


RESEARCH ARTICLE

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

Isabella Lucia Chiara Mariani Wigley¹ | Eleonora Mascheroni²  | Camilla Fontana³ | Roberto Giorda⁴ | Francesco Morandi⁵ | Sabrina Bonichini¹ | Francis McGlone^{6,7} | Monica Fumagalli^{3,8} | Rosario Montirosso²

¹ Department of Developmental and Social Psychology, University of Padua, Padua, Italy

² 0–3 Center for the at-Risk Infant, Scientific Institute IRCCS “Eugenio Medea”, Bosisio Parini, Lecco, Italy

³ Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico, NICU, Milan, Italy

⁴ Molecular Biology Laboratory, Scientific Institute IRCCS “Eugenio Medea”, Lecco, Italy

⁵ Pediatric Unit, Ospedale San Leopoldo Mandic, Merate Lecco, Italy

⁶ School of Natural Sciences and Psychology, Liverpool John Moores University, Liverpool, UK

⁷ Institute of Psychology Health & Society, University of Liverpool, Liverpool, UK

⁸ Department of Clinical Sciences and Community Health, University of Milan, Milan, Italy

Correspondence

Eleonora Mascheroni, 0–3 Center for the at-Risk Infant, Scientific Institute IRCCS “Eugenio Medea”, Bosisio Parini (Lecco), Italy.
Email:

eleonora.mascheroni@lanostrafamiglia.it

Isabella Lucia Chiara Mariani Wigley and Eleonora Mascheroni contributed equally as first authors to this study.

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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 that 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 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-month-old VPT infants.

KEYWORDS

DNA methylation, maternal touch, negative emotionality, serotonin transporter gene, *SLC6A4*, stress response, very preterm infants

1 | 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 socioemotional stress development later in life (Provenzi, Giusti, et al., 2016). 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, Provenzi, Fumagalli, et al., 2016; Provenzi, Fumagalli, et al., 2020). Research on human infants indicates that postnatal maternal touch may buffer the early epigenetic effects of less-than optimal caregiving (Murgatroyd et al., 2015). Although most studies focused on *NR3C1* methylation (a candidate gene related to stress response that codes for glucocorticoid receptor; Conradt et al., 2019; Lester et al., 2018), the association between maternal touch and *SLC6A4* DNA methylation remains unexplored. This 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.

1.1 | Epigenetic variations associated with serotonergic system

The serotonergic system plays a key role in regulating HPA stress reactivity and its negative feedback (Lanfumeijer 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 socioemotional dysregulation and stress susceptibility (Heils et al., 1995). However, the 5-HTTLPR polymorphic variant accounts only partially for differences in socioemotional stress response (Aleknaviciute et al., 2018). 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 mammals, methylation at the fifth 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). Although 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, Giorda, et al., 2016). 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, Giorda, et al., 2016).

1.2 | *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, Giusti, et al., 2016). 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, Fumagalli, et al., 2020). 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 socioemotional regulation throughout infancy and childhood.

1.3 | 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, socioemotional development, and infant stress response. For example, immediate postnatal tactile stimulation and physical contact reduce newborns' crying and distress and support newborn adaptation 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 breastfeeding, considered a proxy of physical contact during the first 5 months of life, showed increased *NR3C1* DNA methylation (Lester et al., 2018). In 5-month-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 et al. 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 nonstress-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 that 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 et al. 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).

1.4 | 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 fur-

ther analysis when: (1) methylation status was significantly changed from birth-to-discharge, (2) *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 versus 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.

2 | METHODS

2.1 | Participants

This study is a post hoc analysis of a larger longitudinal research project that included 32 VPT infants (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, Provenzi, Fumagalli, et al., 2016; Montirosso, Provenzi, Giorda, et al., 2016). Although data in this paper are derived from previously published studies (Montirosso, Provenzi, Fumagalli, et al., 2016; Montirosso, Provenzi, Giorda, et al., 2016; 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.

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 ^a	14.22	14.07
Length of NICU stay ^b	42.48	20.15
Days of mechanical ventilation ^c	11.28	13.78
Sociodemographic 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

^aMedian = 7; range = 1–50.

^bMedian = 38; range = 20–102.

^cMedian = 6; range = 1–55;

Abbreviations: BDI, Beck Depression Inventory; SES, socioeconomic status assessed via the Hollingshead (Hollingshead, 1978); STAI-Y, State-Trait Anxiety Inventory-form Y; VPT, very preterm.

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.

2.2 | 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 corrected age (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 S2 in the Supporting Information 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 socioemotional 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; Montiroso, Provenzi, Giorda, et al., 2016). 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 0.4 m from the infant and adjusted so that her eyes were in 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, 2020; 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.

2.3 | Measures

2.3.1 | Perinatal variables and sociodemographic characteristics

Perinatal variables of VPT infants included GA, birth weight, sex length of NICU stay, and invasive mechanical ventilation (i.e., conventional ventilation and high frequency ventilation). Sociodemographic 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 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).

2.3.2 | NICU pain-related stress

NICU pain-related stress was quantified according to 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.

TABLE 2 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: * $p < .05$, ** $p < .01$. Abbreviations: CpG2, Cytosine–phosphate–Guanine dinucleotides 2 methylation level; GA, gestational age; static touch, maternal static touch.

2.3.3 | 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 = *minimal range*, 14–19 = *mild*, 20–28 = *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 a 4-point scale where higher scores indicate 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.

2.3.4 | SLC6A4 methylation

We analyzed a CpG-rich region of the SLC6A4 promoter (chr17:28562750–28562958, Human hg19 Assembly; see Figure S1 in

in the Supporting Information), between −69 and −213 relative to the transcriptional start site, which contains 20 CpG sites and is adjacent to exon 1A (see Table S1 in the Supporting Information for the specific position of each CpG site). DNA methylation was determined on blood leucocytes using bisulfite modification followed by PCR amplification and next generation sequencing. Procedures for DNA methylation quantification are reported in detail in a previous publication from our group (Provenzi et al., 2015). Only methylation levels at CpG sites that have been found to be significantly different between birth to discharge and significantly associated with NICU pain-related stress were included in the analysis (see below).

2.3.5 | Maternal touch

To capture the main two types of tactile–kinesthetic stimulations (static vs. dynamic) used by mothers with their infants during early mother–infant exchanges (Weiss et al., 2004), we coded maternal touch according to a coding system developed on the basis of well-validated instruments (Provenzi, Rosa, et al., 2020). We coded the amount of dynamic and static touch provided by mothers during the FFSF Play episode. Dynamic touch included affectionate tactile stimulations (e.g., stroking, caressing, massaging), playful touch (e.g., tickling, shaking, squeezing, lifting, moving, or flexing the infant's body) and

tactile stimulations aimed at getting infant's attention (e.g., tapping, patting, squeezing, and pinching). Static touch included light to moderate pressure touch provided to the infant, aimed to maintain physical contact (e.g., holding). Maternal dynamic and static touch were analyzed in each 2-s segment using ELAN. Nonetheless, coders were blind to the aims and hypotheses of the study. The coders were trained with the 25% of videotapes randomly chosen from the study database, obtaining an interrater agreement of Cohen's $\kappa = .80$.

2.3.6 | Infant's negative emotionality during the FFSF paradigm

For each of the five episodes of FFSF, infant's negative emotionality was coded second-by-second by two trained coders and defined as withdrawn, protesting, complaining, being fussy or crying behaviors. Coders had to detect the presence or the absence of negative emotionality-related behaviors for each of the second-by-second time windows. After that, a proportion index of negative emotionality was obtained for each of the five episodes of FFSF. Each index was obtained by dividing the total score of negative emotionality displayed in every FFSF episode for the actual 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 interrater agreement of Cohen's $\kappa = .86$.

2.4 | 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, epiDisplay (Chongsuvivatwong, 2018) package was used to obtain OR and performed Wald's test, rcompanion (Mangiafico, 2021) and ResourceSelection (Lele et al., 2019) packages were used to perform 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 sociodemographic variables between preterm infants included in this study and preterm infants included in previous work but excluded from this one (either because they did not complete the entire still face (SF) procedure or because it was impossible to code maternal touch). Data analysis was carried out by following different steps.

2.4.1 | Preliminary analyses

As the sample included here was slightly different from the original one, we have reanalyzed the data in order to: (1) check if methyla-

tion levels varied between birth and discharge in VPT infants and, (2) test if these changes were linked to pain-related procedures during NICU stay, as highlighted in previous work (Montirosso, Provenzi, Fumagalli, et al., 2016; Montirosso, Provenzi, Giorda, et al., 2016; 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 analysis of variance (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.

2.4.2 | Maternal touch, *SLC6A4* methylation, and infant's negative emotionality

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: (1) *SLC6A4* DNA methylation at discharge; (2) maternal dynamic and static touch during the Play episode separately; (3) the interaction between CpG-specific *SLC6A4* methylation and maternal touch (dynamic or static). Infants' GA 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 (I.L.C.M.W.).

3 | RESULTS

3.1 | Preliminary results

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

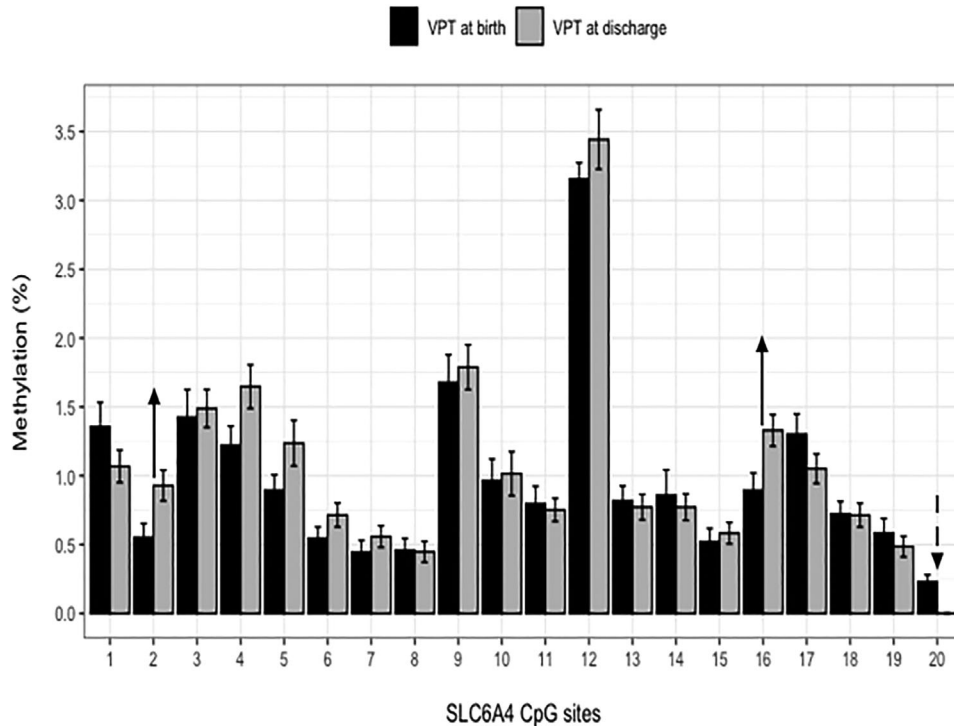


FIGURE 1 Mean methylation percentages of each of 20 Cytosine–phosphate–Guanine (CpG) dinucleotides sites within the *SLC6A4* promoter region at birth and at NICU-discharge VPT ($n = 29$) infants

Note: Black arrows represent significantly increased methylation level while dashed arrow represents significantly decreased methylation level in VPT infants between birth and discharge. Abbreviations: CpG, Cytosine–phosphate–Guanine dinucleotides; VPT, very preterm.

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 S2 in the Supporting Information).

3.2 | Epigenetics data

3.2.1 | *SLC6A4* methylation from birth to discharge in VPT

In accordance with our previous findings (Montirosso, Provenzi, Giorda, et al., 2016), *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, $t(28) = 4.641$, $p < .001$ (Figure 1). As 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, Provenzi, Giorda, et al., 2016), 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 nonsignificant 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.

3.3 | 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 (Figure 2), $t(28) = 4.62$, $p < .001$.

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.

3.4 | 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

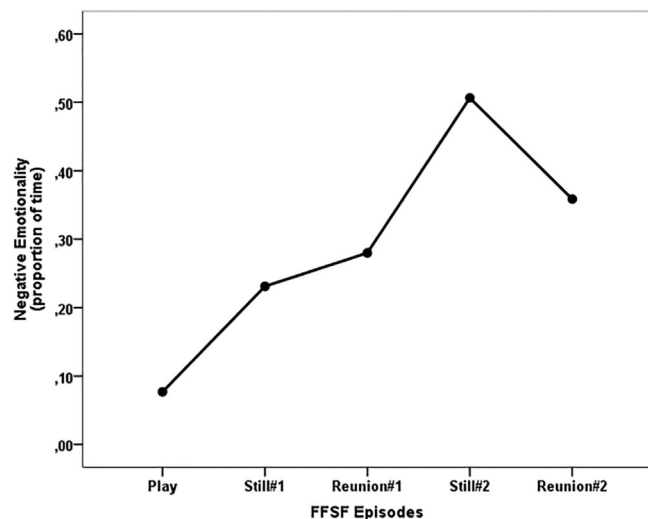


FIGURE 2 Means of negative emotionality through the Face-to-Face Still (FFSF) paradigm in very preterm infants ($n = 29$)

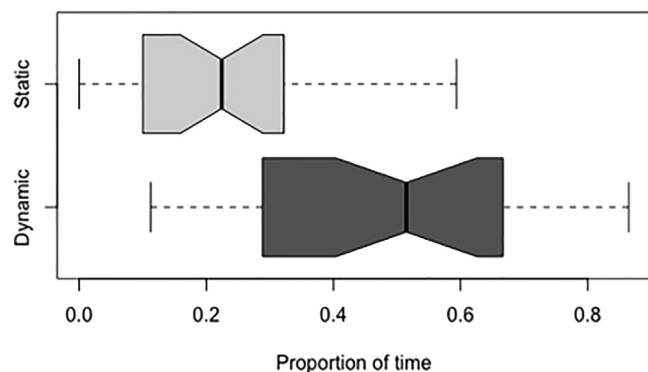


FIGURE 3 Distribution of dynamic and static maternal touch in very preterm infants ($n = 29$)

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

emotionality during Still#1 and methylation level of *SLC6A4* CpG2, maternal static touch assessed during the Play episode of FFSF, infants' GA, 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' GA, and the interaction between *SLC6A4* CpG2 methylation and maternal static touch. Results showed that the change in deviance was not significant in either first or 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' GA, 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 Figures 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' GA 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 infants' 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 Figures 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 [$\chi^2(1, N = 17) = 5.256, p = .729$].

No regression models with maternal dynamic touch as predictor was significant and coefficients are reported in Table S3 in the Supporting Information.

4 | DISCUSSION

The aim of this 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 this 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, Provenzi, Fumagalli, et al., 2016; Montirosso, Provenzi, Giorda,

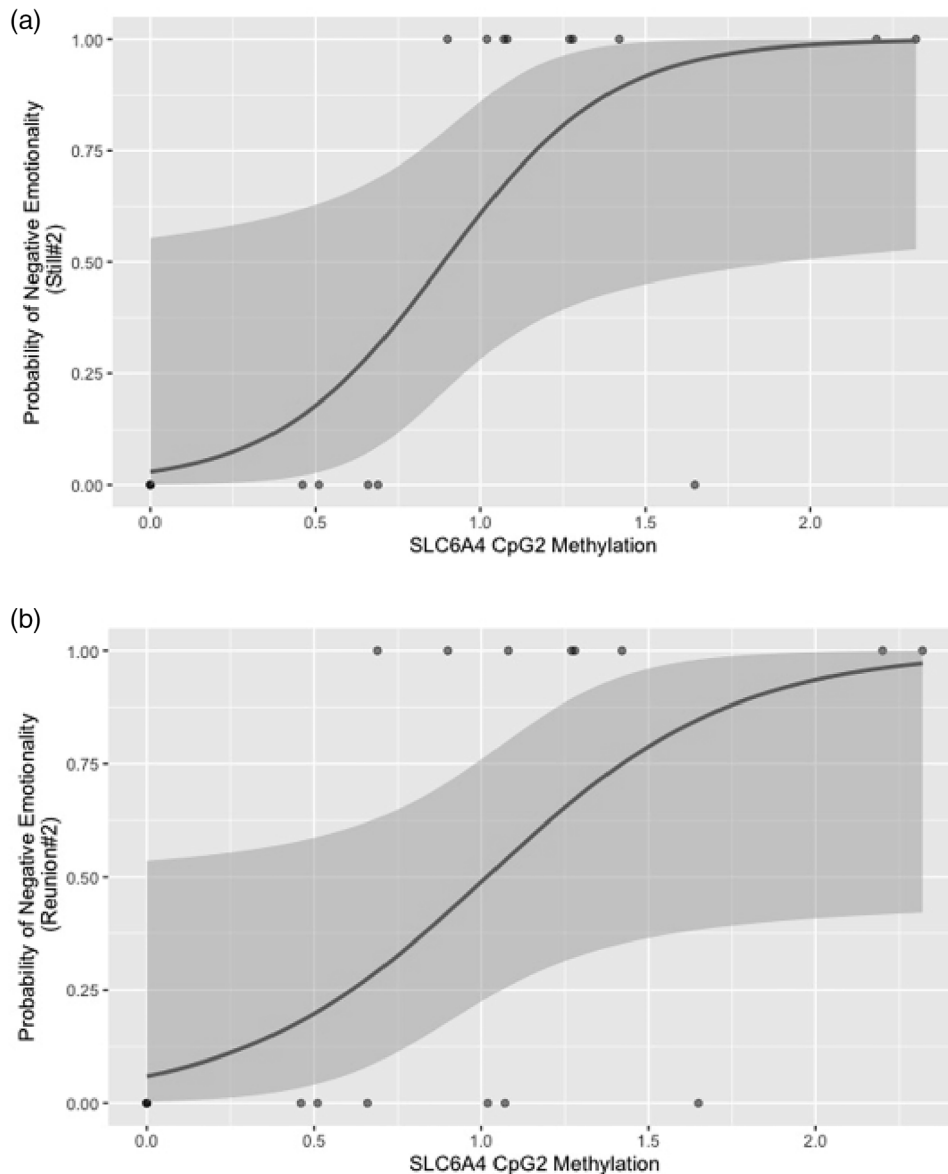


FIGURE 4 Association between *SLC6A4* methylation level and infant's negative emotionality during Still#2 (a) and Reunion#2 (b) for low level of maternal static touch ($n = 17$)

Note: Dark gray line represents the logistic regression curve showing probability of display negative emotionality versus CpG2 *SLC6A4* methylation percentage. Light gray area represents the confidence interval.

et al., 2016; 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, Provenzi, Giorda, et al., 2016). 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, Provenzi, Giorda, et al., 2016; Provenzi, Fumagalli, et al., 2020).

Moreover, VPT infant's DNA methylation of *SLC6A4* CpG2 and maternal static touch during the normal interactive episode of FFSE (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, Conrads et al. (2019) showed that maternal responsiveness/appropriate touch were related

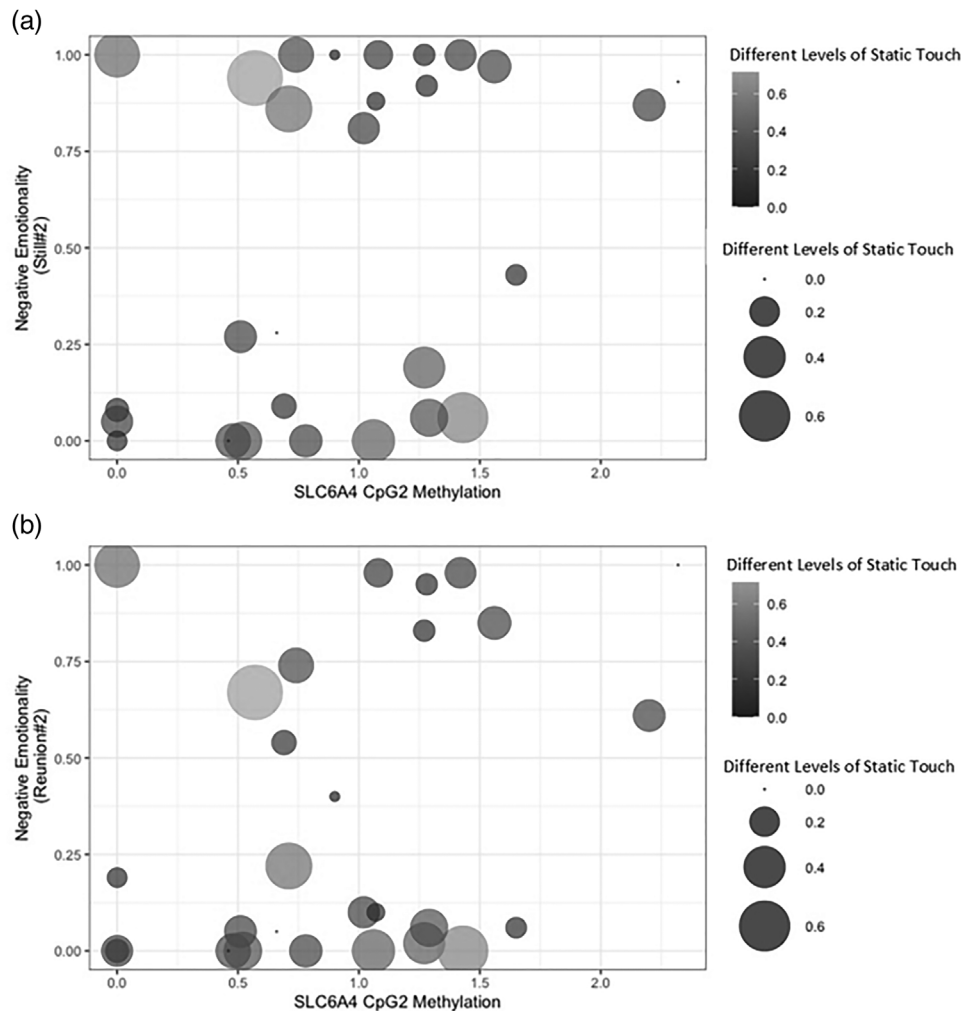


FIGURE 5 The interactive effect of CpG2 *SLC6A4* methylation and low level of maternal static touch on infants' negative emotionality during Still#2 (a) and Reunion#2 (b)

Note: 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.

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: (1) procedural and dynamic touch during standard daily care (e.g., diaper change, repositioning, etc.), medical and/or nursing procedures, and

(2) 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-Agudelo & Díaz-Rossello, 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 this 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 socioemotional stress.

This 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 expo-

sure that may represent a risk factor for behavior outcomes in preterm infants (Steinbauer et 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 meet 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 nonhuman 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. Finally, while the focus of this study is *SLC6A4* methylation, it is important to note that the serotonergic 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 point 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.

5 | CONCLUSIONS

This 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-month-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-month 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 mechanisms associated with the serotonergic system. This leads to the fascinating perspective that a specific approach to NICU-related care might offer an “epigenetic protection” to the neurobehavioral and socioemotional development of preterm infants (Montirosso & Provenzi, 2015; Montirosso et al., 2021).

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

Isabella Lucia Chiara Mariani Wigley, Eleonora Mascheroni, and Roberto Giorda contributed to data analysis, interpretation of findings, and final approval of manuscript. Francesco Morandi, Camilla Fontana, and Monica Fumagalli contributed to data acquisition. Isabella Lucia Chiara Mariani Wigley and Eleonora Mascheroni contributed to study conception and approved the final version of the manuscript. Francis McGlone, Monica Fumagalli, Roberto Giorda, and Sabrina Bonichini contributed to interpretation of findings, drafting of the manuscript, and approval of the final version. Rosario Montirosso contributed to study conception and design, interpretation of findings, drafting of the manuscript, and approval of the final version.

ORCID

Eleonora Mascheroni  <https://orcid.org/0000-0001-8248-7550>

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