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ORIGINAL ARTICLE

Cortisol levels are related to neonatal pain exposure in children born very preterm at age 18 months in two independent cohorts

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Abstract

Exposure to pain-related stress from frequent invasive procedures in the neonatal intensive care unit (NICU) has been associated with altered physiological stress regulation, neurodevelopment, and behavior in children born very preterm (≤32 weeks gestation). Previously, in a cohort born 2003–2006 (Cohort 1), we found that, at 18 months corrected age (CA), children born extremely low gestational age (ELGA; 24–28 weeks) and very low gestational age (VLGA; 29–32 weeks), had higher pre-test cortisol levels and a different pattern of cortisol output across a developmental assessment involving cognitive challenge compared to children born full-term (FT; 39–41 weeks). Also, greater neonatal pain-related stress exposure among the preterm children was related to higher pre-test cortisol levels. Given the adverse long-term effects of neonatal pain in preterm infants and the ensuing rise in clinical concerns to appropriately manage pain in the NICU in recent years, we aimed to examine whether our findings from Cohort 1 would still be evident in an independent cohort (Cohort 2) born 2006–2011 and recruited from the same tertiary NICU in Vancouver, Canada. We also compared the cortisol patterns, clinical and socio-demographic factors, and their interrelationships between the two cohorts. In Cohort 2, our findings using multi-level modeling support and extend our earlier findings in Cohort 1, demonstrating that children born ELGA display higher pre-test cortisol levels than FT. As well, greater cortisol output across assessment was related to more anxiety/depressive behaviors in children born VLGA. Importantly, children born ELGA were exposed to less neonatal pain/stress, mechanical ventilation, and morphine in Cohort 2 than Cohort 1. In both cohorts, however, cortisol levels and patterns were related to neonatal pain/stress and clinical factors (days on mechanical ventilation, overall morphine exposure). Despite less exposure to pain/stress and adverse clinical factors in Cohort 2 compared to Cohort 1, cortisol levels and patterns across cognitive challenge in preterm children at 18-month CA were consistent across the two independent cohorts. These findings highlight that, despite improvements to neonatal care, children born

extremely preterm continue to display altered HPA axis activity, which is associated with their poorer neurodevelopmental and behavioral outcomes.

KEYWORDS
behavior, cortisol, HPA axis, pain, preterm, stress

1 | INTRODUCTION

Children born very preterm (≤32 weeks gestation) display altered stress regulation as indexed by cortisol output (cortisol is the primary human stress hormone released by the hypothalamic–pituitary–adrenal [HPA] axis), which has been related to behavior.[1-4]; Chau et al., 2017.[5] Infants born very preterm spend their first weeks to months in the neonatal intensive care unit (NICU) receiving critical care, during a time when programming of the brain and developing stress systems is occurring. Previous research has found associations between exposure to neonatal pain-related stress (number of invasive procedures during NICU stay) and child physiological stress regulation[6,7] and functional outcomes,[8] in line with animal studies of long-term effects of early life adversity.[9]

In a cohort of children born very preterm during 2003–2006 (Cohort 1), we found that at 18 months corrected age (CA) toddlers born at extremely low gestational age (ELGA; 24–28 weeks) displayed higher cortisol levels after arrival (pre-test) at a clinic for developmental testing involving cognitive challenge, and a different pattern of cortisol output across developmental assessment (during and end of assessment) compared to children born at very low gestational age (VLGA; 29–32 weeks) and at full-term (FT).[1] Furthermore, higher cortisol levels prior to cognitive challenge were associated with higher pain/stress exposure[3] and more behavior problems.

Despite literature on long-term effects of neonatal pain/stress and increased clinical concern regarding pain in very preterm neonates, it is unclear whether the number of painful procedures infants born very preterm are exposed to during the NICU stay has decreased over time, and whether any decrease is related to improvements in their later physiological stress regulation. Moreover, the importance of replication in psychology and medicine research has been emphasized in recent years.[10] A lack of standardized protocols for the assessment of stress reactivity has impeded reproducibility and replication efforts in studies of child physiological stress.[11,12]

In an ongoing program of research studying relationships between early pain/stress and later neurodevelopment, a second cohort of children born very preterm during 2006–2011 was recruited from the same tertiary NICU (Cohort 2). As in Cohort 1, children were seen at 18 months CA for a developmental assessment, and saliva samples for analysis of cortisol were collected with the same methods. To our knowledge, we are the only research group internationally to have collected data on neonatal pain/stress and cortisol at 18 months CA using the same methods and paradigm in two successive cohorts of children born very preterm recruited from the same hospital. In the present study, we capitalized on this unique data to examine whether:

1. Our prior findings that the pattern of cortisol output before, during and after cognitive challenge at 18 months CA differs by gestational age at birth are still present in the later cohort;
2. Relationships between cortisol levels and child behavior are still seen in the later cohort;
3. Pain/stress exposure and clinical factors associated with prematurity differ across the cohorts, and further, whether neonatal pain/stress is related to the *pattern* of cortisol output across cognitive challenge.

2 | METHODS

2.1 | Participants and procedures

A total of 187 children born between 2006 and 2011 comprised the study sample, including 91 born at extremely low gestational age [ELGA; 24–28 weeks], 58 at very low gestational age [VLGA; 29–32 weeks] and 38 healthy full-term [39–41 weeks] infants. Parents of very preterm infants in the Level III NICU at BC Women's Hospital, Vancouver, Canada, were approached by a research nurse to consider enrollment in a longitudinal prospective cohort study of neonatal pain/stress in relation to neurodevelopment and behavior of children born very preterm (GA ≤32 weeks). In Cohort 1, healthy controls were born at the same center and contacted through their pediatricians. In Cohort 2, healthy full-term controls were recruited through community centers and mother–baby exercise and play classes. At 18 months CA, preterm- and full-term children in the current study underwent a developmental assessment administered by experienced physiotherapy or psychology staff in the Neonatal Follow-up clinic at BC Women's Hospital. Study procedures followed that described by [1] Saliva samples were collected using the same protocol as previously.[1] In both cohorts, children with congenital malformations/syndromes, antenatal infections, or large (>2 cm) parenchymal hemorrhagic infarctions were not included at recruitment. Infants were excluded from the present study if they had severe brain injury ($n = 7$), major genetic anomaly ($n = 2$), major motor, sensory and/or cognitive impairment ($n = 6$) and/or were taking medications known to affect cortisol levels ($n = 11$). [1] also excluded children born small for gestational age (SGA) and those exposed to

postnatal dexamethasone. In the current study we included children born SGA to broaden generalizability. Moreover, in Cohort 2, hydrocortisone rather than dexamethasone was routinely used to decrease need for respiratory support in the NICU, following changes in NICU standard practice. Hydrocortisone exposure was unrelated to cortisol levels in the current cohort (r's ~ 0.03, ps > 0.4). All procedures were approved by the University of British Columbia Clinical Research and BC Women's Hospital Ethics Board. Written informed parental consent was obtained at recruitment, then again at the 18-month CA study visit for children born full term and preterm. See Supplement S1 and Supplement 2 for participant flow chart for Cohort 1 and Cohort 2, respectively.

2.2 | Neonatal characteristics

Neonatal chart review was conducted by a research nurse, including but not limited to gestational age, severity of illness on Day 1 (Score of Neonatal Acute Physiology, SNAP-II[13]), number of skin-breaking procedures from birth to term-equivalent age,[14] days on mechanical ventilation, and cumulative dose of morphine.

2.3 | Child cortisol levels

Saliva sampling and cortisol assay procedure were the same for preterm and full-term born children in Cohort 1 and Cohort 2. Child saliva samples were collected at three Time Points (Pre-test [M = 9:45 am, SD = 55 min]), During assessment after cognitive testing (M = 68 mins, SD = 37 min after Pre-test), and End of session (M = 31 min, SD = 9 min after second collection). The first saliva sample was collected about 15 min after arrival at the Neonatal Follow-up clinic. Cortisol levels reflect stress levels 20–30 min after an event. Saliva samples were stored shortly after collection at −20°C, and later assayed using the Salimetrics High Sensitivity Salivary Cortisol Enzyme Immunoassay Kit for quantitative determination of salivary cortisol (Salimetrics LLC, State College, PA) All samples were assayed in duplicate. The majority of samples were assayed within 4 to 6 months of collection (Range 1 month–2.3 years). The intra- and inter-assay coefficients of variation were 3.04% and 6.57% respectively, for Cohort 1, and 5.04% and 6.58% respectively, for Cohort 2.

2.4 | Child behavior

Parent(s) completed the Child Behavior Checklist (CBCL) for ages 1.5–5 years.[15] Parents rate statements regarding the child's behavior from 0 = Not True, 1 = Often True, to 2 = Very True. Consistent with [1] we used T-scores (M = 50, SD = 10) from the Attention Problems subscale and Internalizing subscale scores: Anxious/Depressed, Emotional Reactivity, Somatic Complaints, Withdrawn.

2.5 | Child neurodevelopment

The Bayley Scales of Infant and Toddler Development 3rd Edition (Bayley-III;[18]) was used to assess neurodevelopment in the present study (Cohort 2). We calculated a combined Bayley-III score from the Cognitive Composite and Language Composite scales for analyses, following methods developed by Moore and colleagues.[16]

In Cohort 1, the Bayley Scales of Infant Development (BSID) were administered, with the Mental Developmental Index (MDI) (Bayley, 1993) used to assess combined cognitive and language development.[17] The Bayley-III and BSID test scores are not directly comparable, since the Bayley-III is known to yield higher scores than the BSID. Therefore, for comparison between cohorts, we used "predicted MDI scores" from the Bayley-III following procedures by [16] and Bayley III manual,[18] which includes an adjustment to account for the higher Bayley III scores.

2.6 | Statistical analysis

Analyses were conducted in R version 4.1.6.[19] GA group differences (ELGA, VLGA, FT) on child and socio-demographic characteristics were analyzed using one-way analyses of variance (ANOVA). As food/drink consumption is known to influence salivary cortisol concentration, cortisol data were omitted when the child had consumed food/drink within 30 min prior to the corresponding saliva collection.[20] Due to non-normal distribution of physiological data, cortisol values were log-transformed and outliers were winsorized.[21] Similarly, due to non-normality of morphine exposure and number of invasive procedures, these data were log-transformed for analyses.

To examine independent and interactive effects of predictors on patterns of child cortisol output across assessment, we conducted multilevel models with time-dependent data (Time Point) nested within individuals using lme4[22] and lmerTest[23] R packages. To account for potential effects of circadian rhythm, time of day of cortisol sample collection was controlled for in all analyses. We fitted models using restricted maximum likelihood estimation which estimates parameters of analysis model by maximizing the observed-data likelihood.[24] Model significance of effects was tested using the Satterthwaite approximations for degrees of freedom.[25] An alpha value of <0.05 was considered significant. Power analyses determined that sample sizes of $n = 40$ full-term and $n = 150$ preterm-born infants who had valid cortisol data at 18 months CA (Table 2) would be adequate to detect group differences (power <0.80, $\alpha = 0.05$) based on effect sizes (Cohen's d) of 0.44 in comparing between-group differences in cortisol and 0.41 in internalizing behaviors established in our prior work.[1] For regressions examining predictors of preterm born infants' cortisol, considering a sample size of 150 and maximum 10 predictors, a medium effect size ($f^2 = 0.15$) and an alpha of 0.05, models were powered to 0.99. Here, the use of multilevel model analyses ensures even greater power to detect group differences and predictor estimates than established in power analyses.

3 | RESULTS

3.1 | Descriptive statistics Cohort 2

Neonatal characteristics are presented in Table 1. As expected, neonates born ELGA were exposed to a greater number of invasive procedures and more clinical factors than infants born VLGA. Mothers and fathers (data not shown) of children born ELGA were less likely to hold a post-graduate university degree than mothers of VLGA and FT children. At 18-month CA, ELGA children displayed lower combined Bayley-III scores than VLGA and FT children ($p < 0.001$), but child Internalizing behaviors ($p = 0.377$, and subscales;

TABLE 1 Neonatal and demographic characteristics Cohort 2.

	ELGA ($n = 91$)	VLGA ($n = 58$)	FT ($n = 38$)	p-value
Gestational age at birth (weeks)				
Mean (SD)	26.6 (1.4)	30.6 (0.9)	39.5 (1.0)	**<0.001**
Range (min–max)	24.0–28.9	29.0–32.3	37.9–41.4	
Child sex				
Boy (*N*, %)	48 (53%)	31 (53%)	18 (47%)	0.821
Maternal age at birth (years)				
Mean (SD)	32.1 (5.0)	33.3 (5.9)	34.3 (3.9)	0.072
Range (min–max)	22.1–44.3	22.8–45.9	25.4–41.2	
Maternal marital status				
Married/Common Law	81 (93%)	49 (86%)	35 (92%)	0.334
Single/Divorced/Separated	6 (7%)	8 (14%)	3 (8%)	
Maternal ethnicity				
Other	39 (45%)	20 (36%)	17 (45%)	0.519
White Caucasian	48 (55%)	36 (64%)	21 (55%)	
Maternal level of education				
Secondary School partial or complete	14 (16%)	7 (12%)	1 (3%)	
Undergraduate Degree partial or complete	61 (70%)	34 (61%)	22 (58%)	
Post-Graduate University degree	12 (14%)*	15 (27%)	15 (39%)*	
Neonatal clinical factors				
Neonatal pain-related stress[a]				
Mean (SD)	144.5 (75.4)	61.7 (32.6)	–	**<0.001**
Range (min–max)	26.0–339.0	14.0–181.0	–	
Days on mechanical ventilation/ oscillation				
Mean (SD)	66.9 (29.9)	11.1 (12.1)	–	**<0.001**
Range (min–max)	1.0–109.0	0.0–47.0	–	
Presence of postnatal infection[b]				
Positive (*N*, %)	59 (66%)	11 (19%)	–	**<0.001**
SNAP-II score				
Mean (SD)	16.3 (14.3)	7.3 (8.5)	–	**<0.001**
Range (min–max)	0.0–57.0	0.0–42.0	–	
Morphine exposure[c]				
Mean (SD)	6.1 (11.9)	0.6 (4.0)	–	**0.003**
Range (min–max)	0.0–58.3	0.0–29.9	–	

Note: 4 ELGA dyads missing demographic information (Maternal marital status, Maternal Ethnicity, Maternal Level of Education). The p-values are the significance values and are bolded it indicate $p < .05$

[a]Number of invasive procedures from birth to term.

[b]Positive culture confirmed.

[c]Cumulative dose [mg] adjusted for daily body weight.

*Expected scores differed to predicted scores.

data not shown) did not differ across GA groups, Table 2. Time of day for Pre-test saliva sample collection ($p=0.336$) and total time of developmental assessment did not differ by GA group, $p=0.356$.

3.2 | Aim 1: Cortisol, GA group, and child behavior in Cohort 2

There was a strong trend for elevated cortisol levels in preterm born (ELGA, VLGA) children compared to FTs, B=0.08, SE=0.04, $p=0.057$. A significant interaction between GA Group and Time Point ($p=0.01$) was also evident, Figure 1. Follow-up analyses revealed that FT children displayed lower cortisol at Pre-test than children born ELGA ($B=-0.11$, $SE=0.05$, $p=0.04$) but not VLGA ($p=0.21$). During Assessment, FT children displayed lower cortisol levels than both ELGA ($B=-0.14$, SD=0.05, $p=0.01$) and VLGA children ($B=-0.16$, SD=0.06, $p=0.007$). By End of assessment, cortisol levels did not differ by GA group (p's > 0.700).

Perhaps even more telling are the cortisol patterns across assessment. Children born ELGA displayed a significant decrease in cortisol output from Pre-test to During Assessment ($B=-0.09$, $SE=0.03$, $p\leq 0.001$) but showed no significant change from During to End of Assessment ($B=0.02$, $SE=0.03$, $p=0.532$), while levels of cortisol for children born VLGA were flat across assessment ($B=0.00$, $SE=0.04$, $p=0.967$). By contrast, while cortisol levels in FT children also decreased from Pre-test to During ($B=-0.13$, $SE=0.04$, $p=0.001$), they showed a return to Pre-test levels by End, $B=0.16$, $SE=0.04$, $p<0.001$. Interestingly, ELGA and FT children displayed a similar cortisol decrease from Pre-test to During Assessment ($p=0.502$), whereas FT children displayed a significantly greater change from During to End compared to ELGA ($B=-0.14$, $SE=0.05$, $p=0.004$) and VLGA children ($B=-0.14$, $SE=0.05$, $p=0.008$). Results remained stable after controlling for child combined Bayley-III scores (data not shown). Model fixed effects accounted for 4% variance in outcome; 63% of model variance explained was attributable to between-participant variance (random intercept).

Importantly, GA group interacted with CBCL Anxious/Depressed Behaviors to predict Average Cortisol Level across assessment, $F(2, 148.4)=3.44$, $p=0.035$. For children born VLGA only, greater maternal-reported child anxiety and depression ($B=0.022$, $SE=0.01$, $p=0.023$) was related to greater average total cortisol across assessment, Figure 2. Region of significance analysis demonstrates that at high (T-score ≥ 64) Anxious/Depressed scores, VLGA children display significantly higher average cortisol levels than their FT and ELGA counterparts.

3.3 | Aim 2: Cortisol Levels, clinical, and socio-demographic factors in Cohorts 1 and 2

In statistical analyses to directly compare cortisol levels and patterns in our current and prior cohort, we applied [1] more restrictive exclusion criteria (see Methods). The interaction of Cohort × Time Point × GA group was not significant, $p=0.573$, suggesting no cohort differences in cortisol levels (see Figure 3).

There was no significant difference in overall GA between the two cohorts. Compared to the ELGA group in Cohort 2, the ELGA group in Cohort 1 was exposed to more invasive procedures (Cohort 1: Median 181, IQR=159; Cohort 2: Median 97, IQR=47), more days on mechanical ventilation (Cohort 1: Median 26, IQR=31; Cohort 2: Median 5, IQR=14), and received more morphine (Cohort 1: Median 1.1, IQR=4.3; Cohort 2: Median 0.1, IQR=0.8), p's < 0.05, but SNAP-II scores on Day 1 (illness severity) did not differ. There were no differences in the clinical factors for the VLGA group across cohorts.

Socio-demographic characteristics (maternal age at birth, years of education) and child behavior scores did not differ between cohorts. Predicted MDI scores in Cohort 2 were similar to those of Cohort 1 in FTs, but VLGA (B=4.29, SE=1.81, $p=0.02$) and ELGA (B=13.22, SE=1.82, $p<0.001$) children displayed higher predicted MDI scores in Cohort 2 than Cohort 1.

3.4 | Aim 3: Neonatal pain-related stress in relation to cortisol pattern at 18-month CA

Given that no differences in cortisol levels were evident between cohorts, we examined whether the pattern of cortisol across

TABLE 2 Cortisol levels and neurodevelopment at 18 month CA in Cohort 2.

	ELGA ($n=91$)	VLGA ($n=58$)	FT ($n=38$)
Cortisol, pretest (μg/dL)[a]			
Mean (SD)	0.20 (0.32)	0.23 (0.45)	0.15 (0.20)
Range (min–max)	0.04–2.30	0.03–2.93	0.04–1.05
Cortisol, during assessment (μg/dL)[a]			
Mean (SD)	0.19 (0.36)	0.17 (0.28)	0.09 (0.09)
Range (min–max)	0.02–2.41	0.01 (2.04)	0.01–0.57
Cortisol, end of assessment (μg/dL)[a]			
Mean (SD)	0.19 (0.36)	0.15 (0.13)	0.12 (0.06)
Range (min–max)	0.03–2.81	0.01–0.51	0.03–0.27
Combined language and cognitive bayley-III			
Mean (SD)	101.4 (11.7)[b]	107.5 (9.1)	107.0 (8.4)
Range (min–max)	78.5–121.0	91.3–125.6	92.9–121.4
CBCL anxious/depressed behaviors			
Mean (SD)	45.4 (8.9)	43.7 (9.6)	43.1 (7.9)
Range (min–max)	29.0–66.0	29.0–62.0	29.0–59.0
Missing	10	8	3

[a]Raw, nonwinsorized data.

[b]ELGA significantly lower than VLGA and FT.

assessment differed by the number of invasive procedures and clinical factors associated with prematurity between cohorts. For these analyses, we combined cohorts ($N=212$) to increase sample size and robustness of findings.[10] After controlling for gestational age (weeks) at birth, time of cortisol collection, and clinical factors associated with prematurity (illness severity, morphine exposure, days on mechanical ventilation), there was a clear "dose–response" pattern evident. Children who were exposed to the greatest level of pain/stress (≥200 invasive procedures) in the NICU demonstrated the highest cortisol levels at Pre-test, followed by a decrease through During, and were the lowest at the End ($p<0.001$), see Figure 4. These findings were the same when considering interactions between Time Point and days on mechanical ventilation ($p=0.002$) or morphine exposure ($p=0.012$) instead of pain/stress.

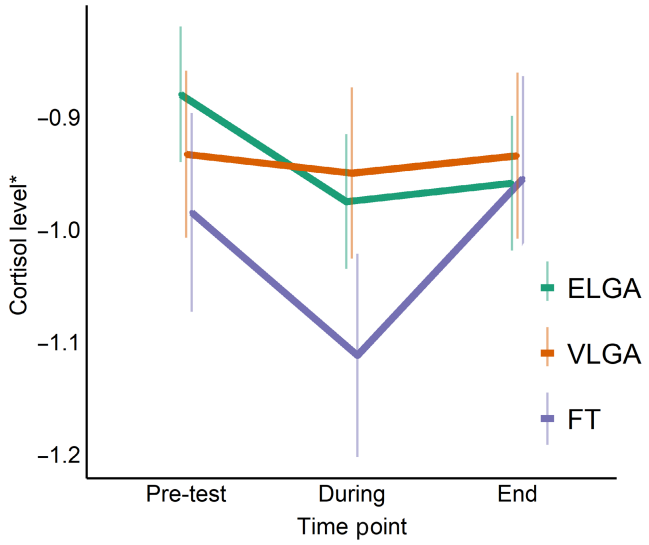

FIGURE 1 Cortisol levels across developmental assessment by GA group in Cohort 2. *Note*: *Predicted log-transformed, winsorized cortisol level.

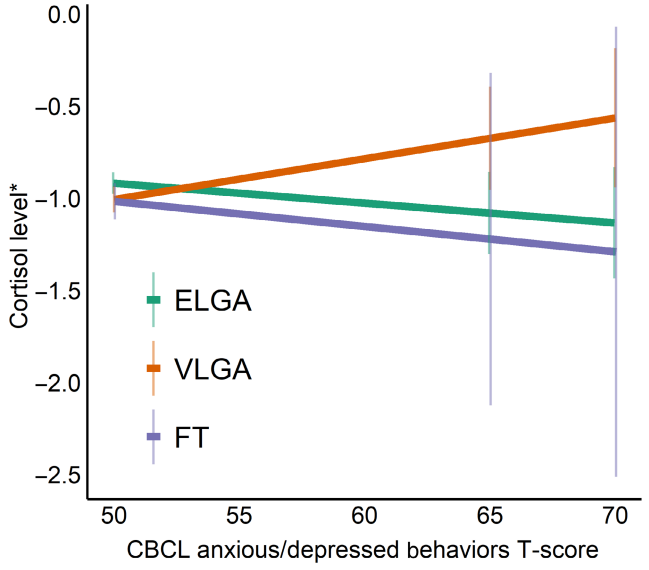

FIGURE 2 Greater Anxious/Depressed behaviors were related to higher average cortisol levels across assessment for children born VLGA only. *Note*: *Log-transformed and winsorized cortisol level.

4 | DISCUSSION

In an independent prospective longitudinal cohort study of children born during 2006–2011, we confirm and extend our findings from a prior cohort born during 2001–2006,[1] showing that, compared to children born full term, children born very preterm display altered

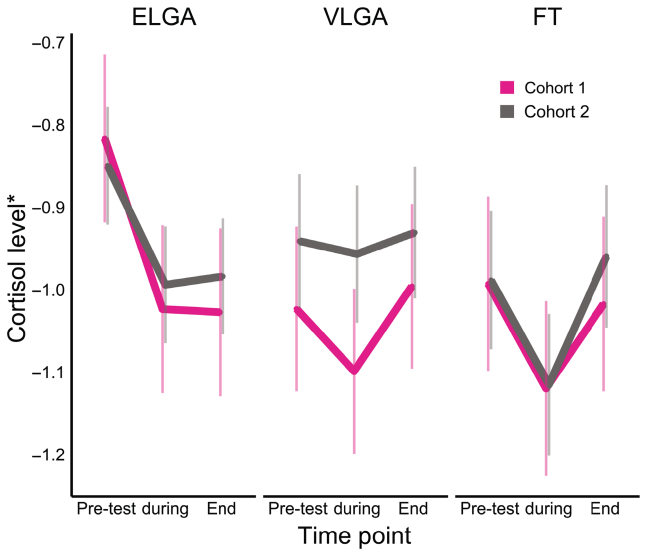

FIGURE 3 Cortisol levels across developmental assessment did not differ between Cohort 1 and Cohort 2 for children born ELGA, VLGA and FT. *Note*: *Log-transformed and winsorized cortisol level.

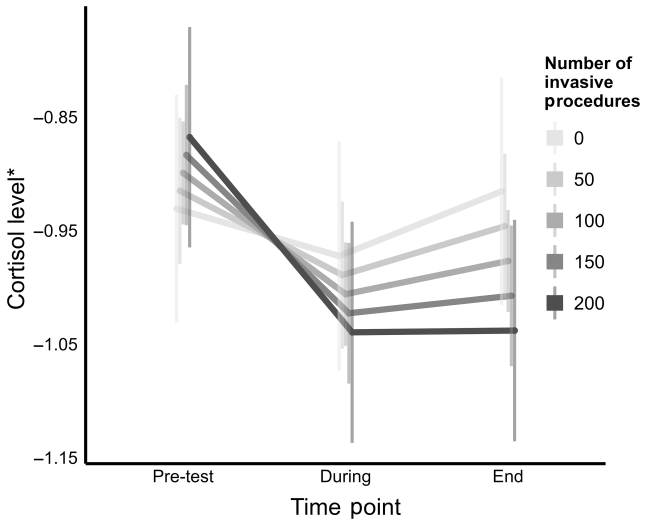

FIGURE 4 Greater decrease in cortisol across assessment was related to greater exposure to neonatal pain-related stress in the combined Cohorts 1 and 2. *Note*: *Log-transformed and winsorized cortisol level.

HPA axis activity and responsiveness across cognitive challenge. In both cohorts, children born ELGA display elevated cortisol levels prior to developmental assessment, especially compared to those born FT. Our most striking finding is that the pattern of cortisol across the developmental assessment was similar across the cohorts and was related to exposure to neonatal pain/stress and clinical factors.

In line with our prior work,[1] in the later cohort, compared to infants born FT, those born ELGA and VLGA similarly displayed altered salivary cortisol levels and patterns in response to a cognitive challenge. Importantly, infants exposed to more pain/stress of invasive procedures during NICU stay showed elevated pre-test cortisol, after accounting for gestational age at birth and clinical risk factors. The FT children displayed lower levels at beginning of the session, followed by a decline after developmental assessment and a return to pre-test levels at the end of assessment. In contrast to those born FT, cortisol levels in ELGA children were significantly elevated at pre-test, declined through assessment, and remained low until the end of the session, whereas VLGA children displayed a slightly elevated, flatter cortisol pattern across assessment. Our finding of higher pre-test cortisol levels followed by a decrease during assessment was also evident at 8 months, 18 months, and 8 years in Cohort 1.[1,6,26] Despite time for familiarization with the clinic setting prior to collection of the first cortisol sample, elevated cortisol levels prior to the developmental assessment session in children born very preterm may in-part reflect a heightened wariness to an unfamiliar environment. Indeed, prior work by Grunau and colleagues reported that children born extremely preterm find developmental assessment challenging, displaying more withdrawal behaviors during cognitive challenge at age 3 years[27] and in late childhood.[28] Moreover, prior work in this cohort demonstrates that elevated cortisol output is related to more sensory processing difficulties at age 4.5 years.[29] Such behaviors have been linked to anticipatory stress in unfamiliar situations.[30,31] The subsequent decrease in cortisol levels suggests increased engagement during cognitive challenge.[32,33] Despite reports that children born VLGA may have more mature capacity than those born ELGA to self- (or co-) regulate (given parental presence) in unfamiliar situations,[2,34] in Cohort 2 we found a flatter cortisol pattern in the VLGA compared to FT group. Lower cortisol levels in full-term children potentially reflect a lower set-point, and better ability to effectively modulate stress responses to unfamiliar situations or to challenges, in addition to fewer problem behaviors and increased attention and cognitive engagement during a cognitive task, returning to Pre-test levels at End of Assessment.[35,36]

Importantly, our findings suggest that beyond degree of prematurity, patterns of cortisol change across developmental assessment at 18 months are related to clinical factors during NICU stay, including exposure to more pain/stress of invasive procedures, longer mechanical ventilation, and higher morphine exposure. In line with animal and human research across the perinatal period,[9] very preterm infants may be especially vulnerable to early postnatal programming of the HPA axis, as it continues to mature across the late second through third trimester.[37] Children born extremely preterm, in particular, are born during a period of extreme brain/neuroendocrine immaturity, displaying greater susceptibility to adversity during the NICU stay.[38] During the NICU stay, it is well established that preterm neonates respond to invasive procedures displaying changes in behavioral, autonomic, and electrophysiological signals.[39] As demonstrated here and in prior work in the field, greater exposures to clinical factors associated with prematurity, including exposure to pain-related stress, during fetal life ex utero may contribute to altered brain maturation in stress-sensitive regions (for review see [8,37,40]) and physiological stress dysregulation in these children.[3,7,26] Findings in the current study confirm and further demonstrate the role of exposure to neonatal pain/stress and associated clinical factors in self-regulation and long-term outcomes for children born very preterm.[8]

Similar to [1], children born VLGA who displayed more anxious and depressive behaviors, as reported by their primary caregiver, displayed elevated cortisol levels across assessment. Relationships between cortisol hyper-reactivity and anxiety in childhood[41] may be indicative of less capacity to regulate stress. Higher levels of internalizing behaviors are evident in this population, in toddlerhood through adolescence.[42-44] In our earlier cohort, cortisol levels at Pre-test were related to greater anxiety/depressive behaviors, emotional reactivity, withdrawal behaviors and attention problems in children born ELGA. In the current cohort, we confirmed the association between Pre-test cortisol levels and anxiety/depressive behaviors, while associations with other outcome measures were no longer significant. It is possible that changes in NICU protocols or differences in interventions needed for the ELGA group (as discussed in Results and below) between the two cohorts might, in part, be responsible for this difference in outcome, but further work is needed to understand this fully. Future studies could look to examine relationships between child behaviors during assessment and other environmental influences on outcome as they relate to physiological markers of HPA axis regulation in this population.

Importantly, we found that the number of invasive procedures, morphine dosage and days on mechanical ventilation decreased greatly for ELGA infants from Cohort 1 to Cohort 2. While morphine and number of procedures both decreased across the two cohorts, one of the most important findings in the present study is that use of morphine did not improve physiological stress regulation. Despite these changes in clinical care, that is, lower exposure to pain and adverse clinical factors in the recent cohort, no clear differences in cortisol levels or pattern were evident between these cohorts. However, our examination of differences in clinical care across cohorts is limited. In this study, we did not examine use of less-invasive ventilatory support or nonpharmacologic pain management across cohorts. Importantly, findings in the present study are in line with the broader literature suggesting that despite advances in neonatal care, risk of poor neurodevelopment remains largely unchanged in this population (Anderson et al., 2003; Synnes et al., 2010).[45,46]

The most important change to clinical practice in our NICU was in the last 5 years, when single room care was introduced. Future research is needed to examine whether mother and infant cortisol levels for infants undergoing single room care differs to that of open bay NICUs. At 18 months of follow-up in mothers of preterm infants enrolled in FICare,[47] a family-integrated care program delivered in single room care, hair cortisol was reduced relative to those receiving standard care.[48] Further, lower maternal cortisol was associated with better concurrent child behavior. However, infant cortisol levels in relation to family-integrated care programs have not been studied to our knowledge. FICare encourages parents to be the primary carer of their preterm infants, increases parent education and support, and promotes parent-newborn skin-to-skin contact, the latter of which has been previously shown to improve child stress regulation in the long run.[49] More research examining modifiable stress prevention and protection strategies for cortisol regulation in children born very preterm is needed. In recent years, researchers have argued that rather than examining the effects of specific risk factors, consequences of early adversity are better conceptualized as domain specific.[50] Neonates born very preterm represent a particularly vulnerable population, and whether domain-specific models may by applicable to this exposure is unknown. As a result of preterm birth, these infants are outside the protective intrauterine setting at a developmental stage when they should still be in utero, and exposed to varying levels of developmentally unexpected environmental exposures that map onto multiple theorized dimensions of adversity, including but not limited to, *deprivation* in the form of parent separation as well as the *threat* and *unpredictability* of frequent invasive procedures,[51] and fragmented, disorganized sensory input.[52] We encourage research to progress from examining models of specificity to evaluating cumulative-risk and dimensional models that consider the multifactorial exposures, pathways and outcomes associated with preterm birth. Such studies could be informative for the development of timely and efficacious interventions.

There are limitations and strengths to consider in the present study. We successfully recruited 38 full-term controls in Cohort 2. Post hoc power analyses showed that we had sufficient power (0.8) to detect differences in cortisol pattern between ELGA, VLGA, and FT groups established in the current study. It is possible that our finding of a strong trend for elevated average cortisol levels between preterm and full-term children was due to a decrease in power when examining between-group differences in average cortisol levels. We do not have data on prenatal stress exposure. Antenatal corticosteroid exposure was not taken into account, given that this is very commonly administered in this population. Limited prior research has examined relationships between antenatal corticosteroids and child basal and reactive cortisol beyond early infancy, with mixed findings (for summary see [53]). Furthermore, we did not examine our data by small for gestational (SGA) status, since we did not have a sufficient sample of infants born SGA. It is a strength that we excluded participants on medications that affect cortisol in our analyses. Additionally, we were able to combine data across two cohorts with the same saliva sampling protocol and developmental assessments, increasing sample size and robustness of effects shown.[10] Scheduling of developmental assessments aligned with clinic availability; therefore, we controlled for time of collection in all analyses to account for potential confounding. While in adults, diurnal cortisol levels typically decrease from midmorning through early afternoon,[54] a recent meta-analysis showed no relationship between time of day and cortisol reactivity in infancy.[12] Eliciting a stress response in infancy is difficult, and a lack of standardized protocols for the assessment of stress reactivity is a limitation of the broader literature.[11,12] We considered it to be ethically problematic to apply a pain or specific stress stimulus to young children for research purposes. Importantly, the protocol used here could be adopted by researchers interested in stress physiology in children born very preterm, given that it occurs at routine neurodevelopmental follow-up visits (e.g., [55]). Considerable between-participant variation unexplained by factors considered here encourages future research to consider postnatal (e.g., parent environment) and individual susceptibility factors (e.g., genetic variation) in the development of physiological stress regulation.

In conclusion, across two independent cohorts admitted to the same tertiary NICU in BC, Canada, we found that, compared to children born full term, children born very preterm displayed altered HPA activity and responsiveness to cognitive challenge. Importantly, while exposure to pain/stress and clinical factors was reduced from Cohort 1 to Cohort 2, findings were similar across these two independent cohorts. Exposure to early adverse exposures during NICU stay, including neonatal pain/stress may program HPA development, resulting in dysregulation through infancy. Future research should examine cumulative-risk and dimensional models that consider the multifactorial exposures, pathways and outcomes associated with preterm birth. Such studies could further our understanding of the etiology of altered stress regulation in children born very preterm and potentially be informative for the development of timely and efficacious interventions.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

Data are not available.

ETHICS STATEMENT

All procedures were approved by the University of British Columbia Clinical Research and BC Women's Hospital Ethics Board.

PATIENT CONSENT STATEMENT

Written informed parental consent was obtained at recruitment, then again at the 18-month CA study visit.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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