Pain-related increase in serotonin transporter gene methylation associates with emotional regulation in 4.5-year-old preterm-born children

Livio Provenzi, PhD[1], Monica Fumagalli, MD[2,5],Giunia Scotto di Minico, PsyD[1], Roberto Giorda, PhD[3], Francesco Morandi, MD[4], Ida Sirgiovanni, MD [2], Paola Schiavolin, MD [2], Fabio Mosca, MD[2,5], Renato Borgatti, MD[6], Rosario Montirosso, PsyD[1]

[1] Scientific Institute IRCCS E. Medea, 0-3 Center for the at-Risk Infant, Bosisio Parini, Lecco, Italy

[2] Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico

[3] Scientific Institute IRCCS E. Medea, Molecular Biology Lab, Bosisio Parini, Lecco, Italy

[4] Pediatric Unit, Sacra Famiglia Hospital, Erba, Como, Italy

[5] University of Milan, Department of Clinical Sciences and Community Health, Milan, Italy

[6] Scientific Institute IRCCS E. Medea, Neuropsychiatry and Neurorehabilitation Unit, Bosisio Parini, Lecco, Italy

Short Title: *SLC6A4* methylation and preterm children emotion regulation

Corresponding Author: Rosario Montirosso, Scientific Institute IRCCS E. Medea, 0-3 Center for the at-Risk Infant, via Don Luigi Monza 20, 23842 Bosisio Parini, Lecco, Italy. Tel: +39-031-877464. E-mail: rosario.montirosso@lanostrafamiglia.it.

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ABSTRACT

Aim. The main goal of this study was to assess the association between pain-related increase in serotonin transporter gene (*SLC6A4*) methylation and emotional dysregulation in 4.5-year-old preterm children compared to full-term matched counterparts.

Methods. Preterm (n=29) and full-term (n=26) children recruited from two Italian hospitals were followed-up from October 2011 to December 2017. *SLC6A4* methylation was assessed from cord blood at birth from both groups and peripheral blood at discharge for preterm ones. At 4.5 years, emotional regulation (i.e., anger, fear, sadness) was assessed through an observational standardized procedure.

Results. Preterm children (18 females; mean age = 4.5, range = 4.3 – 4.8) showed greater anger display compared to full-term controls (14 females; mean age = 4.5, range = 4.4 – 4.9) in response to emotional stress. Controlling for adverse life events occurrence from discharge to 4.5 years and *SLC6A4* methylation at birth, CpG-specific *SLC6A4* methylation in the neonatal period was predictive of greater anger display in preterm children but not in full-term ones.

Conclusion. These findings contribute to highlight how epigenetic regulation of serotonin transporter gene in response to NICU pain exposure contributes to long-lasting programming of anger regulation in preterm children.

KEYWORDS: methylation, pain, preterm birth, serotonin, emotion regulation

KEY POINTS

- Early exposure to adverse events may contribute to the behavioural phenotype of preterm infants *via* epigenetic mechanisms.
- Pain-related increase in serotonin transporter gene methylation associated with increased anger response to emotional stress at 4.5 years of age in preterm children.
- These findings highlight the importance of limiting early pain exposure in preterm infants to reduce the risk of later emotional dysregulation.

INTRODUCTION

Preterm birth accounts for 11% of all livebirths worldwide[1] and it represents the leading cause of mortality and morbidity in human infants and children.[2] Preterm infants need long-lasting specialized care in the neonatal intensive care unit (NICU), which exposes their immature brain to different sources of stress.[3] NICU life-saving procedures still entail invasive and painful stimulations, such as skin-breaking procedures, and NICU-related stress has been associated with emotional and neuroendocrine disruptions later in life.[4–7] Emotional dysregulation during early childhood is one of the most reported detrimental outcomes of preterm birth and it is sensitive to early exposure to NICU-related stress and pain.[8,9]

The serotonin system is a key regulator of emotional development and stress regulation[10] and the *SLC6A4* gene encoding for the serotonin transporter is known to be sensitive to epigenetic regulation through changes in DNA methylation in response to environmental exposures.[11] In preterm infants, early exposure to pain-related stress during NICU stay is linked with increased methylation of the *SLC6A4* gene from birth to discharge, compared to full-term controls.[12] Additionally, controlling for neonatal and clinical confounders, increased *SLC6A4* methylation was found to be predictive of behavioural development and emotional regulation in preterm infants at three months corrected age.[6,13] These findings suggest that gene-specific epigenetic regulation might play a key role in mediating the effect of early NICU pain-related stress exposure on preterm infants' outcomes in emotion regulation. Nonetheless, longitudinal data on emotional regulation in children born preterm are lacking. Prospective studies assessing the long-term consequences of stress-linked epigenetic regulation in preterm infants are warranted for both researchers and clinicians to comprehend the impact of early intensive care procedures on at-risk infants and to promote the necessary evidence-based support for targeted prevention and interventions.[14–16]

In the present study, we followed-up preterm infants from birth to 4.5 years and we assessed their emotional regulation during an observational laboratory procedure (Pre-schooler Regulation of

Emotional Stress; PRES[17]), in comparison to full-term age-matched controls. Based on previous research[6,13], we hypothesized that increased methylation related to early pain exposure would result in altered emotional regulation profile in preterm children compared to full-term counterparts. As DNA methylation is susceptible to additional environmental exposures later in life,[18,19] we controlled for cumulative exposure to adversities that might have occurred from birth to 4.5 years of age.

Patients and methods

This was a follow-up study (Figure 1) of a longitudinal cohort of 56 preterm and 32 full-term infants.[12] Preterm infants were enrolled from the NICU of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy and they had gestational age lower than or equal to 32 weeks and/or birth weight lower than or equal to 1500 grams. Full-term infants were enrolled at the Pediatric Unit of Sacra Famiglia Hospital, Erba, Como, Italy and they had gestational age higher than or equal to 37 weeks and birth weight higher than or equal to 2500 grams. In both groups, infants with major brain lesions, neuro-sensorial deficits, genetic syndromes, and major malformations were excluded. Specific exclusion criteria were: major brain lesions as documented by cerebral ultra-sound (intraventricular haemorrhage > grade 2 according to Papile, cystic periventricular); neuro-sensorial deficits (retinopathy of prematurity ≥ stage2); any genetic syndromes and/or major malformations. The mothers in both groups were at least 18-year-old, with a good comprehension of Italian language, no mental retardation, and no reported psychiatric disorder. Single-parent families were not included in the study. In the original cohort, mothers of eligible infants were enrolled from October 1st, 2011 to April, 30th 2014. For the follow-up study, mothers were re-contacted from June, 1st 2016 to October, 31st 2018, when children were approximately 4.5 years of age. All mothers provided informed consent and the study was approved by the Ethics Committees of the Scientific Institute, IRCCS Eugenio Medea, Bosisio Parini (Lecco, Italy) and of the participating hospitals.

Please, insert Figure 1 here

Epigenetic procedures and measures

Cord blood samples were obtained at birth for preterm and full-term infants, whereas peripheral blood was collected at hospital discharge for the preterm group only. All blood samples were obtained by trained nurses and immediately stored at -20°C at the hospital facilities. DNA methylation of 20 CpG sites adjacent to *SLC6A4* exon 1a (Figure 2) was determined on blood leukocytes using bisulphite modification followed by Next Generation Sequencing. Procedures for DNA methylation quantification are reported in detail in a previous publication from our group.[12] Only methylation levels at CpG sites that have been previously found to be significantly associated with NICU pain-related stress were included in the analysis (CpG2, Chr17: 28562786-28562787; CpG5, chr17: 28562847-28562848; CpG6, chr17: 28562849-28562850; CpG9, chr17: 28562861-28562862).[6,12,13]

Please, insert Figure 2 here

Preschool-age procedures and measures

Mothers were re-contacted by phone calls when their child was approaching 4.5-year-age and a lab session was scheduled according to parents' preferences. At the lab, both preterm and full-term children took part in the PRES procedure[17], an observational paradigm designed to assess preschool children's emotional regulation in response to four age-appropriate stress-inducing sequential episodes (Table 1). Each episode has a three-phase structure: baseline, reactivity, and recovery. For the present study, three emotion-specific responses (anger, fear, sadness) were coded on a 10-sec basis, for each episode-phase (Table 2). Cross-episode phase mean scores (i.e., frequency of all the PRES items grouped under each emotional specific response weighted by actual duration of each episode and phase) were computed for each emotion-specific response, resulting in three indexes for each target emotion (anger-baseline, anger-reactivity, anger-recovery; fear-baseline, fear-

reactivity, fear-recovery; sadness-baseline, sadness-reactivity, sadness-recovery). Higher scores of the emotion-specific indexes were reminiscent of greater emotional display. Additionally, the mother was asked to fill in a socio-demographic form, depressive (Beck Depression Inventory – II, BDI-II)[20] and anxious (State-Trait Anxiety Inventory – Y, STAI-Y)[21] symptomatology questionnaires and a checklist of life adverse events (Coddington Life Events Scales, CLES).[22] An overall *cumulative adversity index* (CAI) was obtained from the CLES, with higher scores reflecting greater exposure to different potential sources of distress after NICU discharge up to 4.5 years of age.

Please, insert Table 1 and Table 2 about here

Plan of analysis. The three emotion-specific scores were compared between preterm and full-term children using a General Linear Model (GLM) with PRES phases (baseline, reactivity, and recovery) as within factor and group (preterm *vs.* full-term) as the between factor. Correlational analyses were used to determine potential confounding effects of perinatal and post-natal variables (i.e., gestational age, length of NICU stay, mother-reported symptomatology and CAI) on the PRES emotion-specific indexes. The GLM was then repeated by including significant potential confounders as covariates. Second, for emotion-specific indexes that showed significant differences between the two groups, a hierarchical regression model was used only for the preterm group to estimate the relative contribution of *SLC6A4* methylation at NICU discharge, by contrasting its effect on birth weight, *SLC6A4* methylation at birth and *CAI*. The model was repeated for each of the CpG sites included in the study (CpG2, CpG5, CpG9, and CpG16). All analyses were performed with $\alpha < 5\%$.

Results

Descriptive statistics are reported in Table 3. No differences emerged between the two groups in age, gender distribution, maternal symptomatology reports, family socioeconomic status and CAI.

Please, insert Table 3 about here

A significant phase-effect emerged ($F(2,104) = 27.53$, $p < 0.001$, $\eta^2_p = 0.35$): anger displays increased from baseline to reactivity and reached back baseline-levels during recovery. Additionally, a significant group-effect was detected ($F(1,52) = 9.53$, $p = 0.003$, $\eta^2_p = 0.16$): overall during the PRES procedure, preterm children exhibited higher displays of anger compared to full-term counterparts. The two effects were further qualified by a significant phase*group effect, ($F(2,104) = 14.38$, $p < 0.001$, $\eta^2_p = 0.22$): anger displays were significantly more frequent in preterm children, compared to controls, specifically during the reactivity phase (Figure 3A). Only a phase-effect emerged for fear ($F(2,104) = 6.23$, $p = 0.003$, $\eta^2_p = 0.11$), and for sadness ($F(2,104) = 17.14$, $p < 0.001$, $\eta^2_p = 0.25$): for both emotion-specific responses, displays increased from baseline to reactivity, and reached back the baseline-values during recovery (Figure 3B and 3C).

Please, insert Figure 3 about here

The summary and coefficients for the regression models assessing the association between CpG-specific *SLC6A4* methylation and anger in response to PRES are reported in Table 4.

Please, insert Table 4 about here

For what pertains CpG5, the model only reached statistical significance when adding *SLC6A4* methylation at discharge in the last hierarchical step ($F = 5.39$, $p = 0.029$): higher level of CpG5 methylation at discharge was significantly associated with greater anger response to PRES at 4.5 years in preterm children, even controlling for confounders (Figure 4A). Adding CpG9 *SLC6A4* methylation at discharge in the last hierarchical step resulted in a significant association (F change $= 8.86$, $p = 0.007$) with greater anger response to PRES at 4.5 years in preterm children (Figure 4B). No significant effects of CpG2 and CpG6 were documented on *anger*.

Please, insert Figure 4 about here

DISCUSSION

Previous studies reported that preterm children have a higher risk of behavioural and emotional problems during preschool-age.[7] Spittle et al[23] reported that 9% of 2-year-old preterm children *versus* 3% of full-term peers were in the clinical range for externalizing problems. Higher prevalence of emotional dysregulation was also reported at age four[24] and five.[25] Here, we further advance the field by providing observational – rather than parental report – evidence of emotional regulation in preschool-age preterm children compared to full-term age-matched counterparts. Specifically, we show that preterm children displayed greater anger response to emotional stress at 4.5 years compared to full-term age-matched controls. Importantly, the degree of anger expression was significantly predicted by increased *SLC6A4* methylation at the same CpG sites previously associated with behavioural and socio-emotional outcomes at three months of age. In this cohort, we have previously reported that pain-related stress exposure during the NICU hospitalization associated with increased methylation at specific CpG sites of the serotonin transporter gene,[12] which in turn was predictive of short-term outcomes in behavioral[13] and socio-emotional stress regulation[6] at 3 months corrected age. Evidence of long-term outcomes of *SLC6A4* methylation status linked with early exposure to NICU pain-related stress was reported in retrospective research.[26] The present manuscript provides further support through evidence from a prospective and longitudinal study.

As for the epigenetic contribution to stress regulation in preschool-age preterm children, both CpG5 (position: chr17-28562847-28562848) and CpG9 (position: chr17-28562861-28562862) sites were significantly associated with reactivity anger in response to the PRES procedure. More specifically, the larger the increase in CpG-specific *SLC6A4* methylation at NICU discharge, the greater the display of anger in response to emotional stress at 4.5 years of age. This significant association was maintained when controlling for potential confounders, including neonatal and post-discharge factors. Consistently, it is plausible that not only the short-term but also the long-term consequences of early pain-related stress exposure in the NICU may be embedded in the developing phenotype of

preterm children emotional regulation, at least partially through functional regulation of the *SLC6A4* gene methylation. In conclusion, one might advance the hypothesis that early epigenetic signatures might result in relatively stable contributions to the establishment of early developmental trajectories of less-than-optimal emotional dysregulation during infancy and early childhood. Nonetheless, in absence of research assessing the epigenetic vestiges of precocious supportive interventions during the NICU stay, this speculation should be considered carefully.

The present study had limitations. First, the focus on specific *SLC6A4* CpG sites is justified to increase the power of the study, is grounded on significant associations reported in previous research on this cohort and has been previously adopted in relatively low sample-size studies on human behavioural epigenetics.[27–29] Nonetheless, the biological plausibility of methylation changes observed at the level of a specific CpG site is presently unknown and it is possible that additional CpG sites may be involved in the long-term, rather than short-term consequences of NICU-related stress on emotional regulation of preschool preterm children. It should also be noted that we did not obtain a blood cell count and characterization for our sample. Therefore, we could not rule out any differences in blood cell populations, due to prematurity or environmental factors that could be associated with differential DNA methylation. We tested for variation in gestational age as a potential confounder and findings did not differ from the model without confounders testing. In the light of these limitations, although – to the best of our knowledge – the present study reports for the first time evidence on the long-term consequences of NICU-induced epigenetic regulation in formerly preterm infants, the findings should be considered preliminary and replication is warranted.

CONCLUSION

PT infants are known to be at higher risk for emotional dysregulation later in life and especially during preschool- and school-age childhood.[8] Increasing our knowledge of the biochemical mechanisms involved in setting greater risk for emotional dysregulation is key to inform evidence-

based effective prevention and intervention programs. The present study contributes to highlighting the epigenetic pathways through which early exposure to stress in the NICU might get under the skin and contribute to the emergence of poor adjustment and emotional dysregulation later in life in preterm born children. As preterm behavioural epigenetic research is accumulating to date,[30] showing how early NICU-related stress is embedded in the developing psychobiology of preterm infants and children, future studies should be likewise dedicated to unveiling the epigenetic correlates of early preventive and developmental care interventions.[16] These studies hold the potentials of revealing the biological underpinnings of effective neuroprotective care programs and might inform in the future more effective and efficient interventions tailored to the needs of preterm infants and their parents.

LIST OF ABBREVIATIONS

CAI, Cumulative Adversity Index

CpG, Cytosine-Guanine dinucleotides

NICU, Neonatal Intensive Care Unit

PRES, Pre-schooler Regulation of Emotional Stress

SLC6A4, Serotonin transporter gene

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TABLE LEGEND

Table 1. Description of the episodes included in the Pre-schooler Regulation of Emotional Stress (PRES) procedure.

Table 2. Micro-analytic codes included in the emotion-specific indexes of the Pre-schooler Regulation of Emotional Stress (PRES) procedure.

Table 3. Socio-demographic descriptive statistics and comparisons between preterm and full-term groups. Note. NICU, Neonatal Intensive Care Unit; SD, Standard Deviation; BDI, Beck Depression Inventory; STAI-Y, State-Trait Anxiety Inventory; ns, $p > .05$; ***, $p < .001$.

Table 4. Summary coefficients of regression models for CpG5 (A) and CpG9 (B).

FIGURE LEGEND

Figure 1. Study design schematic representation. Note: PT, preterm; FT, full-term; NICU, Neonatal Intensive Care Unit; SLC6A4, gene encoding for the serotonin transporter; GA, gestational age. The drop-out rate for PT group was 48%, including 22 parents that refused to participate in the 4.5-year follow-up, 4 parents that could not be contacted, and 1 child with neurodevelopmental impairment. The drop-out rate for FT group was 20%, including 4 parents that refused to participate in the 4.5-year follow-up and 2 parents that could not be contacted.

Figure 2. Representation of SLC6A4 gene portion examined in the present study. Note: CpG sites originally assessed for methylation status are numbered; target CpG sites in the present study are CpG2, CpG5, CpG6, and CpG9.

Figure 3. Emotion-specific indexes (A. Fear, B. Anger, C. Sadness) in response to the PRES procedure in PT and FT children. Note: PT, very preterm; FT, full-term; PRES, Pre-schooler Regulation of Emotional Stress. Error bars represent Standard Errors.

Figure 4. Association of anger index during the reactivity phase of the PRES with (A.) CpG5 methylation at birth in preterm (PT) and full-term (FT) children, (B.) CpG5 methylation at NICU discharge in PT children, (C.) CpG9 methylation at birth in PT and FT children, and (D.) CpG9 methylation at NICU discharge in PT children. Note: PT, very preterm; FT, full-term; PRES, Pre-schooler Regulation of Emotional Stress. Segmented lines report non-significant associations, continuous lines report significant associations.