the glucocorticoid receptor gene, *NR3c1* exon 1F. Infant cortisol reactivity was assessed in response to the still-face paradigm. Greater levels of maternal responsiveness and appropriate touch were related to less DNA methylation of specific regions in *NR3c1* exon 1F, but only for females. There was no association with maternal responsiveness and appropriate touch or DNA methylation of *NR3c1* exon 1F on prestress cortisol or cortisol reactivity. Our results are discussed in relation to programming models that implicate maternal care as an important factor in programing infant stress reactivity.

**KEYWORDS**
DNA methylation, epigenetics, maternal responsiveness, sex differences

## 1 | INTRODUCTION

Caregivers play a vital role in supporting infant biobehavioral development (Sroufe, 2005). Decades of behavioral research with humans has suggested that caregivers who are more responsive and sensitive have infants who exhibit less stress reactivity and more social and emotional competence (Conradt & Ablow, 2010). However, the precise mechanisms by which sensitive caregiving behavior is related to infant stress reactivity is unclear. Research with animal models has suggested that one mechanism may be epigenetic in nature. Epigenetics is defined as the study of molecular processes occurring on and around the genome that regulate gene activity without changing the underlying DNA sequence. While a growing literature has identified that we can measure epigenetic processes in humans (Conradt, 2017; Lester, Conradt, & Marsit, 2016), and that these processes are sensitive to early life stress (Oberlander et al., 2008; Romens, McDonald, Svaren, & Pollak, 2015), there has been no published evidence of whether observed maternal caregiving in humans relates to epigenetic processes in infancy. In this study, we sought to identify whether maternal caregiving behavior was associated with epigenetic modification of a gene implicated in the neuroendocrine

  

response to stress, the glucocorticoid receptor gene, *NR3c1* exon 1F.

## 2 | DEVELOPMENTAL PROGRAMMING AND EPIGENETICS

Developmental programming models suggest that the young child makes adjustments to physiological stress response systems in response to cues received prenatally (Barker, 2002; Glover, O'Connor, & O'Donnell, 2010) and during early postnatal life (Cameron et al., 2008; Mueller & Bale, 2008). An example comes from the prenatal programming literature. Sandman and Davis (2012) and Sandman, Glynn, and Davis (2016) examined whether prenatal exposure to the maternal stress hormone cortisol alters the "gestational clock" and increases risk for preterm birth. They found that high levels of fetal exposure to corticotropin releasing hormone, a cue which may forecast the quality of the postnatal environment, were related to decreased gestational length, and in the extreme, preterm birth. Sandman, Davis, Buss, and Glynn (2012) argued that the fetus may "hedge its bets" and risk being born earlier rather than remaining in an inhospitable prenatal environment.

One mechanism by which these programming processes are thought to occur is via epigenetics. While our genetic code is largely fixed and does not change in response to environmental experiences, our epigenetic code is mutable (Meaney, 2010). It is thought that environmental exposures experienced during sensitive periods of development alter the way in which certain genes are expressed. There are a variety of epigenetic mechanisms, but the one most commonly studied in humans is DNA methylation (Lester et al., 2016). DNA is made up of four nucleotides: cytosine, guanine, adenine, and thymine. Gene expression typically occurs in a region of the gene called a *promoter*, where cytosine and guanine nucleotides tend to cluster. DNA methylation—the most commonly examined epigenetic mechanism in humans—takes place when a methyl molecule is added to a cytosine preceding a guanine along the gene promoter (Bird, 2007). This serves to block transcription, which alters gene expression and subsequent protein development. DNA methylation is therefore thought of as a silencer of anticipated gene activity.

In human behavioral epigenetic research, the most widely studied gene is *NR3c1*, in part because the first epigenetic studies to be implicated in infant development had focused on this gene in rodents (Liu et al., 1997; Meaney, 2010). *NR3c1* is thought to be involved in regulation of the hypothalamic–pituitary–adrenal (HPA) axis by modulating availability of the stress hormone cortisol. Specifically, greater DNA methylation of *NR3c1* should be related to less glucocorticoid receptor gene expression. Less gene expression, in turn, should be associated with fewer glucocorticoid receptors in the hippocampus and, as a result, fewer receptors to which cortisol can bind. This, accordingly, should be related to greater circulating cortisol in the bloodstream, and presumably increases in cortisol in response to stress. In support of this hypothesis, Oberlander et al. (2008) found that prenatal exposure to maternal depression was related to greater DNA methylation of *NR3c1* exon 1F in infants and, in turn, greater cortisol reactivity in infancy. Conradt et al. (2015) also found that greater methylation of *NR3c1* exon 1F was related to greater cortisol reactivity in response to a social stressor, the still-face paradigm. There is thus initial support for the notion that DNA methylation of *NR3c1* is sensitive to early environmental exposures in humans. To our knowledge, only one study has demonstrated that DNA methylation of *NR3c1* is sensitive to early caregiving in humans (Lester et al., 2018). Additional support for this hypothesis comes from early research with animal models.

## 3 | DNA METHYLATION OF *NR3c1* AND MATERNAL CAREGIVING BEHAVIOR

Initial evidence for epigenetic modification of *NR3c1* by maternal caregiving behavior comes from the work of Liu et al. (1997) and Meaney (2010). They found that high levels of licking and grooming and arched-back nursing in rodents was related to low methylation levels of *NR3c1* and, subsequently, low corticosterone responses to stress (Weaver et al., 2004). This research has led to a great deal of excitement for psychologists interested in the molecular underpinnings of early childhood development because it provided initial evidence that caregiving behaviors may *program* infant stress responses.

Efforts to translate this research to humans have been undertaken by many independent research groups. In addition to the work by Oberlander et al. (2008) reviewed earlier, Murgatroyd, Quinn, Sharp, Pickles, and Hill (2015) examined the effects of pre- and postnatal exposure to maternal depression, DNA methylation of *NR3c1*, and modifications by maternal touch. Infants exposed to low levels of prenatal maternal depression, but high levels of postnatal maternal depression, exhibited greater methylation of the glucocorticoid receptor gene, *NR3c1*, as compared to infants exposed to concordant pre- and postnatal depression. This effect, however, was reversed by maternal-report of maternal stroking of the infant during the first few weeks of life (Murgatroyd et al., 2015). Moore et al. (2017) examined whether a daily diary report of maternal–infant tactile contact and infant distress during Week 5 of life was related to DNA methylation of *NR3c1*, along with a variety of other candidate genes. While no main effects emerged, there was an association between epigenetic age, a measure of epigenetic aging

relative to chronological age, infant distress, and infant tactile contact. Infants who were highly distressed and who received low tactile contact from caregivers had a younger epigenetic age relative to their chronological age (Moore et al., 2017).

Only one study, to our knowledge, has examined direct associations between breast-feeding and DNA methylation of *NR3c1* in infancy (Lester et al., 2018). Using data from the present sample, Lester et al. (2018) found that DNA methylation of *NR3c1* was significantly greater in infants who experienced little to no breast-feeding during the first 5 months of life. This study is a partial translation of the licking and grooming research by Liu et al. (1997) and Weaver et al. (2004), and suggests that DNA methylation of *NR3c1* may be sensitive to caregiving experiences in human infants as well as rodents.

Despite burgeoning evidence that early rearing experiences may affect an infant's epigenetic profile, less is known about whether all infants are affected to the same degree. There has been a recent resurgence in investigating the importance of sex differences in DNA methylation in humans. Animal research has indicated that high licking and grooming mothers had female (but not male) offspring with low DNA methylation of estrogen receptor (ER)—$\alpha$, and this methylation was in turn related to more licking in grooming by these females when they reached adulthood (Champagne et al., 2006). In rats, therefore, female offspring of high licking and grooming mothers were more likely to be high licking and grooming mothers themselves if these offspring showed low methylation of ER$\alpha$. In humans, Ostlund et al. (2016) found that prenatal exposure to maternal stressful life events was related to greater DNA methylation of *NR3c1*, and methylation was associated with infant fearful temperament, but only in females (Ostlund et al., 2016). Therefore, it is important that we test whether there may be a sex-specific pathway by which early exposures relate to DNA methylation outcomes.

## 4 | CURRENT STUDY

While there is a growing body of research relating early life exposures such as exposure to maternal depression or stressful life events to DNA methylation of *NR3c1* in humans, there are no studies that have examined the main effects of observed (vs. self-reports) of parenting behavior on DNA methylation of *NR3c1*. Our goal was to attempt to translate animal epigenetic research on caregiving to humans by directly observing whether maternal sensitive care was associated with DNA methylation of *NR3c1*.

We examined whether sensitive caregiving behaviors assessed during free play were associated with DNA methylation of *NR3c1*. We hypothesized that greater levels of maternal sensitive caregiving behavior would be associated with lower levels of DNA methylation of *NR3c1* and more cortisol reactivity. We also examined whether any main effects were moderated by infant sex, but this aim was exploratory, and we propose no specific hypotheses.

## 5 | METHOD

### 5.1 | Participants

Mothers ($N = 128$) and their 5-month-old infants ($n = 58$ girls) were part of the Rhode Island Child Health Study, an existing cohort recruited through Brown University. Mothers from the original study were recruited in accordance with the Institutional Review Board of Brown University. All mothers gave written informed consent prior to participation.

Only singleton, full-term (≥37 weeks' gestational age) infants were included in the study. Other exclusion criteria included maternal age <18 years or a life-threatening medical complication of the mother, and congenital or chromosomal abnormality of the infant. The current sample consisted of mother–infant dyads for whom we had complete data on all variables of interest. Infants who received a diagnosis of intrauterine growth restriction were excluded from analysis. Mothers in the current sample identified as European American (61.2%), African American (10.1%), Hispanic (9.7%), Asian (3.1%), or "other" (9.3%), and had a mean age of 30.9 years, range = 20–40. Infants were on average 19.3 weeks old, range = 15–26, at the time of assessment.

### 5.2 | Measures

#### 5.2.1 | Maternal responsiveness and appropriate touch

Maternal responsiveness and appropriate touch were computed based on behaviors assessed during a 5-min mother–infant free play using a coding scheme developed by Tronick and adapted from Gunning, Fiori-Cowley, and Murray (1999). We chose these scales to limit the number of comparisons run, given that they had been previously combined using a factor analysis and used to predict DNA methylation of *NR3c1* in a separate context, the still-face paradigm (Conradt et al., 2016). Maternal *responsiveness* was operationalized as both the mother's awareness of her infant's signals and her response to them (regardless of the appropriateness of the response), and appropriate *touch* was defined as the mother's ability to touch her infant in a gentle and affectionate manner versus a more intrusive style. These behaviors were coded on a scale of −1, 0, and 1. Higher scores reflected more responsiveness (e.g., the mother picks up the majority of infants' signals) and more appropriate touch (e.g., the mother rarely touches the infant in a way that causes the infant distress or avoidance).

Coders trained to reliability against a set of 10 training tapes coded by three experts in the field of maternal sensitivity. Coders then coded an additional 20% of tapes for reliability. The intraclass correlations were .92 for responsiveness and .85 for touch. Maternal responsiveness and appropriate touch were significantly and positively correlated, $r(126) = .29$, $p < .001$. Values therefore were averaged to create a single score.

#### 5.2.2 | Cortisol

Prestress cortisol samples were taken from infants upon entry into the laboratory. Two poststress cortisol samples were taken following the still-face paradigm (Tronick, Als, Adamson, Wise, & Brazelton, 1978). Following Haley and Stansbury (2003), the first poststress saliva sample was taken 30 min after the end of the first still-face episode, and the second poststress saliva sample was taken 40 min after the end of the first still-face episode (Haley & Stansbury, 2003). Salivary cortisol was collected from the infant 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 min prior to sample collection, their mouths were first swabbed with a wet paper towel. Samples were analyzed by Salimetrics (Arizona) for analysis.

### 5.3 | DNA methylation of glucocorticoid receptor gene (*NR3c1*) at 5 months

#### 5.3.1 | Buccal sample collection, DNA isolation, and bisulfite modification

Buccal-derived DNA was collected from saliva samples using the Oragene-DNA saliva collection system. Buccal cells were taken from the infants' cheeks using a small swab. The swabs were then placed in a collection tube and sealed, releasing a stabilizing solution into the collected sample to allow for processing of the sample at a later period. Batches of sample collection tubes were sent to Dartmouth College for DNA isolation. DNA was isolated from the collection tubes following the Oragene methods. Purified DNA was quantified using an ND-1000 spectrophotometer (Nanodrop, Wilmington, DE), and DNA samples (500 ng) were bisulfite-modified using the EZ DNA Methylation Kit (Zymo Research, CA) and stored at −20°C.

#### 5.3.2 | Bisulfite pyrosequencing DNA methylation analysis

Pyrosequencing, which allows for quantitative assessment of DNA methylation in short sequence regions, was performed on PCR product amplified from bisulfite modified DNA as described previously (Conradt et al., 2016). The primers for amplification were Forward: 5′-TTT TTT TTT TGA AGT TTT TTT A-3′ and Reverse: 5′-Biotin-CCC CCA ACT CCC CAA AAA-3′. The first sequencing primer was designed to sequence the first five CpG sites (5′-GAG TGG GTT TGG AGT-3′), and the second sequencing primer was designed to sequence the following eight CpG sites (5′-AGA AAA GAA TTG GAG AAA TT-3′), for a total of 13 sites sequenced.

The percent methylation at each of the 13 CpG sites of *NR3c1* was quantified using the Pyro Q-CpG software, Version 1.0.11 (Qiagen). Bisulfite conversion controls were included on each sequencing read. For the sample's methylation extent to be called, the bisulfite conversion rate must be >93%, and for all samples examined, the conversion rate was >95%. All assays were performed in triplicate on the same bisulfite converted DNA template on all samples; if any of the repeats differed by >10%, those assays on that sample were repeated. To prevent batch effects from bisulfite treatments interfering with the analysis, samples were randomized across batches.

#### 5.3.3 | Missing data

There were 128 infants who completed the 5-month assessment, and 109 infants with complete maternal responsiveness and appropriate touch and 5-month methylation data. One participant's responsiveness and appropriate touch data could not be coded because of equipment failure, and 18 methylation samples could not be computed because of insufficient saliva volume. Tests for birth and demographic differences between infants with and without missing data revealed that there were no differences in birth weight, infant sex, gestational age, maternal education, or maternal age among infants with and without missing methylation data, $p$s > .17. Infants with missing methylation data were more likely to be non-White, $\chi^2$ (5, $N = 111$) = 11.51, $p = .04$. Mothers of infants with missing methylation data were less likely to be responsive, $t(126) = 2.16$, $p = .03$.

## 6 | RESULTS

### 6.1 | Preliminary analyses

Data were examined for outliers and violations of normality. The raw cortisol values ($\mu$g/dL) were positively skewed and normalized using a log transformation. Outliers above or below 3 *SD*s from the mean were winsorized by replacing the value with the value at 3 *SD*s; <1% of methylation values were affected. There was less methylation at the lower CpG sites (e.g., Sites 1–4) as compared to the later CpG sites. To minimize the number of comparisons, we conducted a factor analysis with the methylation data, which revealed three factors explaining 78.40% of the variance: A factor of CpG Sites 1 to 2, a factor for Sites 3 to 6, and a factor for Sites 7 to 13. All factor loadings were above 0.82.

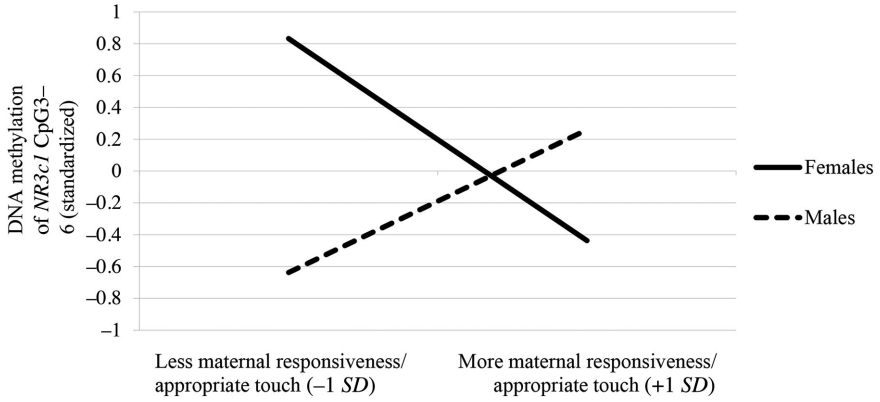


**FIGURE 1** The interaction between infant sex and maternal responsiveness/appropriate touch predicts DNA methylation of *NR3c1* CpG Sites 3 to 6

## 6.2 | Tests of covariates

We examined whether DNA methylation of *NR3c1* or maternal responsiveness and appropriate touch was related to infant age and ethnicity. None of these covariates were significant predictors of DNA methylation of *NR3c1* or maternal responsiveness/appropriate touch, *p*s > .17.

Because of the diurnal rhythm of cortisol, all assessments took place in the morning between 8:00 and 11:30 a.m., range = 8:11–11:20 a.m. We examined whether the time of each of the three assessments was associated with each measure of cortisol (e.g., whether time of the prestress measurement was correlated with the prestress cortisol value). Time of measurement was not significantly related with the time-specific measurement of cortisol, *p*s > .35. We also examined whether either infant or maternal prescription and/or nonprescription steroid medication, or maternal use of caffeine impacted cortisol concentrations. Steroid use within the last 12 hr by either mother or infant was not significantly associated with the cortisol values, *p*s > .40, and neither was maternal consumption of caffeine that morning, *p*s > .11. As nap times also may affect cortisol values, we examined whether time of nap and/or time of awakening affected cortisol; neither was related to our cortisol values, *p*s > .18.

## 6.3 | Maternal responsiveness/appropriate touch, infant sex, and *NR3c1* methylation

We ran a hierarchical linear regression to examine the main and interactive effects of maternal responsiveness/appropriate touch, infant sex, and DNA methylation of *NR3c1*. We ran three regressions, one for each of the three methylation outcome measures. There were no main effects or interactions between maternal responsiveness/appropriate touch, infant sex, and DNA methylation of *NR3c1* CpG Sites 1 to 2 or 7 to 13, *p*s > .20. There was a significant main effect of maternal responsiveness/appropriate touch (but not infant sex) on DNA methylation of *NR3c1* CpG Sites 3 to 6, $b = -.74$, $p = .02$. This main effect was qualified by a significant interaction between maternal responsiveness/appropriate touch and infant sex on DNA methylation of *NR3c1* CpG Sites 3 to 6. We examined differences in DNA methylation of *NR3c1* CpG Sites 3 to 6 for mothers ±1 *SD* from the mean for maternal responsiveness, separately by infant sex. As seen in Figure 1, less maternal responsiveness/appropriate touch was related to greater DNA methylation of *NR3c1* CpG Sites 3 to 6, but only for females, $t(104) = -2.01$, $p = .05$. There were no differences in DNA methylation of *NR3c1* CpG Sites 3 to 6 for males, regardless of their mother's level of maternal responsiveness/appropriate touch, $t(104) = 1.31$, $p = .19$.

## 6.4 | Maternal responsiveness/appropriate touch, *NR3c1* methylation, and cortisol reactivity

We found no significant associations between maternal responsiveness/appropriate touch and infant cortisol reactivity nor did we find significant associations between *NR3c1* methylation and infant cortisol reactivity.

# 7 | DISCUSSION

Animal models indicate that maternal caregiving behavior may program infant stress responses via epigenetic processes. We attempted to translate this research to humans by examining whether maternal responsiveness/appropriate touch was associated with DNA methylation of *NR3c1* and infant cortisol reactivity. We found that maternal caregiving behaviors, as assessed during free play, were related to DNA methylation of *NR3c1*, but this effect varied by infant sex. We did not find significant associations between DNA methylation of *NR3c1* and infant cortisol reactivity.

This study compliments the findings by Lester et al. (2018), also from this sample, who showed differences in DNA methylation of *NR3c1* depending on whether infants were breast-fed. In the rodent literature, both licking and grooming and arched-back nursing were related to differences in glucocorticoid receptor gene methylation. Our research therefore serves as a partial replication of this rodent research and

suggests that DNA methylation of *NR3c1* in infancy may be sensitive to maternal caregiving. Caution is warranted, given that we cannot infer direction of effect with these data. While we cannot randomly assign infants to different caregiving experiences for obvious ethical reasons, we may be able to come closer to inferring direction of effect through intervention, a hypothesis being explored by O'Donnell et al. (2018).

Our findings support developmental programming models that suggest that one mechanism by which programming processes occur is via DNA methylation. In this study, the association between maternal caregiving behavior (responsiveness and appropriate touch) and DNA methylation of *NR3c1* was significant only for females. These findings partially contradict the work of Braithwaite, Kundakovic, Ramchandani, Murphy, and Champagne (2015), who found specific associations between DNA methylation of *NR3c1* and prenatal exposure to maternal depression, but only in males. They also are inconsistent with a small body of research demonstrating that male fetuses may be more vulnerable to the effects of prenatal stress exposure (Kinney, Munir, Crowley, & Miller, 2008). It is consistent with other published research from this sample that has found associations between prenatal exposure to maternal stress and *NR3c1* exon 1F methylation, but only in females, and published literature with rats that has found associations between maternal caregiving and DNA methylation of ER$\alpha$ in females (Champagne et al., 2006; Ostlund et al., 2016). Whereas the male fetus is more sensitive to prenatal stress, perhaps females are more sensitive to the effects of maternal caregiving behavior. More research that is adequately powered is warranted to better understand these effects.

Both animal (Liu et al., 1997) and human (Albers, Riksen-Walraven, Sweep, & Weerth, 2008; Kaplan, Evans, & Monk, 2008) models demonstrate that parental insensitivity is a form of early life stress related to greater expressions of physiological reactivity. However, we did not find significant associations between maternal responsiveness and appropriate touch, components of maternal sensitivity, and cortisol reactivity in our sample, nor did we find significant associations between *NR3c1* exon 1F methylation and cortisol reactivity. In our work and others (Haley & Stansbury, 2003), we have found that the still-face paradigm elicited a neuroendocrine stress response, but like others (Haley & Stansbury, 2003), this finding was not associated with maternal responsiveness. We examined maternal responsiveness/appropriate touch in a context different from a context that was designed to elicit a neuroendocrine stress response. Our test of associations between cortisol reactivity and maternal responsiveness/appropriate touch was arguably more stringent. However, it also could be that the infant HPA axis is more sensitive to maternal responsiveness and appropriate touch in response to distress (vs. the nondistress context of the free play; Leerkes, Blankson, & O'Brien, 2009). It also could be that maternal neuroendocrine responses to stress are better predictors of infant neuroendocrine reactivity than maternal behavior, and important hypothesis that should be explored in future research.

The present study was limited by the correlational nature of the study design. We therefore cannot infer directionality of the effect with these data. Our free-play session, while consistent with the length of free-play sessions in other studies, was short (5 min; Teti, Nakagawa, Das, & Wirth, 1991). It is unclear whether a longer length of time would have yielded significant different maternal responsiveness and appropriate touch data. In addition, like all human behavioral epigenetic studies, we are limited by our ability to use peripheral tissues, such as infant cheek cells, to infer what processes may be occurring in the infant brain.

In sum, this research adds to the small literature documenting associations between maternal behavior and DNA methylation of *NR3c1* in early childhood. It is the first study to find evidence that DNA methylation of *NR3c1* exon 1F is sensitive to observed maternal caregiving behavior, but only for infant girls. This is a partial replication of the seminal work by Liu et al. (1997) and Weaver et al. (2004), who found that caregiving behavior in the form of licking and grooming and arched-back nursing also was related to DNA methylation of *NR3c1*. Following replication of this work, it could ultimately be used in conjunction with early intervention, or home-visiting programs, to measure the strength of the intervention effect at the epigenetic level.

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## CONFLICT OF INTEREST

The authors report no conflict of interest.

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