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Running title: Maternal touch and *SLC6A4* methylation pattern

The role of maternal touch in the association between *SLC6A4* methylation and stress response in very preterm infants.

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Abstract

Very preterm (VPT) infants requiring hospitalization in the Neonatal Intensive Care Unit (NICU) are exposed to several stressful procedural experiences. One consequence of NICU-related stress is a birth-to-discharge increased serotonin transporter gene (*SLC6A4*) methylation which has been associated with poorer stress regulation at 3-months of age. Maternal touch is thought to support infants' stress response, but its role in moderating the effects of *SLC6A4* methylation changes is unknown. The aim of this study was to assess the role of maternal touch in moderating the association between increased *SLC6A4* methylation and stress response in 3-month-old VPT infants. Twenty-nine dyads were enrolled and at 3-months (age corrected for prematurity), participated in the Face-to-Face Still-Face (FFSF) paradigm to measure infants' stress response (i.e., negative emotionality) and the amount of maternal touch (i.e., dynamic and static). Results showed that low level of maternal touch is associated with high level of negative emotionality during social stress. Furthermore, during NICU stay *SLC6A4* methylation in VPT exposed to low level of maternal touch at 3 months was associated with increased negative emotionality. Thus, low levels of maternal static touch can intensify the negative effects of *SLC6A4* epigenetic changes on stress-response in 3-months-old VPT infants.

Keywords: Very preterm infants, DNA methylation, maternal touch, negative emotionality, serotonin transporter gene, *SLC6A4*, stress response.

Introduction

Very preterm (VPT) infants (e.g., <32 weeks Gestational Age, GA) need long-lasting hospitalization in the Neonatal Intensive Care Unit (NICU) during which they are exposed to stressful experiences, such as frequent invasive and potentially painful practices (e.g., skin-breaking procedures), as well as the emotional consequences of touch deprivation due to maternal separation (Grunau et al., 2005). This early exposure to adverse experiences has an impact on hypothalamic-pituitary-adrenal (HPA) axis regulation of VPT infants, which in turn leads to an altered pattern of socio-emotional stress development later in life (Provenzi et al., 2016a). Epigenetic mechanisms, functional modifications of the DNA that regulate gene activity without changing the DNA sequence, may explain, at least partially, how early NICU-related stressful experiences can affect the developmental trajectories of preterm infants (Maddalena, 2013). Emerging evidence suggests a link between variation in the serotonin transporter gene (i.e., *SLC6A4*) and altered developmental trajectories of stress responses in VPT infants (Montirosso et al., 2016a, Provenzi et al., 2020a). Research on human infants indicates that postnatal maternal touch may buffer the early epigenetic effects of less-than optimal caregiving (Murgatroyd et al., 2015). While most studies focused on *NR3C1* methylation (a candidate gene related to stress response which codes for glucocorticoid receptor; Conradt et al., 2019; Lester et al., 2018), the association between maternal touch and *SLC6A4* DNA methylation remains unexplored. The present study was designed to explore the role of maternal touch in moderating the association between during NICU stay altered *SLC6A4* methylation and stress response in 3-month-old VPT infants.

Epigenetic variations associated with serotonergic system

The serotoninergic system plays a key role in regulating HPA stress reactivity and its negative feedback (Lanfumey et al., 2008; Porter et al., 2004). Serotonin (5-HT) receptors are broadly spread throughout the central nervous system and develop early during gestation, with the serotonergic system maturing during the first year of life (Gaspar et al., 2003). This system is regulated by feedback processes through the serotonin transporter (5-HTT), which is encoded by the

SLC6A4 gene. The transcriptional activity of *SLC6A4* is regulated by genetic variants and epigenetic mechanisms. Previous research has explored the role of a transporter-linked polymorphic region (i.e., 5-HTTLPR) in infants' stress response (Pauli-Pott et al., 2009). The 5-HTTLPR has short (S) or long (L) allelic variants, with the former linked to reduced 5-HTT transcription and augmented risk of adverse developmental outcomes, such as socio-emotional dysregulation and stress susceptibility (Heils et al., 1995). However, the 5-HTTLPR polymorphic variant accounts only partially for differences in socio-emotional stress response (Mayer et al., 1999). During the last decade the field of epigenetics has provided a new perspective to explore DNA transcriptional changes due to the interaction between genes (e.g., *SLC6A4*) and early environmental adversity conditions including neonatal pain (Chau et al., 2014). In mammalians, methylation at the 5th carbon of cytosine (5-methylcytosine; 5-mC) is the most predominant DNA modification. It occurs when a methyl group is inserted in the cytosine residue of specific 5′- cytosine guanine-3′ dinucleotides (CpG sites), often clustered in CpG-rich regions (CpG islands), which are prominently found within the promoter region of a gene (Hyman, 2009). While increased methylation of the cytosine residues (i.e., hypermethylation) often leads to a decreased expression of the mRNA and the protein of interest, decreased methylation (i.e., hypomethylation) increases gene expression (Jones, 2012). Accordingly, the methylation status of different CpG sites within the *SLC6A4* promoter region has been inversely associated to the degree of 5-HTT expression (Duman & Canli, 2015). An increasing number of studies reported that increased *SLC6A4* methylation might be a marker of early adverse experiences and might play a role in altered developmental trajectories of stress response and susceptibility (Provenzi et al., 2016b). For instance, prenatal exposure to maternal depression, childhood maltreatment and poor socioeconomic conditions have been associated with CpG-specific patterns of altered methylation within the *SLC6A4* promoter region (Provenzi et al., 2016b).

SLC6A4 epigenetic variations and stress response in VPT infants

Even when controlling for perinatal and medical confounds, greater methylation of the *SLC6A4* predicted poor stress regulation in VPT infants. For instance, one study has documented that *SLC6A4* promoter region methylation is associated with NICU-related stress in VPT infants' development, highlighting that the number of painful skin-breaking procedures during the NICU stay was linked to altered methylation of specific *SLC6A4* CpG sites at discharge (Provenzi et al., 2015). Moreover, at 3-months of age, *SLC6A4* methylation status was associated with temperamental difficulties (Montirosso et al., 2015) and higher stress susceptibility during a social stress procedure (i.e., Face-to-Face Still-Face (FFSF) paradigm; Provenzi et al., 2016a). Additionally, a recent study, found that VPT children displayed greater anger in response to an emotional stress procedure at 4.5 years compared with full-term age-matched controls. Remarkably, in the VPT children sample, the degree of anger expression was significantly predicted by increased *SLC6A4* methylation measured at NICU discharge (Provenzi et al., 2020a). Furthermore, higher exposure to pain-related stress during NICU stay predicted an increased *SLC6A4* methylation in 7-year-old VPT children (Chau et al., 2014), which in turn was related to internalizing behaviors. In sum, there is evidence that early NICU-related stressful events lead to altered methylation status of the gene encoding the serotonin transporter, with consequences for socio-emotional regulation throughout infancy and childhood.

Maternal touch and epigenetic status

Along with other components of parenting (e.g., sensitivity, responsiveness), maternal proximity, including touch, influences infant behavioral and physiological stability, socio-emotional development and infant stress response. For example, immediate post-natal tactile stimulation and physical contact reduce newborns' crying and distress and support newborn adaption to life outside of the womb (Winberg, 2005). In 6-month-old infants, the presence of maternal touch during the FFSF paradigm reduces infants' physiological reactivity to social stress (e.g., maternal unavailability) (Feldman et al., 2010). Recent evidence suggested that epigenetic mechanisms could be associated with tactile contact experience in full-term infants (Mariani Wigley et al., 2022). One

study found that infants who experienced little to no breast-feeding, considered a proxy of physical contact during the first 5-months of life, showed increased *NR3C1* DNA methylation (Lester et al., 2018). In 5-months-old infants, maternal nurturing touch (i.e., gentle and affectionate touch) and higher parental responsiveness (i.e., mother's sensitivity to infant's signals) were related to reduced DNA methylation of *NR3C1* (Conradt et al., 2019). Moore and colleagues conducted a longitudinal study during which mothers filled out a diary reporting infants' status throughout the day and corresponding caregiving behaviors, including the amount of physical contact during week 5 of life. Results showed a significant difference in five non-stress related genes involved in metabolic and immunologic pathways (Moore et al., 2017). A very recent study investigated the effect of preterm birth, and of an early intervention program based on enhanced maternal care and positive multisensory stimulation (i.e., infant massage and visual interaction), on Long Interspersed Nuclear Element-1(LINE-1) retrotransposons (Fontana et al., 2021). LINE-1 are a class of transposable DNA elements which contribute to genomic somatic mosaicism of the brain and are deregulated in several neurological disorders that often occur in individuals born preterm (Lapp & Hunter, 2019). In their study Fontana and colleagues found that while LINE-1 elements were hypomethylated at birth, early intervention, but not standard care, restored LINE-1 methylation to levels comparable to healthy newborns. Importantly, LINE-1 methylation increased proportionally to maternal care received through early intervention, which was quantified as the average number of massages that infants received per week, suggesting a strong association between maternal touch and epigenetic variations in preterm infants (Fontana et al., 2021).

Present study

Despite the above-mentioned findings suggesting that DNA methylation might be sensitive to caregiving touch in human infants, to the best of our knowledge, no study has investigated whether maternal touch interacts with epigenetic modification of the *SLC6A4* gene. Here, we explored the potential contribution of maternal touch in moderating the relationship between CpG-specific *SLC6A4* methylation at discharge from the NICU and infants' stress response,

operationalized as negative emotionality at 3-months. *SLC6A4* CpGs were selected for further analysis when: a) methylation status was significantly changed from birth-to-discharge, b) *SLC6A4* CpGs methylation were found to be significantly associated with pain-related stress exposure in NICU. First, we examined the association between NICU-related stress and *SLC6A4* methylation at NICU discharge in order to evaluate how this is associated with infant's negative emotionality during FFSF paradigm. Second, we questioned whether maternal touch would moderate the association between *SLC6A4* methylation and negative emotionality. Previous full-term infant studies suggested that modalities of maternal touch (i.e., different types characterized by specific stimulation features) may be more relevant than touch frequency (Hertenstein et al., 2006; Moszkowski et al., 2009; Tronick, 1995). As for mothers of preterm infants, one study found that during face-to-face interaction with their 3-month-old infants, mothers used static touch (i.e., contact without movements) for the 60% of the time and dynamic touch (i.e., caressing actions or repositioning their infant involving vestibular sensations, such as lifting) for 40% of the time (Weiss et al., 2004). Accordingly, we analyzed whether maternal dynamic *vs.* static touch assessed during the first episode of FFSF paradigm interacted with *SLC6A4* DNA methylation in explaining infants' negative emotionality across the subsequent stressful and recovery episodes of the observational procedure. Although specific hypotheses regarding the role of type of touch (dynamic *vs.* static touch) could not be formulated based on existing research, we expected that maternal touch *per se* would play a relevant role together with *SLC6A4* DNA methylation in explaining VPT infant's negative emotionality.

Methods

Participants

The present study is a post-hoc analysis of a larger longitudinal research project that included 32 VPT infants (gestational age (GA) < 32 weeks and/or birth weight ≤ 1500 g), recruited between October 2011 and April 2014 and who had complete data at 3 months (age corrected for prematurity). The original project probed the link between NICU pain-related stress and epigenetic

status in VPT infants. In previous work, we have also reported data about *SLC6A4* methylation and infants' behavioral development during the first months of life (Montirosso et al., 2016a; Montirosso et al 2016b). Although data in the current paper are derived from previously published studies (Montirosso et al., 2016a; Montirosso et al., 2016b; Provenzi et al., 2015; Provenzi et al., 2017), the current sample is not identical to previous ones due to unavailable touch coding information during mother-infant video-coded interactions (i.e., the mother's hands were covered from view most of the time). Therefore, from the initial sample three VPT infants were excluded due to unavailable maternal touch coding information, leaving a group of 29 VPT and their mothers for which outcomes were analyzed. Procedures for infants' and mothers' recruitment and eligibility criteria for VPT infants are reported in detail in previous work (Provenzi et al., 2015). Sample characteristics are reported in Table 1.

All parents provided informed consent. The present project has been conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki, 2013) and has been approved by the Ethics Committees of Scientific Institute IRCCS Eugenio Medea (Bosisio Parini, Italy) and participating hospital.

Procedure

In accordance with previous studies, cord blood samples were obtained at birth whereas peripheral blood was collected at hospital discharge (Provenzi et al., 2015). All blood samples were obtained by trained nurses and immediately stored at -20°C at the hospital facilities. Infants' perinatal data and pain-related stress in NICU were obtained from medical records. At 3 months CA, during a home visit, mother-infant dyads participated in a double-exposure FFSF paradigm to measure infants' stress response (i.e., negative emotionality). The double FFSF paradigm consists of three 2-min interaction episodes (Play, Reunion#1 and Reunion#2) and two 2-min Still episodes (Still#1 and Still#2). During interaction episodes mothers were instructed to play with their infants as they usually would at home (Play and Reunion), whereas during the Still episodes they were instructed to pose a neutral expressionless face to their infants, to look at them but not to smile, talk,

or touch them (see Figure S4 in Supplementary Materials for a visual representation of the paradigm). During these episodes, infants exhibit the typical *still-face effect*, which consists of increased negative emotionality displays, enhanced gaze aversion, reduced positive emotionality and decreased social and communicative behaviors (Adamson & Frick, 2003). In Reunion episodes infants show a *carryover effect*, which consists of a partial recovery of positive emotionality and both social and communicative behaviors and by enduring negative emotionality from the Still-Face episode, which represent a context of socio-emotional stress recovery (Mesman et al., 2009). The double-exposure version of the original FFSF paradigm has been found to be especially useful to obtain information about cumulative stress-response capacities, given that infants are exposed twice to *still-face effect* and *carryover effect* (DiCorcia et al., 2016; Montirosso et al., 2016b). Mothers and infants were videotaped during the FFSF procedure using two cameras: one focused on the infant, the other on the mother who was approximately 0.4m from the infant and adjusted so that her eyes were level with her baby's eye. For coding purposes, the signals from the two cameras were edited offline to produce a single video with simultaneous frontal view of the face, hands, and torso of infant and mother. These videos were then used to encode infants' negative emotionality and maternal touch off-line via the Eudico Linguistics Annotator (ELAN; Max Planck Institute for Psycholinguistics, The Language Archive, Nijmegen, The Netherlands; Lausberg & Sloetjes, 2009). Finally, during the home visit mothers were asked to fill out questionnaires about their emotional state (depressive and anxious symptoms) and a sociodemographic survey that included the collection of neonatal variable and sociodemographic characteristics.

Measures

Perinatal variables and socio-demographic characteristics

Perinatal variables of VPT infants included gestational age, birth weight, sex length of NICU stay and invasive mechanical ventilation (i.e., conventional ventilation and high frequency ventilation). Socio-demographic data included maternal age, years of study and occupation. According to Hollingshead's classification, the more prestigious occupation level between mother

and father was selected to indicate socioeconomic status (SES) of the family (Hollingshead, 2011). Hollingshead scores can range from 0 (occupations that do not require high school graduation) to 90 (occupations that require high level of education and specialization).

NICU pain-related stress

NICU pain-related stress was quantified according to Grunau and colleagues (Grunau, 2013) as the total number of skin-breaking procedures throughout the NICU stay including arterial and venous punctures, heel lance, peripheral venous line insertion. In the present sample, no VPT infants underwent surgery and chest tube insertion.

Maternal emotional state

Maternal depression symptomatology was evaluated with the Beck Depression Inventory (BDI), a 21-item self-report. Each item is rated on a 4-point scale indicating the presence or absence and the severity of depressed feeling, symptoms and behavior (Beck et al., 1961). Higher scores correspond to higher depressive symptomatology. Specifically, a total score of 0-13 is considered minimal range, 14-19 is mild, 20-28 is moderate and 29-63 severe. Second, maternal anxiety symptomatology was assessed by the State-Trait Anxiety Inventory-form Y (STAI-Y) which is a 40-item Likert scale that measures the severity of state (1-20 items) and trait anxiety (21-40 items). Items rated on 4-points scale where higher scores indicates higher presence of anxiety (Spielberger, 2010). To detect clinically significant symptoms, a total score of 39-40 is considered. We considered depressive and anxious symptoms in VPT infants' mothers in order to test if the variables of interest (i.e., infants' negative emotionality and maternal touch) would be influenced by maternal depression and anxiety.

SLC6A4 methylation

We analyzed a CpG-rich region of the *SLC6A4* promoter (chr17:28562750-28562958, Human hg19 Assembly; see Figure S1 in Supplementary Materials), between -69 and -213 relative to the transcriptional start site, which contains 20 CpG sites and is adjacent to exon 1A (see Table S2 in Supplementary Materials for the specific position of each CpG site). DNA methylation was

299 determined on blood leucocytes using bisulphite modification followed by PCR amplification and
300 next generation sequencing. Procedures for DNA methylation quantification are reported in detail in
301 a previous publication from our group (Provenzi et al., 2015). Only methylation levels at CpG sites
302 that have been found to be significantly different between birth to discharge and significantly
303 associated with NICU pain-related stress were included in the analysis (see below).

304 ***Maternal touch***

305 In order to capture the main two types of tactile-kinesthetic stimulations (static *vs.* dynamic)
306 used by mothers with their infants during early mother-infant exchanges (Weiss et al., 2004), we
307 coded maternal touch according to a coding system developed on the basis of well-validated
308 instruments (Provenzi et al., 2020b). We coded the amount of dynamic and static touch provided by
309 mothers during the FFSF Play episode. Dynamic touch included affectionate tactile stimulations
310 (e.g., stroking, caressing, massaging), playful touch (e.g., tickling, shaking, squeezing, lifting,
311 moving or flexing the infant's body) and tactile stimulations aimed at getting infant's attention (e.g.,
312 tapping, patting, squeezing, and pinching). Static touch included light to moderate pressure touch
313 provided to the infant, aimed to maintain physical contact (e.g., holding). Maternal dynamic and
314 static touch were analyzed in each 2-sec segment using ELAN. Nonetheless, coders were blind to
315 the aims and hypotheses of the study. The coders were trained with the 25% of videotapes randomly
316 chosen from the study database, obtaining an inter-rater agreement of Cohen's kappa = .80.

317 ***Infant's negative emotionality during the FFSF paradigm***

318 For each of the five episodes of FFSF, infant's negative emotionality was coded second-by-
319 second by two trained coders and defined as withdrawn, protesting, complaining, being fussy or
320 crying behaviors. Coders had to detect the presence or the absence of negative emotionality-related
321 behaviors for each of the second-by-second time windows. After that, a proportion index of
322 negative emotionality was obtained for each of the five episodes of FFSF. Each index was obtained
323 by dividing the total score of negative emotionality displayed in every FFSF episode for the actual
324 length of the episode, resulting in five negative emotionality indexes. For off-line coding purposes

ELAN has been used by two researchers blind to demographic of infants and mother and to research hypothesis. The coders were trained with the 25% of videotapes randomly chosen from the study database, obtaining an inter-rater agreement of Cohen's kappa = .86.

Data analysis

Statistical analyses were performed using R software version 1.3.1056 (R Development Core Team, 2012). Specifically, stats (R Core Team, 2020) package was used for testing regression models, epiDislpay (Chongsuvivatwong, 2018) package was used to obtained OR and performed Wald's test, rcompanion (Mangiafico, 2021) and ResourceSelection (Lele et al., 2019) packages were used to performed Nagelkerke and GOF test respectively, ggplot2 (Wickham, 2016) was used for graphical representations of the data. Prior to data analysis, included variables (i.e., methylation, maternal touch, infant's negative emotionality) were examined for normal distributions (Hair et al., 2010). No significant differences were found for infants' characteristics and socio-demographic variables between PT included in the present study and PT included in previous work but excluded from this one (either because they did not complete the entire SF procedure or because it was impossible to code maternal touch). Data analysis was carried out by following different steps.

Preliminary analyses

As the sample included here was slightly different from the original one, we have reanalyzed the data in order to: a) check if methylation levels varied between birth and discharge in VPT infants and, b) test if these changes were linked to pain-related procedures during NICU stay, as highlighted in previous work (Montirosso et al., 2016a; Montirosso et al., 2016b; Provenzi et al., 2015). First, paired sample *t*-tests were performed in order to analyze possible *SLC6A4* changes from birth to NICU discharge in VPT infants. Second, bivariate correlations were run to test associations between significantly different birth-to-discharge methylated *SLC6A4* CpGs and pain-related stress exposure in NICU. Similarly, bivariate correlations were run to test whether maternal anxiety and depression were associated with infants' negative emotionality and maternal touch. A repeated measures ANOVA was performed to examine the trend of infants' negative emotionality

throughout FFSF paradigm. Finally, to evaluate possible differences in the amount of dynamic and static touch provided by mothers during the Play episode, paired sample *t*-tests were performed.

Maternal touch, SLC6A4 methylation and infant's negative emotionality

In order to assess the role of maternal touch in the relationship between *SLC6A4* methylation levels at those CpGs highlighted from preliminary analyses and infants' negative emotionality, a set of multivariate logistic regressions were run. Although we planned to analyze infant's negative emotionality in the FFSF episodes as it was measured (i.e., on a continuous scale), visual inspection of graphed data strongly suggested a low and high negative emotionality group; thus, we dichotomized infant's negative emotionality into a low and high group using mean-split and run logistic regression models to analyze infant's negative emotionality as a binary outcome variable. In regression models, predictors were: (a) *SLC6A4* DNA methylation at discharge; (b) maternal dynamic and static touch during the Play episode separately; (c) the interaction between CpG-specific *SLC6A4* methylation and maternal touch (dynamic or static). Infants' gestational age at birth was included as a potential confounder in each of the multivariate logistic regression models. The goodness of fit of the regression models was assessed using maximum likelihood estimates and the Hosmer-Lemeshow test to compare the overall significance of the models, and the Wald χ^2 statistic to compare the statistical significance of the regression coefficients. Nagelkerke's adjusted coefficient of determination was computed to assess the overall validity of the models. All the regression models were built manually by one of the authors (ILCMW).

Results

Preliminary results

Infant perinatal variables, number of skin-breaking procedures, socio-demographical characteristics and maternal emotional state variables are reported in Table 1.

Please insert Table 1 about here.

No significant associations emerged between the variables of interest (i.e., infants' negative emotionality and maternal touch) and depressive and anxious symptoms in VPT infants' (see Table S3 in the Supplementary Materials).

Epigenetics data

SLC6A4 methylation from birth to discharge in VPT

In accordance with our previous findings (Montirosso et al., 2016b), *t*-tests showed that from preterm birth to discharge *SLC6A4* methylation significantly increased at CpG2, $t(28) = -2.206$, $p = .036$, and CpG16, $t(28) = -2.598$, $p = .015$, while it decreases at CpG20, $t(28) = 4.641$, $p < .001$. Since methylation levels were found to be significantly different from birth to discharge, reflecting a potential effect of NICU environment, associations between the methylation level of *SLC6A4* CpG2, CpG16 and CpG20 and skin-breaking procedures were tested. In line with previous work (Montirosso et al., 2016b), bivariate correlations highlighted a positive and significant association between the methylation level of *SLC6A4* CpG2 and pain-related stress exposure in NICU ($r = .44$, $p = .034$) and a non-significant correlation with days of mechanical ventilation ($r = .32$ p = .090). Moreover, the methylation status of *SLC6A4* CpG2 was not associated with the duration of hospitalization ($r = .307$ $p = .105$), indirectly suggesting that DNA methylation changes were not simply related to time elapsed from birth, but the NICU experience. As a result, the methylation status of *SLC6A4* CpG2 was considered for further analysis.

Please insert Figure 1 about here.

Infant's negative emotionality during the FFSF paradigm and maternal touch

Regarding maternal touch assessed during the FFSF Play episode, dynamic touch was found to be significantly higher than static touch (Fig. 2), $t(28) = 4.62$, $p < .001$.

Please insert Figure 2 about here.

The repeated measures ANOVA revealed that negative emotionality was significantly different among FFSF episodes, $F(4, 112) = 11.045$, $p < .001$, $\eta^2 = .283$. Figure 3 highlights the trend of infants' negative emotionality through FFSF episodes.

Please insert Figure 3 about here.

The effect of maternal touch on the association between *SLC6A4* CpG2 methylation and infants' negative emotionality

In the following regression models infants' negative emotionality was split into low and high levels and coded as 0 (low negative emotionality) and 1 (high negative emotionality). The first logistic regression model examined the relationship between infants' negative emotionality during Still#1 and methylation level of *SLC6A4* CpG2, maternal static touch assessed during the Play episode of FFSF, infants' gestational age and the interaction between *SLC6A4* CpG2 methylation and maternal static touch. The second regression model examined the relationship between infants' negative emotionality during Reunion#1 and methylation level of *SLC6A4* CpG2, maternal static touch assessed during the Play episode of FFSF, infants' gestational age and the interaction between *SLC6A4* CpG2 methylation and maternal static touch. Results showed that the change in deviance was not significant in either first and second regression model, [$\chi^2(4, N = 29) = 1.187$, $p = .880$] and [$\chi^2(4, N = 29) = 7.679$, $p = .104$].

The third regression model examined the relationship between infants' negative emotionality during Still#2 and methylation level of *SLC6A4* CpG2, maternal static touch assessed during the Play episode of FFSF, infants' gestational age and the interaction between *SLC6A4* CpG2 methylation and maternal static touch. Results showed that the change in deviance was significant [$\chi^2(4, N = 29) = 16.889$, $p = .002$] and confirmed by the Hosmer-Lemeshow test [$\chi^2(4, N = 29) = 7.192$, $p = .516$]. Among the included variables, methylation level of *SLC6A4* CpG2 and the interaction between methylation level of *SLC6A4* CpG2 and maternal static touch were significant. Higher CpG2 methylation levels at NICU discharge were predictive of heightened infants' negative emotionality during Still#2. These main effects were qualified by a significant interaction between maternal touch and CpG2 methylation. We tested the association between CpG2 methylation (predictor) and negative emotionality in the Still#2 (outcome), considering two level of static touch (high and low). As summarized in Figure 4a and 5a, results showed that VPT

infants of mothers characterized by low maternal static touch showed a significant positive association between *SLC6A4* methylation of CpG2 (OR = 51.82, 95% CI [1.14, 2350.26]) and negative emotionality during Still#2, [$\chi^2(4, N = 17) = 10.168$, $p = .001$] and confirmed by the Hosmer-Lemeshow test [$\chi^2(4, N = 17) = 9.181$, $p = .327$].

The last regression model examined the relationship between infants' negative emotionality during Reunion#2 and methylation level of *SLC6A4* CpG2, maternal static touch assessed during the Play episode of FFSF, infants' gestational age and the interaction between *SLC6A4* CpG2 methylation and maternal static touch. Results showed that the change in deviance was significant [$\chi^2(1, N = 29) = 13.271$, $p = .010$] and confirmed by the Hosmer-Lemeshow test [$\chi^2(1, N = 29) = 5.059$, $p = .751$]. Among the included variables, methylation level of *SLC6A4* CpG2, static touch and the interaction between methylation level of *SLC6A4* CpG2 and maternal static touch were significant. Higher CpG2 methylation levels at NICU discharge were predictive of heightened infans' negative emotionality during Reunion#2. These main effects were qualified by a significant interaction between maternal touch and CpG2 methylation. Therefore, we controlled for the association between CpG2 methylation and negative emotionality in the Reunion#2 for high and low level of maternal static touch. As shown in Figure 4b and 5b, results highlighted a positive and significant association between *SLC6A4* methylation of CpG2 (OR = 15.11, 95% CI [1.1, 229.98]) and negative emotionality during Reunion#2 in VPT infants of mothers characterized by low maternal static touch, [$\chi^2(1, N = 17) = 7.209$, $p = .007$] and this is confirmed by the Hosmer-Lemeshow test [$\chi^2(1, N = 17) = 5.256$, $p = .729$].

Insert Figure 4 and 5 about here.

No regression models with maternal dynamic touch as predictor was significant and coefficients are reported in Table S5 in Supplementary Materials.

Discussion

The aim of the present study was to assess the moderating role of maternal touch on the association between *SLC6A4* methylation at NICU discharge and VPT infants' negative

emotionality. As a first step, considering the sample included in the present study, we checked whether methylation levels varied between birth and NICU discharge in VPT infants. Although the sample had a slightly different composition, the results were similar to findings we obtained in previous studies (Montirosso et al., 2016a; Montirosso et al., 2016b; Provenzi et al., 2015). Specifically, DNA methylation level at three CpG specific sites (i.e., CpG2, CpG16 and CpG20) was significantly different from birth to discharge. In addition, we found that the methylation level of *SLC6A4* CpG2 was significantly correlated with the number of skin-breaking procedures (i.e., a proxy of the NICU-related stress) that occurred during the hospitalization, confirming results from previous studies (Montirosso et al., 2016b). Overall, these results corroborated evidence from our previous work suggesting that the altered methylation status of the serotonin transporter gene is not necessarily just a consequence of premature birth *per se*. Rather, NICU-related stress altered the transcriptional functionality of *SLC6A4* in VPT infants, which, in turn, impacted on infant stress response (i.e., negative emotionality) at 3-months of age (Montirosso et al., 2016b; Provenzi et al., 2020a).

Moreover, VPT infants DNA methylation of *SLC6A4* CpG2 and maternal static touch during the normal interactive episode of FFSF (i.e., Play), explained infant's negative emotionality in subsequent episodes. Specifically, a low amount of maternal static touch appeared to negatively moderate the relationship between high levels of CpG2 *SLC6A4* methylation and high levels of infant's negative emotionality during the second episode of maternal unresponsiveness (i.e., Still#2) and the second reunion episode (i.e., Reunion#2). To date, different studies explored associations between maternal touch and DNA methylation in early childhood. For example, Conradt and colleagues (2019) showed that maternal responsiveness/appropriate touch were related to DNA methylation in a stress-related gene (i.e., *NR3C1*) in 5-month-old FT infants (Conradt et al., 2019). One study focusing on the oxytocin receptor gene (i.e., *OXTR*) found that, along with other behaviors indicative of maternal engagement, maternal touch was associated with a reduction in methylation levels between 5 and 18 months of age in full-term infants (Krol et al., 2019).

Importantly, a recent paper found that LINE-1 methylation status in preterm infants was sensitive to the level of maternal care received through early intervention in NICU (Fontana et al., 2021). Therefore, our results expand these previous findings by suggesting that maternal touch may not only predict DNA methylation changes, but also interact with already altered methylation patterns thereby buffering the negative effects of the time spent in NICU on child neurodevelopmental outcomes.

Our findings are consistent with diathesis-stress/dual-risk models (Pluess & Belsky, 2010). According to these models, in risk conditions (e.g., preterm birth) less-than-optimal maternal behavior (e.g., low level of maternal touch) is associated with poorer stress regulation (e.g., high level of negative emotionality during social stress) than the same risk condition supported by nurturing maternal behaviors (e.g., high level of maternal touch). Furthermore, *SLC6A4* DNA methylation in VPT exposed to less-than-optimal maternal behavior was associated with increased stress susceptibility. Taken together, these findings highlight the fact that an infant's epigenetic status operates with respect to environmental factors so that infant's negative emotionality across FFSF appears to be affected by the interplay between maternal touch behavior and the infant's epigenetic status.

Additionally, our findings also highlight that maternal static touch, but not dynamic touch, had an impact on infants' negative emotionality across FFSF in VPT infants. How could we interpret this specificity? Could this finding be associated with touch experiences that preterm infants experienced in NICU? Preterm infants during NICU-stay receive mainly two tactile-kinesthetic stimulations: a) procedural and dynamic touch during standard daily care (e.g., diaper change, repositioning, etc.), medical and/or nursing procedures, and b) soothing touch, such as still touch without stroking or massage, skin-to-skin contact, kangaroo mother care, administered in order to reduce stress during painful procedures (e.g., heel lance, see Gursul et al., 2018) and/or to promote infant's well-being (Conde et al., 2016). Clinical studies have found that in preterm infants some procedural touch can be unpleasant and/or overstimulating, with potentially negative impact

on an infant's physiologic stability and behavioral responses (Harrison et al., 2000). Consequently, in order to minimize these undesirable effects, some NICUs have adopted a minimal handling/touch approach. Importantly, while physiological and/or behavioral stress responses increase significantly even when preterm infants are handled during standard nursing caregiving such as diaper change (Holsti et al., 2005; Holsti et al., 2006; Zeiner et al., 2016), comforting static touch may have soothing neurophysiological effects suggesting several benefits of this kind of touch on fragile VPT infants (Herrington & Chiodo, 2014; Smith, 2012). For example, facilitated tucking, a kind of static touch, has been shown to be effective in relieving procedural pain in VPT infants (Axelin et al., 2009; Gursul et al., 2018). Thus, during routine nursing and medical interventions in NICU, a static touch is effective in promoting a calm response by increasing parasympathetic activity (i.e., vagal activity; Field et al., 2006). Therefore, we speculate that physiologically fragile premature infants, such as those involved in the present study, may benefit from static touch when they face stressful procedures (Harrison et al., 2000).

Animal studies suggest that there is interplay between the HPA axis function and the serotonergic system. In this context, the serotonergic system has been identified as a one of the systems involved in developmental programming of the HPA axis (Andrews & Matthews, 2004). Exposure to stress during the NICU stay increases methylation of the *SLC6A4* which may have functional consequences, possibly reflecting variations in serotonin transporter expression and altering regional serotonin reuptake. In the developing brain, this serotonergic tone deficit might lead to a permanent modification of glucocorticoid receptor expression in the hippocampus. Thus, considering the serotonergic regulation of glucocorticoid receptor expression in hippocampal neurons, this model suggests a mechanism whereby early life events might predispose preterm infants to vulnerability to stress during infancy. Thus, going back to our results, maternal static touch during an interactive episode (Play) could recall the soothing touch experienced by these infants in NICU, which could be more effective in sustaining the infant's capacity to regulate socio-emotional stress.

The present study has some limitations. First, not having performed a power analysis and due to the small sample size, the robustness of the results and the possibility to test additional contributing factors (e.g., infants' sex) are limited. Future studies in this field should therefore include a proper power analysis and a larger number of participants in order to provide more generalizable data. Second, having no data regarding the quantity and quality of early touch experiences during NICU stay, we can only speculate about the role of early experiences in the perception of maternal touch at 3-months of age. Research in the field should collect this kind of data in order to test this hypothesis. Third, as we did not collect data about pharmacological sedation, we were not able to control for a potentially important clinical factor such as opiate exposure which may represent a risk factor for behavior outcomes in preterm infants (Steinbauer e al., 2021). Incidentally, protracted sedation is usually associated with severe clinical factors such as need for surgery, necrotizing enterocolitis, severe respiratory failure, which did not met inclusion criteria adopted in our study. Thus, although we are not able to rule out a potential role of sedation, it is reasonable to assume that it could have had a very limited impact on our findings. Fourth, unlike in non-human animal studies, DNA methylation markers in humans can only be tested in peripheral tissues, as access to brain tissue is limited to postmortem samples. Moreover, *SLC6A4* methylation has been obtained from two different peripheral tissues: cord-blood at birth and peripheral blood at discharge. As a result, the difference in CpG methylation could just be due to differences between tissues. Nonetheless, recent findings suggest, first, that cord blood methylation is maintained in peripheral blood cells during childhood and second, that peripheral methylation levels correlate with the those measured centrally (Agha et al., 2016; Braun et al., 2019). Fifth, one may wonder that differences in the methylation level would be related to the passage of time rather than to NICU related experiences. Sixth, considering the prospective nature of our study, we cannot exclude that *SLC6A4* methylation status might have been changed in post-discharge period, that is before the mother-infant interaction observation at 3-months. Therefore, future studies are warranted to employ a research design that includes different time points of DNA methylation

assessment in order to study the trend of epigenetic changes and its stability over time. Seventh, leukocytes consist of a mixture of different cell types. As we did not perform any immunologic analysis to ascertain the white blood cell distribution in our peripheral blood samples, we are unable to correct our results for cell content. Lastly, while the focus of this study is *SLC6A4* methylation, it is important to note that the serotoninergic system is just one of many systems affected by early adverse experiences. For example, there is a growing literature demonstrating the impact of early caregiving on the epigenetic modification of the glucocorticoid receptor gene in offspring (Murgatroyd et al, 2015; Conradt et al., 2019; Lester et al., 2018). Besides, it should pointed out that maternal touch is strongly associated with the oxytocin system, which is crucially involved in adult and infant brain responses to social information (Maud et al., 2018). Therefore, future work focused on DNA methylation of social affiliative behavior candidate genes, such as *OXTR*, would further elucidate the role of maternal touch on infants' epigenetics.

Conclusions

The present study provides preliminary evidence that low levels of maternal static touch can intensify the negative effects of *SLC6A4* epigenetic changes on stress-responses in 3-months-old VPT infants. Our findings could have substantial implications for understanding the role of tactile stimulation in NICU setting, such as touch-based interventions to alleviate pain and stress in preterm infants. This finding provides further evidence that during routine nursing and medical interventions gentle, holding touch would be preferable to dynamic touch in very fragile preterm infants during their stay in NICU. It could also be useful for supporting parenting programs. Indeed, mothers of preterm infants who took part in an early parental intervention in NICU (i.e., Family Nurture Intervention, PremieStart) showed not only a greater amount of touch, but particularly static, calming touch during face-to-face interaction with their premature infants at 4-months CA (Beebe et al., 2018). In sum, our findings indirectly suggest that touch may play a protective role against the risk of long-lasting programming of an altered stress response involving epigenetic

582 mechanisms associated with the serotoninergic system. This leads to the fascinating perspective that
583 a specific approach to NICU-related care might offer an "epigenetic protection" to the
584 neurobehavioral and socio-emotional development of preterm infants (Montirosso et al., 2021).
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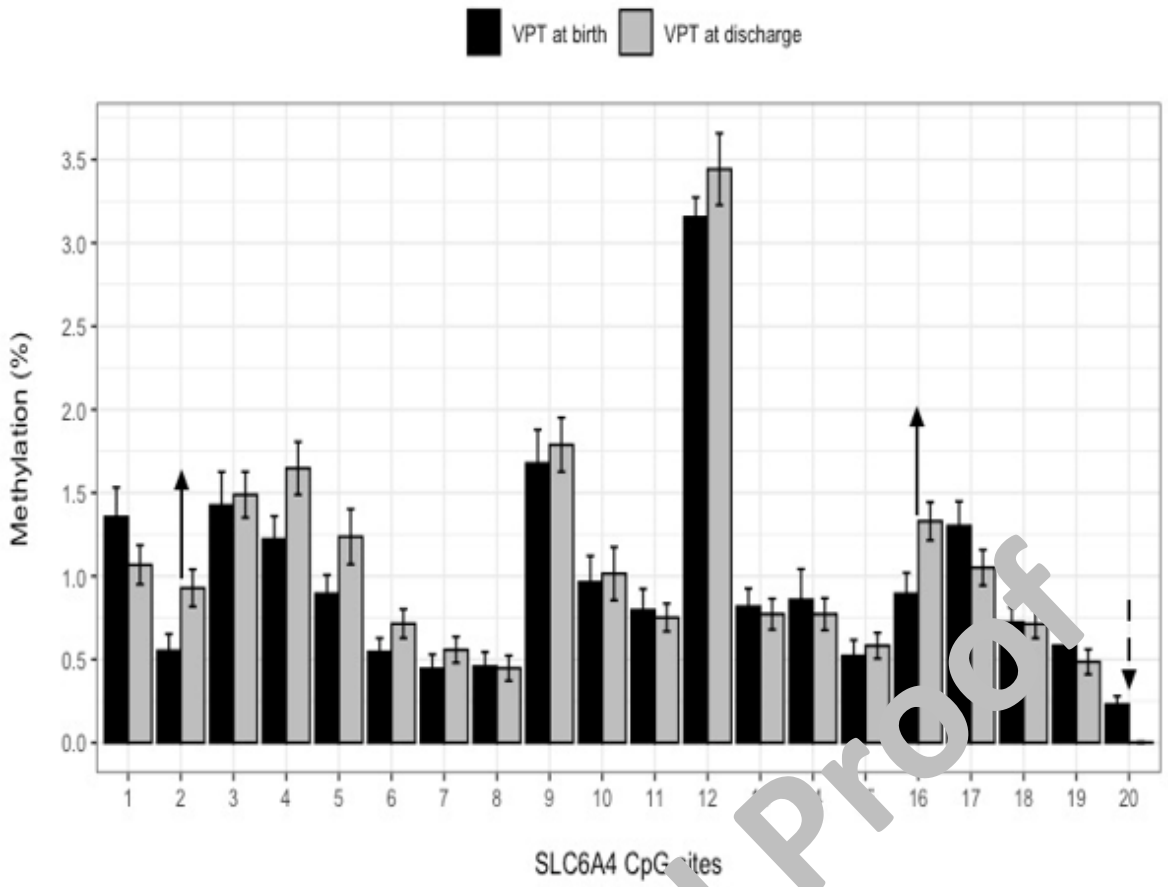
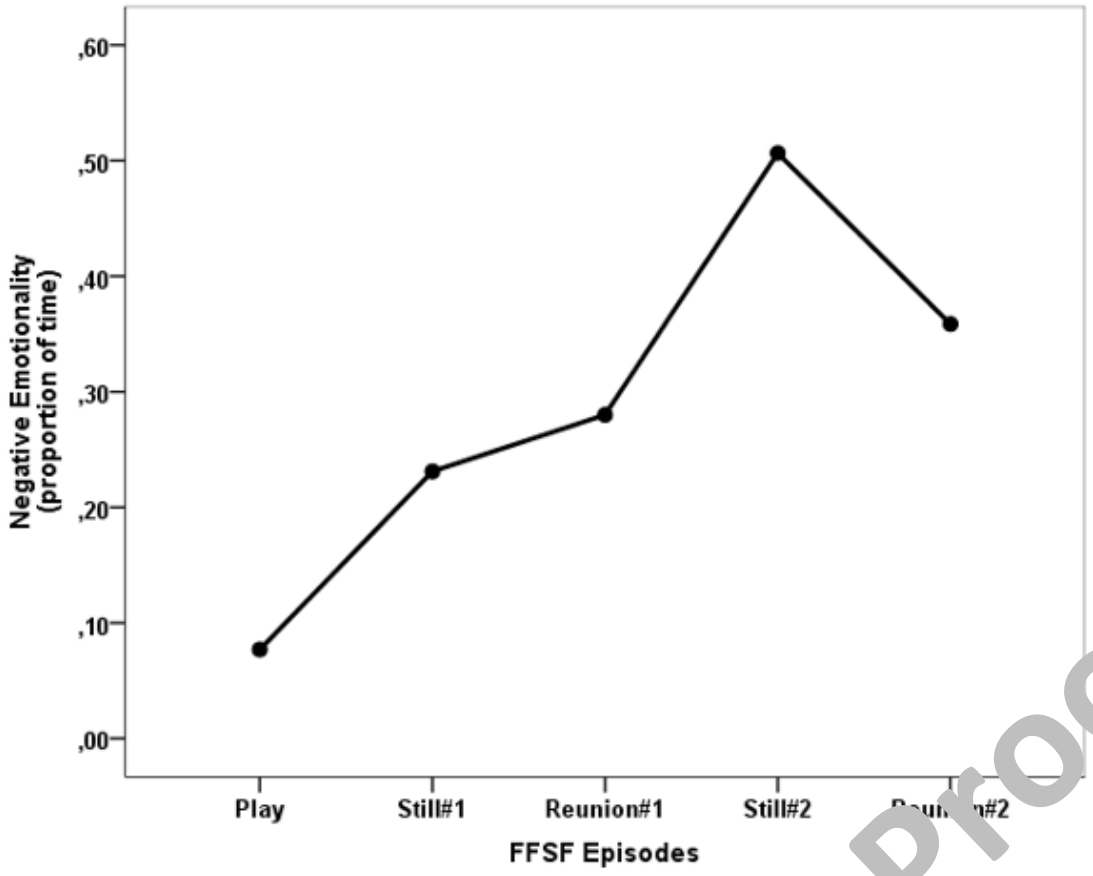
Figure 1. Mean methylation percentages of each of 20 Cytosine-phosphate-Guanine (CpG) dinucleotides sites within the *SLC6A4* promoter region at birth and at NICU-discharge VPT (*n* = 29) infants. Black arrows represent significantly increased methylation level while dashed arrow represents significantly decreased methylation level in VPT infants between birth and discharge.

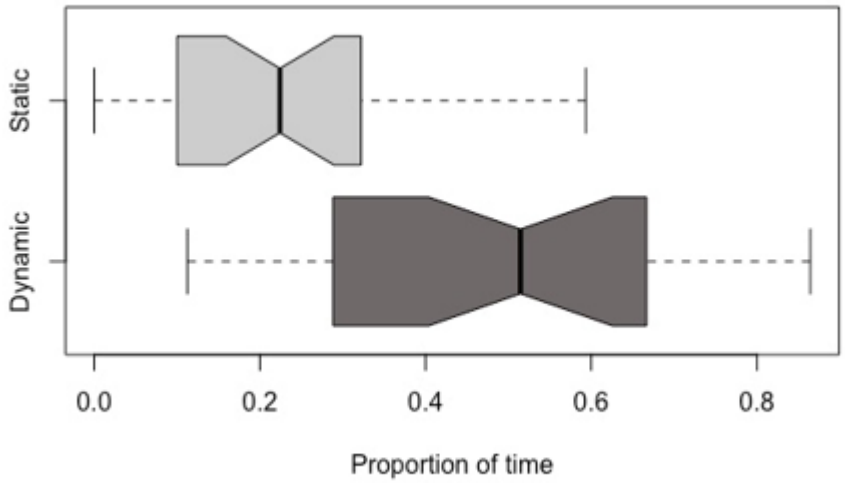


Note: *CpG, Cytosine-phosphate-Guanine dinucleotides; VPT = very preterm.*

Figure 2. Means of negative emotionality through the Face-to-Face Still (FFSF) paradigm in very preterm infants (n = 29).



820 *Figure 3*. Distribution of dynamic and static maternal touch in very preterm infants (*n* = 29).

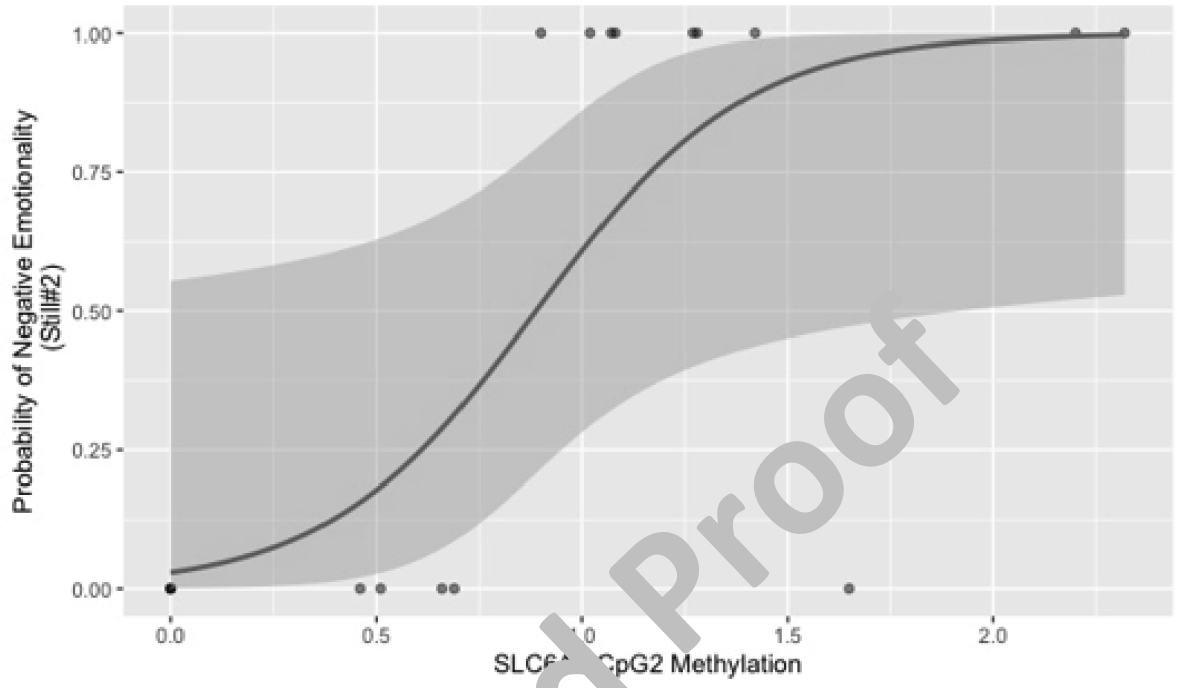


821

822 Note: *Boxes represent data distribution with interquartile range and horizontal black lines as the*
823 *median.*

Figure 4a. and *b.* Association between *SLC6A4* methylation level and infant's negative emotionality during Still#2 (a) and Reunion#2 (b) for low level of maternal static touch (n = 17). Dark grey line represents the logistic regression curve showing probability of display negative emotionality versus CpG2 *SLC6A4* methylation percentage. Light grey area represents the Confidence Interval.

a.



b.

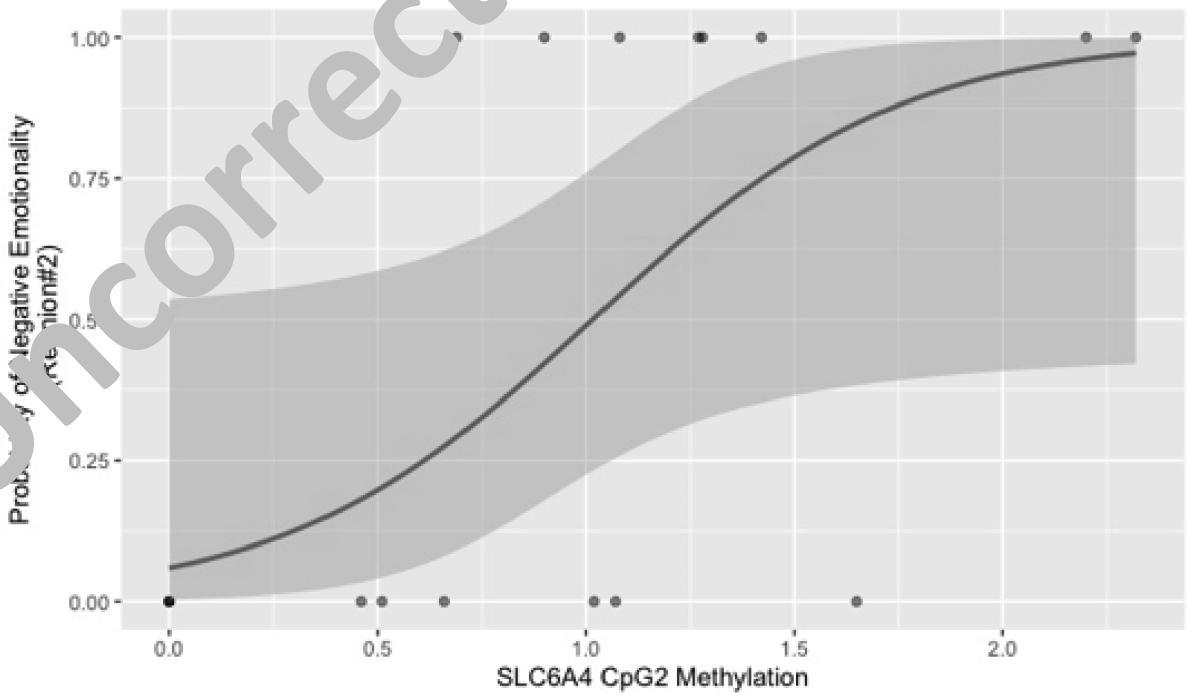


Figure 5a. and *b.* The interactive effect of CpG2 *SLC6A4* methylation and low level of maternal static touch on infants' negative emotionality during Still#2 (a) and Reunion#2 (b). Both the size and color of the circles indicate different levels of maternal static touch. Larger circles and lighter shade of gray indicate higher levels of maternal static touch. Smaller circles and darker shade of gray indicate lower levels of maternal static touch.

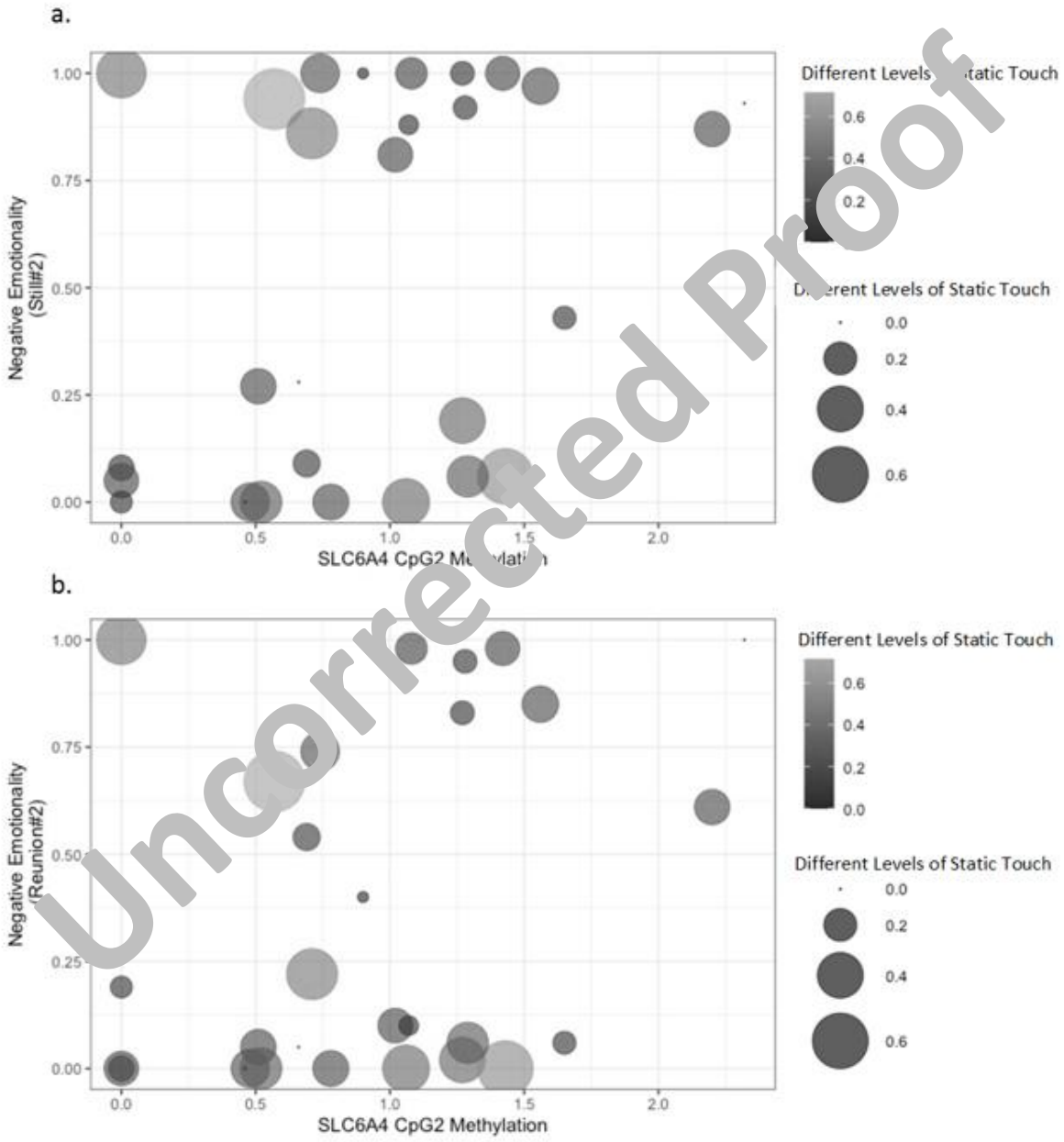


Table 1. Descriptive statistics of the sample.

	VPT infants (N = 29, Female = 16)	
	Mean	SD
Infant perinatal variables		
Gestational age (weeks)	30.86	1.84
Birth weight (grams)	1477.06	350.65
NICU-related variables		
Number of Skin-breaking procedures[#]	14.22	14.07
Length of NICU-stay[##]	42.48	20.15
Days of Mechanical Ventilation[###]	11.28	13.78
Socio-demographic characteristics		
Maternal age (years)	36.24	4.61
Maternal Education (years)	15.72	2.40
Family SES	60.00	18.65
Maternal emotional state		
STAI-Y state score	29.64	6.70
STAI trait score	35.50	6.13
BDI score	7.20	4.58

Note: VPT = very preterm; [#]Median = 7; range = 1-50*;* [##]Median = 38; range = 20-102; [###]Median = 6; range = 1-55;*SES = socioeconomic status assessed via the Hollingshead (Hollingshead, 1978); STAI-Y = State-Trait Anxiety Inventory-form Y; BDI = Beck Depression Inventory.*

Table2. Multivariate Logistic Regressions Analysis.

Predictors	χ^2	χ^2 Hosmer-Lemeshow	R^2 Nagelkerke	*B*	*Wald*	*OR (95%CI)*
Model 1	1.187	8.775	0.058			
CpG2				1.254	0.309	1.77 (0.44; 7.13)
Static Touch				3.107	0.525	0.77 (0.01; 79.31)
GA				0.061	0.803	0.95 (0.61; 1.49)
CpG2* Static Touch				-3.455	0.470	-
Model 2	7.679	3.095	0.317			
CpG2				3.579	0.065	1.82 (0.49; 6.72)
Static Touch				8.170	0.167	0.05 (0; 6.59)
GA				0.034	0.907	0.94 (0.62; 1.42)
CpG2* Static Touch				-14.838	0.062	-
Model 3	16.889**	7.192	0.589			
CpG2				8.547*	0.020	4.18 (0.91; 19.19)
Static Touch				26.959	0.065	1.33 (0.02; 81.24)
GA				0.538	0.263	1.04 (0.7; 1.56)
CpG2* Static Touch				-28.870*	0.049	-
Model 4	13.271**	5.059	0.495			
CpG2				6.060*	0.012	3.68 (0.84; 16.21)
Static Touch				16.647*	0.032	0.57 (0.01; 38.79)
GA				0.487	0.216	1.07 (0.71; 1.62)
CpG2* Static Touch				-19.537*	0.027	-

*Note. Regression coefficients are reported with level of significance: *, p < .05; **, p < .01; CpG2, Cytosine-phosphate-Guanine dinucleotides 2 methylation level; Static Touch, Maternal static touch; GA, gestational age.*