Towards improving neurodevelopmental outcomes in preterm infants

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Chapter 16

General discussion and future perspectives

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The overall aim of this thesis was to move towards improving neurodevelopmental outcomes in preterm infants. This thesis provided insight into

(a) detection of adverse neurodevelopmental outcomes following preterm birth using developmental milestones in preventive child healthcare (PCHC),
(b) understanding adverse neurodevelopmental outcomes following preterm birth, with stress during neonatal intensive care unit (NICU) stay as an underlying factor and epigenetic alterations as a pathophysiological molecular mechanism involved,
(c) directions for efforts towards preventing adverse neurodevelopmental outcomes through a new promising intervention: live-performed music therapy during NICU stay.

In this chapter, we summarize and discuss the main findings and provide directions for future research.

Three sides of the same triangle

Despite advances in modern health care, including Neonatology and PCHC, adverse neurodevelopmental outcomes following preterm birth are still common and deserve attention. Only when we understand the mechanisms underlying adverse neurodevelopmental outcomes better, can we implement strategies to prevent them as early as possible. Additionally, prediction models and monitoring strategies can be adapted so that they include new underlying factors. With research into mechanisms of action we can deepen our understanding of neurodevelopment. The lessons learned in this thesis for all three sides of the triangle are summarized in **Figure 1**.

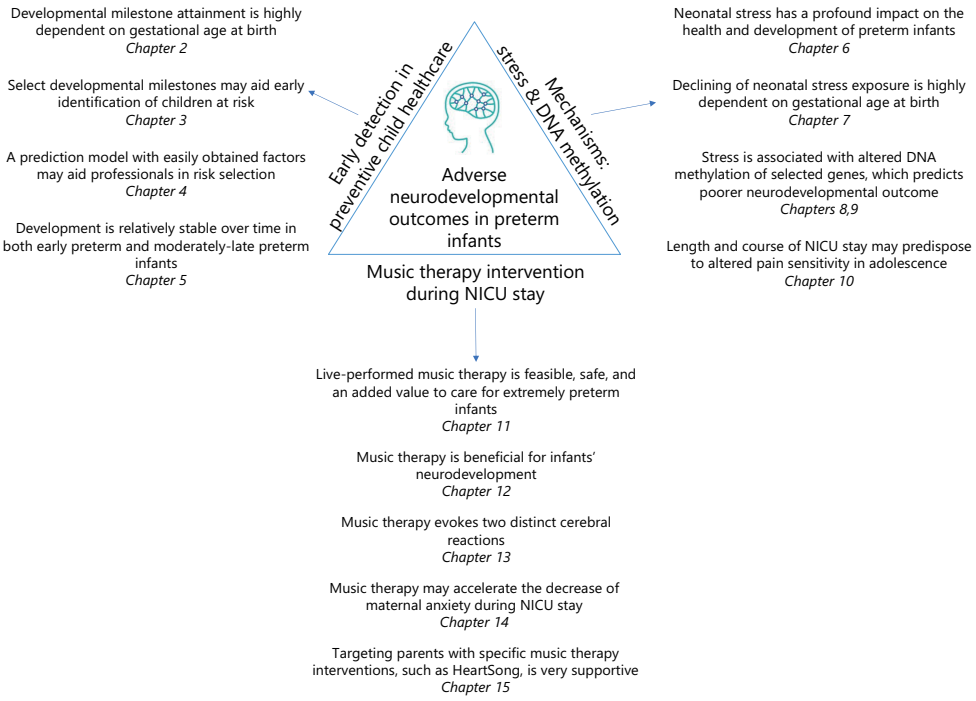

Figure 1. Main findings and lessons learned in this thesis.

Part I: Developmental milestone attainment in PCHC: early detection of adverse neurodevelopment

Of the first part of this thesis, the aim was to provide insight into the early detection of adverse neurodevelopmental outcomes following preterm birth, by gaining more knowledge on the associations between prematurity, developmental milestone attainment and adverse neurodevelopmental outcome in a PCHC setting. In **Chapter 2**, we assessed the association between prematurity and attainment of gross motor milestones, in children with normal development upon school entry. We found that rates of gross motor milestone attainment in the first two years of life were highly dependent on the degree of prematurity. This pattern may be attributed to immaturity of the brain at birth, as well as to a higher prevalence of perinatal complications and the course of NICU stay. In **Chapter 3**, we determined the pattern of attainment of the developmental milestones 'smiling in response' and 'walking independently' and their use as indicators of developmental delay for moderately-late preterm children. We found that each week later attainment of 'smiling in response', corrected for prematurity, increased the likelihood of personal social, problem solving, gross motor problems, and general developmental delay at school entry. Similarly, each month later attainment of 'walking independently', corrected for prematurity, increased the likelihood of

personal-social and gross motor problems, and general developmental delay at school entry. In **Chapter 4**, we present a prediction model for developmental delay at school entry (age 4), using variables that are easily obtainable and applicable for all children in PCHC. The final model entailed male sex, low maternal educational level (less than 12 years), pre-existing maternal obesity, non-attainment of the developmental milestones 'smiling', 'speaking two-to-three-word sentences' and 'standing', and a higher body mass index z-score at one year of age. Strikingly, neither gestational age nor birth weight contributed to the final model. In **Chapter 5**, we describe neurodevelopmental trajectories based on developmental milestones from PCHC practice in two cohorts of preterm infants born 18 years apart. We found similar developmental (D-)score trajectories for children born in 2002-2003 and for children born in 2020-2021.

The role of developmental milestones

Assessments based on developmental milestones are one of the cornerstones of detecting adverse neurodevelopmental outcomes following preterm birth. Several other early life assessments exist that have proven valuable in risk assessment, such as the General Movements Assessment,[1-3] and the Bayley Scales of Infant and Toddler Development.[4] However, these assessments require time and specific expertise, which may not be feasible in routine PCHC assessment. Such assessments based on developmental milestones as outlined in **Chapter 2** and **Chapter 3**, are feasible in a population-based setting and therefore suitable for monitoring and screening. Early identification of children at risk of adverse neurodevelopmental outcomes in early childhood using developmental milestones may be difficult, because of highly variable attainment ages. The evidence shows that preterm infants have a delay in attaining their developmental individual milestones,[5-7] and that individual milestones may predict later functional outcomes.[8-13] Our results add to these earlier reports that selected individual milestones could be used as red flags to consider which infants could benefit from additional follow-up (**Chapter 3**). Still, it may be more accurate to determine deviation of normal patterns of development, rather than making decisions based on individual milestones, because developmental milestone attainment is highly variable between individual infants. Where literature on tests and screening instruments is extensive, literature on attainment trajectories of individual milestones for preterm infants is rather scarce. Our results (**Chapter 2**) add a gross motor trajectory, that could serve as a basis for further trajectory development. The D-score from **Chapter 5** also adds a trajectory that incorporates motor development, as well as cognitive development into one. This score, and therefore the underlying developmental milestone assessments, seem to be robust and stable over decades. This indicates that they are suitable to use in PCHC settings. Close monitoring of preterm children at higher risk of adverse neurodevelopment as well as the offering of early intervention services may improve their outcomes.

Moderately-late preterm children and their follow-up

Not only early preterm children, but also those born moderately-late preterm showed delayed attainment of milestones in their first year of life (**Chapter 2**). Their attainment rates were less different from those of fullterm children when compared with early preterm children and became similar to those of fullterm children faster. Strikingly, those children with abnormal Ages and Stages Questionnaire (ASQ) scores upon school entry still showed delayed attainment in the second year of life, in contrast to the children with normal ASQ scores, who caught up their delay. This coincides with the age when developmental milestones become more complex. Even though (corrected) attainment ages may be within normal ranges valid for fullterm children, we showed that later attainment in moderately-late preterm children is associated with increased likelihood of adverse neurodevelopmental outcomes at school entry (**Chapter 3**). These results are in line with other studies performed in moderately-late preterm children, showing that they are also affected by preterm birth.[14-24] With this knowledge, a structured routine follow-up program for moderately-late preterm children is validly in place in the Dutch PCHC setting.[25] PCHC seems the optimal setting for this follow-up program, because of the large number of children that are born moderately-late preterm, the standardized way follow-up care is provided and the expertise in evaluating development. The program is offered in close collaboration with pediatricians, parents, and other parties. The findings from this thesis may add specific knowledge on milestone attainment to this guideline and program.

Early identification of adverse neurodevelopmental outcomes in PCHC

Early identification of children at risk of adverse neurodevelopmental outcomes, to which end our prediction model (**Chapter 4**) was developed, may open opportunities for timely and targeted interventions. Especially in PCHC, where time for physicians to screen children for adverse neurodevelopmental outcomes may be limited, an additional tool could enhance early detection. In this setting, easily obtainable predictor variables are a must, because this makes early detection more feasible. The variables in our prediction model are already collected as part of standard screening in Dutch PCHC and are therefore easy to use. Remarkably, neither gestational age nor birth weight were predictors in our model, even though in **Chapters 2 and 3** we found gestational age to be an important determinant of neurodevelopmental outcomes, as did others.[26-32] We explain this discrepancy by developmental milestones being a proxy of a combination of several perinatal variables, such as gestational age, being born small-for-gestational age, and perinatal conditions, to which milestone attainment may be highly related. Operationalizing this prediction model into a calculator with good sensitivity and specificity makes it applicable to daily PCHC practice.

Part II: The role of neonatal stress and associated DNA methylation: understanding mechanisms underlying adverse neurodevelopment

In the second part of this thesis, our specific aims were (1) to expand knowledge on the course of neonatal stress in preterm infants during NICU stay and its effects on health and development in early childhood and beyond and (2) to further unravel the molecular mechanism of epigenetic alterations, specifically DNA methylation, involved in neonatal stress. In **Chapter 6**, we present an overview of the current literature on associations of neonatal stress with health and development. We found that neonatal stress is associated with a variety of clinical outcomes, e.g., cognitive delays, motor delays and emotional and behavioral problems. We also identified a distinct association with structural brain development in several areas of the brain that relate to these clinical outcomes. Our findings additionally demonstrated several associations with underlying pathophysiological mechanisms, e.g., the functioning of the hypothalamic-pituitary-adrenal (HPA) axis, and DNA methylation of several stress-related and/or neurodevelopmentally relevant genes. Still, there is also a wide gap in the literature on impact of neonatal stress because we did not find studies on associations with growth, cardiovascular outcomes, language development or parent-infant interactions, nor did we find studies on associations with the neonatal immune system or microbiome. We conclude from this overview that NICU stay may have profound consequences for neurodevelopment, expressed through several pathophysiological mechanisms that may interrelate. In **Chapter 7**, we present the course of neonatal stress during preterm infants' stay in the NICU. We found mean daily scores of 66.4 (SD 8.7), with large variations between infants and within individuals. Scores were highest in the first 7 days after birth for all gestational age groups, but after that, declining of scores highly depended on gestational age. Infants who were mechanically ventilated, had a blood-culture proven sepsis and/or intraventricular hemorrhages had higher cumulative scores at 7, 14 and 28 days after birth. Of note, scores were not higher in infants with necrotizing enterocolitis. Next, **Chapter 8** focuses on placental DNA methylation of genes that may be involved in neurodevelopmental impairment in preterm infants. Using Prechtl's assessment of the early motor repertoire and its associated Motor Optimality Score – Revised (MOS-R), we found that infants with near-optimal MOS-R scores had higher methylation of the *NR3C1* gene, that encodes for the glucocorticoid receptor. We did not find differences between atypical and near-optimal MOS-R groups for other genes. This increased methylation may indicate less exposure to cortisol levels in utero, possibly leading to better neurodevelopment. In **Chapter 9**, we assessed the association between neonatal stress exposure and DNA methylation of several stress-related and neurodevelopmentally relevant genes. Our results suggest that cumulative neonatal stress exposure may inversely be related to DNA methylation upon discharge in several target genes. Importantly, this relation may exist for the *OPRM1* gene, encoding for the mu-opioid receptor

that is involved in pain regulation. In **Chapter 10** we report on the self-reported and parent-reported sensitivity to pain in adolescents. We found that one in five early preterm, one in eight moderately-late preterm and one in fourteen fullterm adolescents report increased sensitivity to pain. In early preterm adolescents, this higher sensitivity to pain was associated with inotrope treatment during NICU stay. These findings on sensitivity to pain underline the hypothesis drawn from this thesis, that NICU stay, and specific treatments, could have consequences later in life, in this case for the sensitivity to pain of preterm-born adolescents.

Defining, measuring, and quantifying neonatal stress: exposure versus experience

In this thesis, we have defined the word stress according to the biological or psychological perspective of the Oxford English Dictionary: *"an adverse circumstance that disturbs, or is likely to disturb, the normal physiological or psychological functioning of an individual"*.[33] Because infants are exposed to many of these adverse circumstances during NICU stay, the cumulative exposure was defined as neonatal stress or NICU-related stress. However, to fully understand the effects of neonatal stress, it is crucial to contemplate this definition and how it is used in measuring and quantifying neonatal stress in research till date.

In most research, only counting skin-breaking procedures is included as the measure of neonatal stress (**Chapter 6**), but it is unanswered whether better alternatives are available to measure stress exposure in neonates. Even though counting skin-breaking procedures may seem objective, definitions of what is a skin-breaking procedure vary between studies.[34] Besides, these studies overlook the non-painful, but potentially stressful exposures, such as routine handling and other medical procedures. The Neonatal Infant Stressor Scale, developed by Newham and colleagues,[35] was specifically designed to incorporate these additional factors in a quantitative measure. The Neonatal Infant Stressor Scale is used increasingly in the field of neonatal stress research. These studies confirm our findings of **Chapter 7**.[34,36,37] The downside of counting skin-breaking procedures as well as using the Neonatal Infant Stressor Scale is that they both measure only exposure to stressful events, rather than how the infants experience these stressful events. It may well be that how infants experience stressful events bears a stronger relation with neurodevelopmental outcomes than the exposure itself.

To quantify experiences of (pain-related) stress in neonates, several indices have been described, including the Neonatal Facial Coding System, the Preterm Infant Pain Profile, the Neonatal Pain, Agitation and Sedation Scale, the COMFORT-Neo Scale, and the Neonatal Infant Pain Scale. All of these typically include physiological indicators such as heart rate, blood pressure, respiratory rate and oxygen saturation, behavioral indicators such as crying, facial expressions and behavioral states, and contextual indicators such as gestational age or postmenstrual age.[38] However, all of these indices have limited applicability in clinical practice because the indicators that they include may not be specific for (pain-related) neonatal stress,

but may also be altered in situations of deteriorating medical conditions.[38] Kappesser and colleagues have compared several of these indices with more subjective numeric rating scales for pain and stress, and found that the indices for (pain-related) stress better discriminate pain than stress.[38] They also reported that the behavioral components in the indices such as facial expressions, better differentiate between stressful and painful procedures than the physiological components, such as heart rate.[38] The downside of using such indices is that they mainly evaluate a single moment or procedure, rather than cumulative experiences during NICU stay. Moreover, individual trajectories of responses to pain seem to be highly variable between infants and within the same infant across procedures.[39] Thus, a suitable and applicable measure of neonatal stress experiences has yet to be developed.

Next to counting exposures to stressful events and measuring the infants' experience of stress with physiological and behavioral clues, experience to stress can also be measured with the hormonal stress response. This relies on the body's stress system producing cortisol in response to a painful or stressful event. Cortisol is considered to be one of the most precise measures of stress.[40] Additionally, cortisol can be measured in a variety of ways, i.e., in serum, urine, and saliva. These samples indeed show a response to acute stressors and correlate well with the Neonatal Infant Stressor Scale.[41] For chronic stressors, a novel and non-invasive method has been developed using skin cortisol measurements with specifically designed tape, which also has proven to correlate well with the Neonatal Infant Stressor Scale.[37] The downside of using cortisol is that the preterm HPA axis may be underdeveloped leading to an inaccurate cortisol response. Cortisol results may thus be unreliable as measure of stress in preterm infants, as is also known from studies examining preterm versus fullterm cortisol responses in later life.[42]

In summary, all measures, whether assessing exposure to neonatal stressors or assessing how infants experience neonatal stress, have their benefits and downsides. Therefore, defining, measuring, and quantifying all aspects of neonatal stress in preterm infants during NICU stay is still an important quest that may need different tools depending on the specific situation.

Gestational age and neonatal stress: double jeopardy

To fully comprehend adverse neurodevelopmental outcomes after preterm birth, we need to acknowledge the importance of both gestational age at birth and neonatal stress, i.e., two hits that may cause double jeopardy.

Gestational age at birth has been suggested to be an important determinant of neurodevelopmental outcomes in childhood and beyond in many studies across several domains.[26,27,29,30,43] In **Chapter 2**, we confirmed this assumption for the attainment of gross motor milestones in early childhood. In **Chapter 10**, we also observed the importance of gestational age at birth regarding pain sensitivity outcomes. Both associations may be

explained by immaturity of the brain at birth. The preterm brain is extremely sensitive and rapidly developing in the first months after birth.[44,45] Developmental processes such as synaptogenesis, neuronal and axonal growth, myelination, maturation of neurochemical and enzymatic processes and focused apoptosis will have to take place in an environment that is highly different from that in utero.[44-46]

Regarding neonatal stress, infants born extremely and very preterm are also exposed to the pain and stressors of NICU stay (**Chapters 6 and 7**), which may increase the likelihood of adverse neurodevelopmental outcomes (**Chapter 6**). The effects of broadly defined neonatal stress, including pain-related stress, but also maternal separation, noise, and light, on subsequent short-term and long-term neurodevelopment that we found in **Chapter 6** are confirmed in several systematic reviews,[47-49] as well as in new studies that have recently been published.[50-52] More knowledge also becomes available on the critical windows of exposure to stressful events during NICU stay. Zhang and colleagues strikingly describe that the third trimester of pregnancy is the most important exposure window affecting brain development, with younger infants being more affected.[53] Following this theory, our findings in **Chapter 10** of altered pain sensitivity that is more pronounced in early preterm adolescents compared with their moderately-late preterm and fullterm peers, would suggest that the NICU environment plays an important role for later life outcomes. In a systematic review by Williams and Lascelles, these mechanisms are further detailed to include altered processing pathways in the developing brain.[54] Additionally, exposure to neonatal pain is reported to be associated with increased connectivity in the brain, between the thalami and bilateral somatosensory cortices, and the right insular cortex and ipsilateral amygdala and hippocampal regions.[54] These changes are more pronounced in preterm infants with higher exposure to pain-related stressful events.[55] Thus, neonatal stress has a profound and long-lasting effect.

Unravelling the role of gestational age and associated brain immaturity from the impact of NICU stay is difficult, and perhaps we should not try to disentangle the two. We propose that both immaturity and neonatal stress may play a crucial role in adverse neurodevelopmental outcomes following preterm birth, with immaturity being the first hit, but NICU stay and associated neonatal stress being the second, leading to double jeopardy. Neonatal stress may affect neurodevelopment through intermediately affecting the already immature brain development, proceeded by biological changes in signaling pathways that connect to the biological stress system.

HPA axis functioning and epigenetic alterations following stress exposure

Neonatal stress may induce epigenetic alterations, specifically altered DNA methylation in stress-related and neurodevelopmentally relevant genes, as our exploratory study in **Chapter 9** suggests. For the two most studied genes in neonatal stress literature, i.e., *NR3C1* encoding

for the glucocorticoid receptor and *SLC6A4* encoding for the serotonin transporter, this has been reported by others as well. For example, Giarraputo and colleagues report that infants at high medical risk, thus experiencing more neonatal stress, have decreased methylation at a single CpG site in *NR3C1*.[56] Altered methylation of *NR3C1* may have major consequences for health and development later in life. Argentieri and colleagues provide evidence for possible associations of altered *NR3C1* methylation with cardiovascular diseases, cancer, inflammatory diseases, and mental health outcomes such as post-traumatic stress disorder.[57] In preterm infants in particular, changes in HPA axis function that are associated with altered DNA methylation of *NR3C1* and other genes, have been associated with cardiovascular and metabolic risks at school age.[58] For *SLC6A4* increased DNA methylation at specific CpG sites are reported, both when comparing preterm infants at NICU discharge with fullterm infants and when comparing preterm infants at NICU discharge with time of birth,[59] and in infants with higher neonatal stress.[60] For the *SLC6A4* gene, an association has been reported between increased methylation and emotion regulation in preterm infants at 4 years of age.[61] Additionally, *SLC6A4* methylation has been found to be associated with socio-emotional stress responses.[62] In this association, maternal sensitivity and maternal touch throughout the life span have been proven influential,[63] highlighting the importance of maternal presence from birth onwards. Maternal separation as a source of neonatal stress has received the least attention in human neonatal stress literature (**Chapter 6**), while in animal studies, separation has proven to be one of the most influential stressors.[64] Lester and colleagues used lack of breastfeeding as a proxy of maternal separation in human fullterm infants. They reported an association between less breastfeeding and decreased methylation in the *NR3C1* gene.[65] It thus seems that maternal care in humans also impacts the stress system that is regulated by the HPA axis. The type of stress that an infant is exposed to may have consequences for their later life outcomes and may even result in brain region specific DNA methylation patterns.[66] Differential DNA methylation in a variety of other genes has been found to be associated with neurodevelopmental outcomes as well. For example, in their epigenome-wide study, Everson and colleagues describe associations regarding neurodevelopmentally relevant genes, some of which being associated with neurological structure and function, others with neurobehavioral disorders, and with neurobehavioral responses during NICU stay.[67] DNA methylation profiles may still be different between extremely low birth weight infants and normal birth weight infants in adult life,[68] which underlines the theory of biological embedding of neonatal stress exposure through DNA methylation.

Importantly, in our exploratory study in **Chapter 9**, we reported a decrease in average DNA methylation in the *OPRM1* gene between the second week after birth and at discharge from the NICU. Average DNA methylation in this gene also correlated with neonatal stress exposure throughout NICU stay. The *OPRM1* gene encodes for the mu-opioid receptor that is involved in pain and reward signaling in the brain. Literature on *OPRM1* methylation is scarce,

only one study examined the effect of noxious stimuli during NICU stay on DNA methylation and reported no relationship.[69] In adults, prescription of short-term opioids resulted in hypermethylation of this gene.[70] This effect might also have impacted the findings in our study, as some neonates received morphine during their NICU stay. However, because of the small sample size of this study, we were unable to investigate the role of treatment with morphine on this specific DNA methylation pattern. This finding of *OPRM1* methylation (**Chapter 9**) may be a mechanism underlying the findings of increased sensitivity to pain as described in **Chapter 10** and by others.[48,71-76] In various areas of pain research, including fibromyalgia or other chronic pain conditions, the *OPRM1* gene was found to play an important role in pain perception and pain sensitivity.[77] This theory also underlines the biological embedding framework of neonatal stress exposure through DNA methylation.

The role of the prenatal environment

As evidenced in **Chapter 8**, adverse prenatal conditions may predispose preterm infants for adverse neurodevelopmental outcomes. The association of fetal programming through placental DNA methylation with neurodevelopment has been confirmed by others.[78,79] The placenta is a key factor in the prenatal condition of the fetus, because it is the link between mother and fetus and serves as a regulator of growth and development through metabolism, neuroendocrine signaling and immunologic control.[80] While in **Chapter 8** we found stress exposure and HPA axis functioning to be the most influential for adverse neurodevelopmental outcomes, perinatal inflammation has been suggested as another important mechanism through which adverse neurodevelopmental outcomes may occur.[80,81] Inflammatory cytokines may be passed through the placenta into the fetal bloodstream. Based on a mouse study examining the contribution of both the prenatal and the postnatal environment, authors conclude that prenatal life experiences have a larger effect on adult mouse brain epigenomes in several genes, including *NR3C1*, than postnatal experiences.[82] Thus both the prenatal and the postnatal environmental circumstances should be taken into consideration when assessing risk for neurodevelopmental impairment in preterm infants.

Additional biological pathways between neonatal stress exposure and neurodevelopment

In **Chapter 6**, we identified substantial gaps in the current evidence regarding the understanding of pathophysiological mechanisms involved in the association between neonatal stress exposure and neurodevelopment. Following the theory of biological embedding of stress exposure by Nist and colleagues,[83] the autonomic nervous system and the neonatal immune system may be two biological pathways involved in this association. Additionally, other epigenetic alterations beyond DNA methylation may be involved as well.

Regarding the autonomic nervous system, activation may follow neonatal stress exposure. The autonomic nervous system encompasses the sympathic and parasympathic nerve branches. These two branches regulate both the respiratory and cardiovascular systems.[84] One of the main biomarkers of autonomic nervous system functioning is heart rate variability (HRV). HRV is defined as the change in the time interval between successive heartbeats and may provide non-invasive information on both the sympathic and parasympathic branches. In experimental settings where pain was induced an increase in sympathic activity was noted through exploring HRV characteristics.[85] Additionally, when interventions such as mindfulness or yoga were applied, the parasympathic activity during pain was increased as well, showing the effect of pain-reducing interventions through exploring HRV characteristics.[85] In preterm neonates, procedural pain has also been reported to increase the sympathic branch activity,[83,86] while skin-to-skin care has proven to increase the parasympathic branch activity.[83] A novel robot intervention specifically designed to calm infants during painful procedures using touch also proved to increase parasympathic branch activity.[87] The sensitization of the nervous system with increased exposure to painful procedures may represent the immaturity of the autonomic nervous system and predispose infants for altered stress reactivity later in life.[83,88]

Additionally, the neonatal immune system, which includes the microbiome, may play an important role in the association between neonatal stress and neurodevelopment. From other areas of early life adversity, we know that neuromodulators, such as microglia in the central nervous system and cytokines, as well as complement factors in the peripheral nervous system, play a role in structural brain development and associated clinical outcomes.[89] This includes synapse plasticity, neuronal apoptosis, and myelination, as well as emotional and behavioral development, particularly anxiety and depression.[89] In children with early life adversity, who had increased pro-inflammatory interleukin-6 cytokine plasma levels, an indirect association was found with internalizing behavioral problems at school age.[90] This supports the theory of biological embedding, where the immune system may have altered brain structure and functioning, leading to adverse neurodevelopment.

Other epigenetic mechanisms beyond DNA methylation may also be implicated in neonatal stress exposure. This includes telomere lengths. The limited evidence available on telomere lengths, demonstrates that preterm infants have longer telomeres at birth compared with fullterm infants, and infants with higher neonatal stress have shorter telomeres at NICU discharge compared with their preterm peers with lower stress exposure.[91] Telomere lengths are reported to associate with HPA axis functioning in preterm infants,[92] but the association of telomere lengths with neurodevelopment is still under debate.[93]

Potential cross-links in these different biological systems may exist as well. For example, the immune system may be cross-linked with the HPA axis, because many immune cells express stress hormones receptors and, in turn, the HPA axis is stimulated by proinflammatory

cytokines.[89] Moreover, several studies suggested that an inflammatory state and infections may influence emotional and behavioral development through altered microbiome compositions.[94] Finally, epigenetic alterations have been proposed to regulate neonatal immune responses.[95] In the biological embedding theory these biological pathways are all represented and they may play an important role in our understanding of the effects of neonatal stress exposure on health and development in preterm infants.

Part III: Improving neurodevelopment: feasibility and effects of live-performed music therapy during NICU stay

In the final part of this thesis, our specific aims were to determine the feasibility, effects, and underlying mechanisms of action of live-performed music therapy for extremely and very preterm infants to improve their neurodevelopment. In **Chapter 11**, we present findings of our feasibility study on the safety and applicability of this music therapy intervention in infants born before 30 weeks' gestation. We demonstrated that music therapy is well-tolerated and does not overstimulate infants. Importantly, parents and nurses evaluated music therapy as an added value to developmental care during NICU stay. In our brief report (**Chapter 12**) we present findings of a pilot study into the effects of music therapy on early neurological functioning, assessed using the General Movement Optimality Score (GMOS) in infants admitted to the NICU. Our most important finding was a distinct improvement in GMOS scores. We found that infants receiving respiratory support in the form of continuous positive airway pressure (CPAP) had substantially lower delta GMOS scores than infants with low or high-flow nasal cannulas or without respiratory support. In **Chapter 13**, we wanted to further elucidate mechanisms of action responsible for this neurodevelopmental improvement in live-performed music therapy in preterm infants. We therefore studied neonatal cerebral hemodynamics using Near-Infrared Spectroscopy (NIRS) and identified two distinct reactions to music therapy. In approximately half of the infants, oxygen saturation increased while oxygen consumption decreased, possibly reflecting a sedative effect. In the other half, oxygen saturation decreased while oxygen consumption increased, possibly reflecting a hyperalert state. Because music therapy is an environmental stimulus, it may well interact with the autonomic nervous system, involved in the regulation of brain oxygenation as well as heart rate. Of note, the clinical significance of these two distinct reactions for music processing and neurological functioning in these infants deserves further investigation. **Chapter 14** focuses on the association between infant stress and maternal anxiety as well as the role of live-performed music therapy for mothers of preterm infants. We found that mothers have high anxiety scores early during NICU stay. Fortunately, this anxiety decreased to levels comparable with the general population at discharge. Anxiety was strongly associated with cumulative neonatal stress exposure, but not with gestational age. We also found that our live-performed music therapy intervention of six sessions had an anxiety reducing effect. This music therapy

intervention may therefore accelerate the reduction of anxiety symptoms in mothers of preterm infants. **Chapter 15** entails a qualitative study that shows caregivers' experiences with a specific music therapy intervention, the 'HeartSong'. In the HeartSong, an infant's heartbeat is paired with the parent-chosen song-of-kin and afterwards provided to parents as a form of support. Both staff members and parents indicated that this intervention is valuable in bereavement support and anticipatory grief, but also in supporting parents throughout and beyond NICU stay, in various areas, including parent-infant bonding. All caregivers recommended this intervention for wider use in the NICU and beyond. Taken together, live-performed music therapy may be an asset to developmental care that deserves further research to unravel its effects and potential mechanisms of action.

Music-based interventions in the NICU: the role of live-performed music therapy

In our feasibility study (**Chapter 11**), we identified the feasibility and applicability of live-performed music therapy for extremely and very preterm infants. Our findings demonstrated that infants tolerate music therapy according to the 'Rhythm, Breath, and Lullaby' method well, without signs of overstimulation. While we did not observe overstimulation, based on literature, signs of overstimulation or stress, e.g., crying, finger spreading, medical instability as indicated by apneas, bradycardias and/or low oxygen saturation, may be contraindications for starting music therapy interventions.[96,97] This may also be the case for infants who were just extubated or are recovering from surgery.[96,97] Another important contraindication may be severe brain injury (i.e., grade 3 or 4 intraventricular hemorrhage or cystic periventricular leukomalacia). Epstein and colleagues reported that infants with these injuries showed physiological and behavioral instability when sung to by their mother.[98] Therefore, caution is always warranted when providing live-performed music therapy to the sickest and smallest infants in the NICU. We believe that it is crucial to only offer live-performed music therapy in the NICU as opposed to recorded music-based interventions, because of the immaturity of these infants, even though recorded music has been proven beneficial for neonates from 32 weeks' gestation.[99-101] An accredited music therapist with NICU training will be able to offer meaningful sounds and adapt to the specific needs of the infant more safely than recorded music, while simultaneously introducing the benefits of interaction with parents and the environment.

In our feasibility study, we found that both parents and nursing staff were very pleased with this addition to neonatal care. In a Norwegian cohort, similar experiences were described, with parents noting live-performed music therapy as beneficial for their infant and for themselves.[102] In that study, nurses also observed that parent-infant interactions improved. One challenge that was reported by nurses was inviting parents to participate in live-performed music therapy at a time of uncertainty, anxiety, and grief.[102] Because music

therapists most often have a background in psychotherapy, dealing with these emotions is familiar to them, and introducing their services at such a time could very well benefit parents. Another reported challenge was integrating music therapists into the NICU's multidisciplinary team.[102] These challenges are inherent to the implementation of a novel intervention in a NICU, but the results of both studies showed that after an implementation period, all parties are convinced of the benefits of music therapy.

Effects of live-performed music therapy on neurological functioning

Live-performed music therapy may be beneficial for early neurological functioning. We indeed identified a substantial improvement in GMOS scores from before to after music therapy (**Chapter 12**). When the whole range of GMOS is subdivided into six percentile categories (<P10, P10-25, P25-50, P50-75, P75-90, >P90), this reflects an improvement of approximately two categories.[103] As far as we know, we were the first to examine the effects of live-performed music therapy on neurological functioning. Our findings as reported in **Chapter 12** also show that for three participants GMOS scores did not improve. We speculate that music therapy improves neurological functioning over multiple sessions, because one of the infants with deteriorating scores showed improved scores over the three recordings. Unfortunately, the other infant was not videotaped multiple times. This hypothesis is supported by research from Qiu and colleagues, who report an increase in endogenous opioid concentrations only after multiple sessions of creative music therapy.[104] Pioneering work in animal research also seems to indicate that cumulative music is key in promoting growth of neuronal networks.[105]

Changes in structural brain development after music exposure

The improved GMOS scores as reported in **Chapter 12** may be explained by the effects of music on changes in neuronal development, because neuronal responses proceed in movements. Studies using functional magnetic resonance imaging (fMRI) assessments report that exposure to recorded music in a NICU environment may induce changes in functional connectivity in brain areas that are associated with music processing.[106] In addition, early postnatal music interventions may increase neural responses related to tempo processing and recognition.[106] Earlier work also demonstrated the neural architecture underlying music processing in infants, showing that the whole brain is activated,[107] and that the neuronal response to music is sensitive to changes in for example the tonal key of music and to differences in the sensory dissonance of musical signals.[108] Importantly, stronger neuronal responses were also seen in maternal singing.[109] Furthermore, changes in activity within limbic structures suggest that infants engage neural resources of emotional processing in response to musical stimuli.[108] Because general movements (GMs) have proven to be predictive for cerebral palsy, and motor and cognitive skills later on in life,[1,2,110,111] our finding of improved scores as early as several weeks after birth may also translate into favorable long-term

outcomes. A direct effect of exposure to music on the brain may therefore be a mechanism leading to neurodevelopmental improvement.

Music and stress reduction

The promising findings of improved GMOS scores after music therapy (**Chapter 12**) may reflect stress reduction signaled by the biological stress system. Because exposure to neonatal stress has a profound impact on brain development (**Chapter 6**), stress reduction could improve neurodevelopmental outcomes. Stress reduction through musical intervention may be achieved via several pathophysiological pathways.

First, music may work through stimulation of neuronal pathways sensitive to stress hormones. For example, Qiu and colleagues reported higher concentrations of beta endorphins, associated with less response to pain and stress, for infants in the musical intervention group.[104] Strikingly, they report no effects of music on cortisol concentrations.[104] This may be due to the immature HPA axis of preterm infants, and the associated inadequate cortisol response. Music therapy as a stress reducing non-pharmacological intervention has also been implemented by Ulsten and colleagues, who report an in-depth musical analysis of three cases, in which they identify the possible benefits of live lullabies during painful procedures.[112]

A second pathway regards an effect of music on the autonomic nervous system, which reacts to environmental stimuli and is wired to regulate the cardiovascular and respiratory systems.[113,114] In several studies, music-based interventions, including music therapy, have indeed been found to be associated with improvements in cardiovascular and respiratory stability.[101,115] In infants with a cardiac disease who underwent surgery and had a need for sedatives, pioneering work has shown promising results, with lowered days on mechanical ventilation, and lowered incidence of delirium.[116] Additionally, music might already have an effect on the developing fetus in utero. Novel work by Massimello and colleagues provide insight into autonomic nervous system modulation in utero, showing that fetuses are more relaxed when listening to music, with a sustained effect.[117] Live-performed music therapy may even enhance these findings, through maternal relaxation and enhanced mother-infant bonding through song-of-kin.

The autonomic nervous system may also be the underlying system explaining our findings of altered cerebral oxygenation, as presented in **Chapter 13**, where we found cerebral oxygenation to reflect either a sedative or a hyperalert reaction to music therapy. These findings are confirmed by others, reporting that altered cerebral oxygenation was induced in the frontal lobes during auditory stimulations of neonates.[118] In a more physiologically stable condition, infants may have more energy to process the offered music, leading to stronger neurophysiological responses.[119] This could also explain why we found a greater improvement in delta GMOS in infants receiving little respiratory support or no respiratory support at all in

comparison with infants receiving CPAP (**Chapter 12**). Alternatively, infants on CPAP may have a delayed auditory maturation, that could delay processing of music.[120] Stress reduction due to music and the effects of this stress reduction on the developing brain may therefore be a mechanism leading to better neurodevelopment.

Parent-infant interaction through music therapy interventions

The promising findings of **Chapter 12** of improved GMOS scores after music therapy, may also reflect enhanced parent-infant interaction during NICU stay. A healthy parent-infant dyad communication and bonding is crucial for infant development.[121] Parental mental health, the ability to care for their infant and reaction of an infant to their voice or touch, are all needed for a healthy attachment in the parent-infant dyad.[121] These are all altered because of preterm birth; in **Chapter 14**, we found that mothers of preterm infants experience high levels of anxiety, strongly related to their infant's exposure to stressful stimuli. The higher the infant's stress exposures, the higher their illness severity (**Chapter 7**) and this makes it the more likely that parents will not be able to take their parental role. This is recognized as the key stressor for mothers, which enhances their anxiety. Our findings presented in **Chapter 14** indicate that music therapy may alleviate this maternal anxiety, most likely through an enhanced feeling of maternal empowerment. Importantly, this alleviated maternal anxiety is achieved both through the 'Rhythm, Breath, and Lullaby' method that we applied as well as through creative music therapy, another form of live-performed music therapy.[122,123] Involving mothers in the music-based intervention may improve maternal responsiveness and sensitivity, in turn improving interaction and bonding with her infant.[124] This maternal proximity and interactive behavior may well have positive effects on the developing brain, regulating neurobiological, sensory and emotional systems.[109,124] This hypothesis is strengthened by research of Hamm and colleagues, who reported improved mother-infant interactions after a music-based intervention program, based on which they suggest improved dyadic communication as the underlying mechanism.[125] Not only mothers, but also fathers may benefit from music therapy interventions, as they themselves report increased stress during NICU stay,[126] but also that contact with their infant is crucial in bonding.[127] Targeting parents with specific music therapy interventions, such as the HeartSong (**Chapter 15**) may improve parent-infant interactions through parent support, bonding support and memory making, following from their own experiences. Especially in countries where social security is less regulated and parents must follow a transition back to working life quite soon, this could be a supporting intervention. Involving parents in a music therapy intervention therefore seems crucial for infants' neurodevelopment.

Music and the environment

A last mechanism of action through which our findings of improved neurodevelopment after music therapy (**Chapter 12**) could be explained regards environmental enrichment. Preterm infants are subjected to a highly different acoustic environment in the NICU compared with fullterm fetuses in the womb, with exposure to loud and high intensity sounds. Even though the American Academy of Pediatrics recommends a sound level of maximum 45 decibels (dBs), sound levels often exceed this level and may even be as high as 120 dBs.[128] Because of the immature auditory system, these sounds may have profound consequences for infants' neurodevelopment. As excessive stimuli, they may cause neurons to form alternative pathways between the cerebral cortex and the brainstem.[128] These stimuli may also trigger the autonomic nervous system along with the HPA axis, diverting resources from brain development.[119] In contrast, deprivation of sounds may also impact the maturation of the brain, as well as subsequent speech and language development.[129] Sounds in utero are of a highly musical nature, e.g., the whooshing sound of maternal blood flow or maternal heartbeat.[129]

In our feasibility study (**Chapter 11**), nurses noticed a distinct effect of live-performed music therapy on the acoustic environment of the NICU, with less noise and lowered voices. This may lower the dBs an infant is exposed to and change the soundscape of the NICU.[123] The NICU soundscape is a factor that is overlooked in most studies, but that signals stress to infants,[130] and may also be perceived as stressful by parents.[131] Changing the soundscape in a NICU to improve infant neurodevelopmental outcomes builds on the concept of environmental music therapy (EMT), in which music is utilized to intentionally change the soundscape of an environment, not by diminishing noxious sounds, but by integrating them and changing the perception of the soundscape.[132] The high-pitched beeping noises in our NICU, which are mainly in musical key A, may be integrated in song-of-kin lullabies or other pieces of environmental music, by transposing these into the same key, so that the noises become less apparent.[133] This may be beneficial for both parental anxiety and infant stress and therefore, environmental enrichment by live-performed music therapy may be beneficial for infants' neurodevelopment.

Future perspectives

Towards improved early detection in PCHC

In Dutch PCHC, the Van Wiechen Developmental Instrument is used to monitor development of all children, including those born preterm. Studies on the validity and applicability of this instrument for preterm infants are still lacking, even though the instrument is used in routine daily practice. The studies in **Chapters 2-5** will hopefully serve as a basis for better use of this instrument for preterm infants in PCHC, and the development of other tools that aid PCHC physicians in the early detection of children at risk of adverse neurodevelopment. To this end,

the prediction model that we developed (**Chapter 4**) could be refined further in an implementation study. In addition, there is a need for studies investigating whether improved detection combined with early intervention strategies after discharge indeed improves neurodevelopment. These studies should be initiated from within the clinical practice of PCHC, for example following consultations when developmental delays are suspected. Intervention studies should focus on both children and their parents in a holistic approach.

Towards a better understanding of neonatal stress and its pathophysiological mechanisms

Our findings from **Chapter 6 and 7** imply that it is important to define neonatal stress broadly, as not only including skin-breaking procedures, but also entailing other potentially stressful stimuli. The neonatal field would benefit from a general definition of neonatal stress in preterm infants. This will make future studies more comparable, eventually leading to better care. Future studies should better distinguish stress exposure from stress experience. The use of specific measures of neonatal stress will depend on the specific research question that is addressed. Experts on neonatal stress should cooperate to form a core outcome set for research and clinical practice, to better evaluate and further tailor care for extremely and very preterm infants.

Regarding pathophysiological mechanisms, DNA methylation as a mechanism underlying the associations between neonatal stress exposure and adverse neurodevelopmental outcomes as evidenced in **Chapters 6, 8 and 9** should be further studied. Such studies should include additional stress-related and neurodevelopmentally relevant genes, such as *NFKBIA*, a gene that encodes for a protein regulating immune and inflammatory responses, and *FKBP5*, a gene that is part of the HPA axis pathway and encodes for a glucocorticoid receptor and binding protein.[134] They should also include gene expression and hormone levels, to provide a full overview of the effects of neonatal stress on preterm infants. Several potential mechanisms involved in neonatal stress exposure could be further investigated, as evidence on them is currently scarce (**Chapter 6**). This includes other epigenetic mechanisms such as telomere lengths and micro-RNA expression that have received less attention, and also the role of the autonomic nervous system and the immune system.

Additionally, it is important to limit neonatal stress exposure and thereby hopefully stress experiences as much as possible, because of its impact on neurodevelopment (**Chapters 6 and 10**). Clinicians should weigh the benefits of diagnostic procedures, handling, and interventions very carefully. Additionally, future studies could focus on improving infant self-regulation regarding specific stressful events. For example, promising research reports that helping infants to self-regulate as much as possible in the case of nursing care, positively relates to neurodevelopment at two years of age.[135] Investigating and implementing such soothing interventions should be a priority in neonatal stress research. For implementing

developmentally appropriate interventions it is important to involve all stakeholders and parents, and to develop setting- and site-specific educational tools to support all involved.[136]

Towards improving neurodevelopment through live-performed music therapy in the NICU

Live-performed music therapy in the NICU to improve the neurodevelopmental outcomes of extremely and very preterm infants is a young and developing field of research and practice. We have established that live-performed music therapy is feasible for extremely and very preterm infants (**Chapter 11**). Next, several directions are promising for further research. First, we need data on a longer follow-up than the current studies, i.e., beyond three months post-term. Second, studies should aim to better understand biological mechanisms of live-performed music therapy. This may regard the role of live-performed music therapy in parent-infant bonding and parental experiences with live-performed music therapy in the NICU. Even though it should be standard practice, involving fathers in these studies is crucial. Further it is promising to assess the protective effects of music therapy on intermediate outcomes such as epigenetic alterations, the autonomic nervous system, the immune system, and brain structure. Third, the field of live-performed music therapy should expand its scope to other infant populations on the NICU and Pediatric Intensive Care Unit (PICU). For example, music therapy could focus on parent-infant dyads experiencing a difficult start in life, cardiac disease, and music therapy could contribute to specific on-demand settings such as during painful procedures. Finally, music therapy may expand its scope to antepartum units, as music therapy may have a profound effect on parent-infant bonding and neurodevelopmental outcomes already in utero.

Implementing live-performed music therapy in the NICU: steps beyond research

Our feasibility study (**Chapter 11**) was the first study to introduce live-performed music therapy into the Dutch NICU environment. This inherently came with implementation challenges, but after the study period, parents and nurses were generally very pleased with this additional care. To integrate this intervention into routine care, it is crucial to educate medical and nursing staff on the role of live-performed music therapy in the NICU and to set expectations. In addition, music therapists should be part of interdisciplinary social rounds to actively identify parent-infant dyads who could benefit from this intervention. That will also integrate them better in the NICU team.

Conclusion

In conclusion, in this thesis we have provided substantial contributions towards improving neurodevelopmental outcomes of preterm infants. First, we showed that routine developmental screening in PCHC can help to detect children at risk of adverse neurodevelopmental outcomes and that this detection can be improved by using neurodevelopmental trajectories and predictive tools. Efforts towards early detection of adverse neurodevelopmental outcomes in PCHC settings could be intensified with the added knowledge. Second, we showed that both gestational age and neonatal stress exposure are important determinants of adverse neurodevelopmental outcomes following preterm birth. Stress exposure in the prenatal and postnatal environments may be biologically embedded at least through altered DNA methylation of stress-related and neurodevelopmentally relevant genes. Efforts towards an even better understanding of neonatal stress can regard further research on these biological pathways. Finally, we showed that live-performed music therapy is feasible for extremely and very preterm infants during NICU stay. Music therapy may improve neurodevelopment through direct effects on the brain, stress reduction, enhanced parent-infant interaction, and environmental enrichment. Early interventions, such as live-performed music therapy during pregnancy and NICU stay that focus on alleviating stress and improving parent-infant bonding and interaction can contribute to improved neurodevelopmental outcomes in this vulnerable population. All future efforts should be focused towards protecting the infant brain and thereby improving neurodevelopment.

References

1. Peyton C, Einspieler C. General movements: A behavioral biomarker of later motor and cognitive dysfunction in NICU graduates. *Pediatr Ann*. 2018;**47**:e159-e164.
2. Einspieler C, Bos AF, Libertus ME, Marschik PB. The general movement assessment helps us to identify preterm infants at risk for cognitive dysfunction. *Front Psychol*. 2016;**7**:e406.
3. Prechtl HF, Einspieler C, Cioni G, Bos AF, Ferrari F, Sontheimer D. An early marker for neurological deficits after perinatal brain lesions. *Lancet*. 1997;**349**:1361-1363.
4. Bode MM, D'Eugenio DB, Mettelman BB, Gross SJ. Predictive validity of the bayley, third edition at 2 years for intelligence quotient at 4 years in preterm infants. *J Dev Behav Pediatr*. 2014;**35**:570-575.
5. Bucher HU, Killer C, Ochsner Y, Vaihinger S, Fauchère J. Growth, developmental milestones and health problems in the first 2 years in very preterm infants compared with term infants: A population based study. *Eur J Pediatr*. 2002;**161**:151-156.
6. Den Ouden L, Rijken M, Brand R, Verloove-Vanhorick SP, Ruys JH. Is it correct to correct? developmental milestones in 555 "normal" preterm infants compared with term infants. *J Pediatr*. 1991;**118**:399-404.
7. Scharf RJ, Scharf GJ, Stroustrup A. Developmental milestones. *Pediatr Rev*. 2016;**37**:25-38.
8. Diepeveen FB, Dusseldorp E, Bol GW, Oudesluys-Murphy AM, Verkerk PH. Failure to meet language milestones at two years of age is predictive of specific language impairment. *Acta Paediatr*. 2016;**105**:304-310.
9. Flensborg-Madsen T, Mortensen EL. Infant developmental milestones and adult intelligence: A 34-year follow-up. *Early Hum Dev*. 2015;**91**:393-400
10. Murray GK, Jones PB, Kuh D, Richards M. Infant developmental milestones and subsequent cognitive function. *Ann Neurol*. 2007;**62**:128-136.
11. Taanila A, Murray GK, Jokelainen J, Isohanni M, Rantakallio P. Infant developmental milestones: A 31-year follow-up. *Dev Med Child Neurol*. 2005;**47**:581-586.
12. Kover ST, Edmunds SR, Ellis Weismer S. Brief report: Ages of language milestones as predictors of developmental trajectories in young children with autism spectrum disorder. *J Autism Dev Disord*. 2016;**46**:2501-2507.
13. Di Rosa G, Cavallaro T, Alibrandi A, et al. Predictive role of early milestones-related psychomotor profiles and long-term neurodevelopmental pitfalls in preterm infants. *Early Hum Dev*. 2016;**101**:49-55.
14. de Jong M, Verhoeven M, van Baar AL. School outcome, cognitive functioning, and behaviour problems in moderate and late preterm children and adults: A review. *Semin Fetal Neonatal Med*. 2012;**17**:163-169.
15. Cserjesi R, Van Braeckel KNJA, Butcher PR, et al. Functioning of 7-year-old children born at 32 to 35 weeks' gestational age. *Pediatrics*. 2012;**130**:e838-e846.
16. Chan E, Quigley MA. School performance at age 7 years in late preterm and early term birth: A cohort study. *Arch Dis Child Fetal Neonatal Ed*. 2014;**99**:F451-F457.
17. Quigley MA, Poulsen G, Boyle E, et al. Early term and late preterm birth are associated with poorer school performance at age 5 years: A cohort study. *Arch Dis Child Fetal Neonatal Ed*. 2012;**97**:F167-F173.
18. Chyi LJ, Lee HC, Hintz SR, Gould JB, Sutcliffe TL. School outcomes of late preterm infants: Special needs and challenges for infants born at 32 to 36 weeks gestation. *J Pediatr*. 2008;**153**:25-31.
19. Huddy CL, Johnson A, Hope PL. Educational and behavioural problems in babies of 32-35 weeks gestation. *Arch Dis Child Fetal Neonatal Ed*. 2001;**85**:F23-F28.
20. Shah P, Kaciroti N, Richards B, Oh W, Lumeng JC. Developmental outcomes of late preterm infants from infancy to kindergarten. *Pediatrics*. 2016;**138**: e20153496.
21. Woythaler M, McCormick MC, Mao WY, Smith VC. Late preterm infants and neurodevelopmental outcomes at kindergarten. *Pediatrics*. 2015;**136**:424-431.

22. Gill JV, Boyle EM. Outcomes of infants born near term. *Arch Dis Child*. 2017;**102**:194-198.
23. de Jong M, Verhoeven M, van Baar AL. School outcome, cognitive functioning, and behaviour problems in moderate and late preterm children and adults: A review. *Semin Fetal Neonatal Med*. 2012;**17**:163-169.
24. Kerstjens JM, de Winter AF, Bocca-Tjeertes IF, ten Vergert EMJ, Reijneveld SA, Bos AF. Developmental delay in moderately preterm-born children at school entry. *J Pediatr*. 2011;**159**:92-98.
25. Van der Pal S, Pols M. *JGZ richtlijn 'te vroeg en/of small for gestational age (SGA) geboren kinderen'*. Utrecht: Nederlands Centrum voor Jeugdgezondheidszorg; 2013.
26. Kirkegaard I, Obel C, Hedegaard M, Henriksen TB. Gestational age and birth weight in relation to school performance of 10-year-old children: A follow-up study of children born after 32 completed weeks. *Pediatrics*. 2006;**118**:1600-1606.
27. Kerstjens JM, de Winter AF, Bocca-Tjeertes IF, Bos AF, Reijneveld SA. Risk of developmental delay increases exponentially as gestational age of preterm infants decreases: A cohort study at age 4 years. *Dev Med Child Neurol*. 2012;**54**:1096-1101.
28. Shah PE, Kaciroti N, Richards B, Lumeng JC. Gestational age and kindergarten school readiness in a national sample of preterm infants. *J Pediatr*. 2016;**178**:61-67.
29. Mathiasen R, Hansen BM, Andersen ANN, Forman JL, Greisen G. Gestational age and basic school achievements: A national follow-up study in Denmark. *Pediatrics*. 2010;**126**:e1553-e1561.
30. Poulsen G, Wolke D, Kurinczuk JJ, et al. Gestational age and cognitive ability in early childhood: A population-based cohort study. *Paediatr Perinat Epidemiol*. 2013;**27**:371-379.
31. Schonhaut L, Armijo I, Pérez M. Gestational age and developmental risk in moderately and late preterm and early term infants. *Pediatrics*. 2015;**135**:e835-e841.
32. Hediger ML, Overpeck MD, Ruan WJ, Troendle JF. Birthweight and gestational age effects on motor and social development. *Paediatr Perinat Epidemiol*. 2002;**16**:33-46.
33. Weissmann G. The experimental pathology of stress: Hans selye to paris hilton. *The FASEB Journal*. 2007;**21**:2635-2638.
34. Nist MD, Harrison TM, Pickler RH, Shoben AB. Measures of stress exposure for hospitalized preterm infants. *Nurs Res*. 2020;**69**:S3-S10.
35. Newnham CA, Inder TE, Milgrom J. Measuring preterm cumulative stressors within the NICU: The neonatal infant stressor scale. *Early Hum Dev*. 2009;**85**:549-555.
36. Abdelmageed RI, Youssef AM, El-Farrash RA, Mohamed HM, Abdelaziz AW. Measurement of cumulative preterm neonatal and maternal stressors during neonatal intensive care unit admission. *J Pediatr Psychol*. 2021:Online ahead of print. doi:10.1093/jpedspsy/jsab124.
37. D'Agata AL, Roberts MB, Ashmeade T, Dutra SVO, Kane B, Groer MW. Novel method of measuring chronic stress for preterm infants: Skin cortisol. *Psychoneuroendocrinology*. 2019;**102**:204-211.
38. Kappesser J, Kamper-Fuhrmann E, de Laffolie J, et al. Pain-specific reactions or indicators of a general stress response?: Investigating the discriminant validity of 5 well-established neonatal pain assessment tools. *Clin J Pain*. 2019;**35**:101-110.
39. Hundert A, Woolcott C, Dorling J, Benoit B, Campbell-Yeo M. Classification of individual pain response trajectories following medically indicated heel lances in preterm infants during their NICU admission. *Clin J Pain*. 2021;**38**:151-158.
40. Mörelius E, He H, Shorey S. Salivary cortisol reactivity in preterm infants in neonatal intensive care: An integrative review. *Int J Environ Res Public Health*. 2016;**13**:e337.
41. Pourkaviani S, Zhang X, Spear EA, et al. Clinical validation of the neonatal infant stressor scale with preterm infant salivary cortisol. *Pediatr Res*. 2020;**87**:1237-1243.
42. Brummelte S, Chau C, Cepeda IL, et al. Cortisol levels in former preterm children at school age are predicted by neonatal procedural pain-related stress. *Psychoneuroendocrinology*. 2015;**51**:151-163.

43. Dueker G, Chen J, Cowling C, Haskin B. Early developmental outcomes predicted by gestational age from 35 to 41weeks. *Early Hum Dev*. 2016;**103**:85-90.
44. Tau GZ, Peterson BS. Normal development of brain circuits. *Neuropsychopharmacology*. 2010;**35**:147-168.
45. Cochard LR. *Netter's atlas of human embryology, updated edition.* Elsevier Inc.; 2012: Chapter 3 "The Nervous System", p51-81.
46. Kinney HC. The near-term (late preterm) human brain and risk for periventricular leukomalacia: A review. *Semin Perinatol*. 2006;**30**:81-88.
47. Valeri BO, Holsti L, Linhares MB. Neonatal pain and developmental outcomes in children born preterm: A systematic review. *Clin J Pain*. 2015;**31**:355-362.
48. van den Bosch GE, van Dijk M, Tibboel D, de Graaff JC. Long-term effects of early exposure to stress, pain, opioids and anaesthetics on pain sensitivity and neurocognition. *Curr Pharm Des*. 2017;**23**:5879-5886.
49. Boggini T, Pozzoli S, Schiavolin P, et al. Cumulative procedural pain and brain development in very preterm infants: A systematic review of clinical and preclinical studies. *Neurosci Biobehav Rev*. 2021;**123**:320-336.
50. Steinbauer P, Deindl P, Fuiko R, et al. Long-term impact of systematic pain and sedation management on cognitive, motor, and behavioral outcomes of extremely preterm infants at preschool age. *Pediatr Res*. 2021;**89**:540-548.
51. Marchal A, Melchior M, Dufour A, Poisbeau P, Zores C, Kuhn P. Pain behavioural response to acoustic and light environmental changes in very preterm infants. *Children (Basel)*. 2021;**8**:e1081.
52. Cassiano RG, Gaspardo CM, Linhares MB. Neonatal stress and behavior problems in preschool children born preterm. *Clin Child Psychol Psychiatry*. 2022:Online ahead of print, doi: 10.1177/13591045221098521.
53. Zhang X, Spear E, Hsu HL, Gennings C, Stroustrup A. NICU-based stress response and preterm infant neurobehavior: Exploring the critical windows for exposure. *Pediatr Res*. 2022:Online ahead of print. doi: 10.1038/s41390-3.
54. Williams MD, Lascelles BDX. Early neonatal pain-A review of clinical and experimental implications on painful conditions later in life. *Front Pediatr*. 2020;**8**:e30.
55. Tortora D, Severino M, Di Biase C, et al. Early pain exposure influences functional brain connectivity in very preterm neonates. *Front Neurosci*. 2019;**13**:e899.
56. Giarraputo J, DeLoach J, Padbury J, et al. Medical morbidities and DNA methylation of NR3C1 in preterm infants. *Pediatr Res*. 2017;**81**:68-74.
57. Argentieri MA, Nagarajan S, Seddighzadeh B, Baccarelli AA, Shields AE. Epigenetic pathways in human disease: The impact of DNA methylation on stress-related pathogenesis and current challenges in biomarker development. *EBioMedicine*. 2017;**18**:327-350.
58. Padbury JF, Do BT, Bann CM, et al. DNA methylation in former extremely low birth weight newborns: Association with cardiovascular and endocrine function. *Pediatr Res*. 2022;**91**:1469-1477.
59. Montirosso R, Provenzi L, Fumagalli M, et al. Serotonin transporter gene (SLC6A4) methylation associates with neonatal intensive care unit stay and 3-month-old temperament in preterm infants. *Child Dev*. 2016;**87**:38-48.
60. Provenzi L, Fumagalli M, Sirgiovanni I, et al. Pain-related stress during the neonatal intensive care unit stay and SLC6A4 methylation in very preterm infants. *Front Behav Neurosci*. 2015;**9**:e99.
61. Provenzi L, Fumagalli M, Scotto di Minico G, et al. Pain-related increase in serotonin transporter gene methylation associates with emotional regulation in 4.5-year-old preterm-born children. *Acta Paediatr*. 2020;**109**:1166-1174.

62. Montirosso R, Provenzi L, Giorda R, et al. SLC6A4 promoter region methylation and socio-emotional stress response in very preterm and full-term infants. *Epigenomics*. 2016;**8**:895-907.
63. Mariani Wigley, Isabella Lucia Chiara, Mascheroni E, Fontana C, et al. The role of maternal touch in the association between SLC6A4 methylation and stress response in very preterm infants. *Dev Psychobiol*. 2021;**63 Suppl 1**:e22218.
64. Watamura SE, Roth TL. Looking back and moving forward: Evaluating and advancing translation from animal models to human studies of early life stress and DNA methylation. *Dev Psychobiol*. 2019;**61**:323-340.
65. Lester BM, Conradt E, LaGasse LL, Tronick EZ, Padbury JF, Marsit CJ. Epigenetic programming by maternal behavior in the human infant. *Pediatrics*. 2018;**142**:e20171890.
66. Catale C, Bussone S, Lo Iacono L, et al. Exposure to different early-life stress experiences results in differentially altered DNA methylation in the brain and immune system. *Neurobiol Stress*. 2020;**13**:e100249.
67. Everson TM, Marsit CJ, Michael O'Shea T, et al. Epigenome-wide analysis identifies genes and pathways linked to neurobehavioral variation in preterm infants. *Sci Rep*. 2019;**9**:e6322.
68. Mathewson KJ, McGowan PO, de Vega WC, et al. DNA methylation profiles in adults born at extremely low birth weight. *Dev Psychopathol*. 2022;**34**:19-36.
69. Hatfield LA, Hoffman RK, Polomano RC, Conley Y. Epigenetic modifications following noxious stimuli in infants. *Biol Res Nurs*. 2018;**20**:137-144.
70. Sandoval-Sierra JV, Salgado García FI, Brooks JH, Derefinko KJ, Mozhui K. Effect of short-term prescription opioids on DNA methylation of the OPRM1 promoter. *Clin Epigenetics*. 2020;**12**:e76.
71. Grunau RE, Whitfield MF, Petrie JH. Pain sensitivity and temperament in extremely low-birth-weight premature toddlers and preterm and full-term controls. *Pain*. 1994;**58**:341-346.
72. Hermann C, Hohmeister J, Demirakca S, Zohsel K, Flor H. Long-term alteration of pain sensitivity in school-aged children with early pain experiences. *Pain*. 2006;**125**:278-285.
73. van Ganzewinkel C, Been JV, Verbeek I, et al. Pain threshold, tolerance and intensity in adolescents born very preterm or with low birth weight. *Early Hum Dev*. 2017;**110**:31-38.
74. Vederhus BJ, Eide GE, Natvig GK, Markestad T, Graue M, Halvorsen T. Pain tolerance and pain perception in adolescents born extremely preterm. *J Pain*. 2012;**13**:978-987.
75. Buskila D, Neumann L, Zmora E, Feldman M, Bolotin A, Press J. Pain sensitivity in prematurely born adolescents. *Arch Pediatr Adolesc Med*. 2003;**157**:1079-1082.
76. Hohmeister J, Demirakça S, Zohsel K, Flor H, Hermann C. Responses to pain in school-aged children with experience in a neonatal intensive care unit: Cognitive aspects and maternal influences. *Eur J Pain*. 2009;**13**:94-101.
77. Landa A, Peterson BS, Fallon BA. Somatoform pain: A developmental theory and translational research review. *Psychosom Med*. 2012;**74**:717-727.
78. Meakin CJ, Martin EM, Santos HP, et al. Placental CpG methylation of HPA-axis genes is associated with cognitive impairment at age 10 among children born extremely preterm. *Horm Behav*. 2018;**101**:29-35.
79. Tilley SK, Martin EM, Smeester L, et al. Placental CpG methylation of infants born extremely preterm predicts cognitive impairment later in life. *PLoS One*. 2018;**13**:e0193271.
80. Bangma JT, Hartwell H, Santos HP, O'Shea TM, Fry RC. Placental programming, perinatal inflammation, and neurodevelopment impairment among those born extremely preterm. *Pediatr Res*. 2021;**89**:326-335.
81. Hodyl NA, Aboustate N, Bianco-Miotto T, Roberts CT, Clifton VL, Stark MJ. Child neurodevelopmental outcomes following preterm and term birth: What can the placenta tell us? *Placenta*. 2017;**57**:79-86.
82. Mattern F, Post A, Solger F, et al. Prenatal and postnatal experiences associated with epigenetic changes in the adult mouse brain. *Behav Brain Res*. 2019;**359**:143-148.

83. Nist MD, Harrison TM, Steward DK. The biological embedding of neonatal stress exposure: A conceptual model describing the mechanisms of stress-induced neurodevelopmental impairment in preterm infants. *Res Nurs Health*. 2019;**42**:61-71.
84. Cardoso S, Silva MJ, Guimarães H. Autonomic nervous system in newborns: A review based on heart rate variability. *Childs Nerv Syst*. 2017;**33**:1053-1063.
85. Forte G, Troisi G, Pazzaglia M, Pascalis VD, Casagrande M. Heart rate variability and pain: A systematic review. *Brain Sci*. 2022;**12**:e153.
86. Lavanga M, Bollen B, Caicedo A, et al. The effect of early procedural pain in preterm infants on the maturation of electroencephalogram and heart rate variability. *Pain*. 2021;**162**:1556-1566.
87. Williams N, MacLean K, Guan L, Collet JP, Holsti L. Pilot testing a robot for reducing pain in hospitalized preterm infants. *OTJR*. 2019;**39**:108-115.
88. Latremoliere A, Woolf CJ. Central sensitization: A generator of pain hypersensitivity by central neural plasticity. *J Pain*. 2009;**10**:895-926.
89. Brydges NM, Reddaway J. Neuroimmunological effects of early life experiences. *Brain Neurosci Adv*. 2020;**4**:e23298212820953706.
90. Flouri E, Francesconi M, Papachristou E, Midouhas E, Lewis G. Stressful life events, inflammation and emotional and behavioural problems in children: A population-based study. *Brain Behav Immun*. 2019;**80**:66-72.
91. Provenzi L, Giorda R, Fumagalli M, et al. Pain exposure associates with telomere length erosion in very preterm infants. *Psychoneuroendocrinology*. 2018;**89**:113-119.
92. Provenzi L, Giorda R, Fumagalli M, et al. Telomere length and salivary cortisol stress reactivity in very preterm infants. *Early Hum Dev*. 2019;**129**:1-4.
93. Feiler MO, Patel D, Li H, et al. The association between early-life relative telomere length and childhood neurodevelopment. *Neurotoxicology*. 2018;**65**:22-27.
94. Foster JA, McVey Neufeld K. Gut-brain axis: How the microbiome influences anxiety and depression. *Trends Neurosci*. 2013;**36**:305-312.
95. Bermick J, Schaller M. Epigenetic regulation of pediatric and neonatal immune responses. *Pediatr Res*. 2022;**91**:297-327.
96. Haslbeck FB. Creative music therapy with premature infants: An analysis of video footage. *Nordic J Music Therapy*. 2013;**23**:5-35.
97. Haslbeck FB. The interactive potential of creative music therapy with premature infants and their parents: A qualitative analysis. 2013;**23**:36-70.
98. Epstein S, Bauer S, Levkovitz Stern O, et al. Preterm infants with severe brain injury demonstrate unstable physiological responses during maternal singing with music therapy: A randomized controlled study. *Eur J Pediatr*. 2021;**180**:1403-1412.
99. van der Heijden MJE, Oliai Araghi S, Jeekel J, Reiss IKM, Hunink MGM, van Dijk M. Do hospitalized premature infants benefit from music interventions? A systematic review of randomized controlled trials. *PLoS ONE*. 2016;**11**:e0161848.
100. Foroushani SM, Herman CA, Wiseman CA, Anthony CM, Drury SS, Howell MP. Evaluating physiologic outcomes of music interventions in the neonatal intensive care unit: A systematic review. *J Perinatol*. 2020;**40**:1770-1779.
101. Bieleninik L, Ghetti C, Gold C. Music therapy for preterm infants and their parents: A meta-analysis. *Pediatrics*. 2016;**138**:e20160971.
102. Janner C, Gaden TS, Nakstad B, Solevåg AL. Implementing music therapy in a Norwegian neonatal intensive care unit. *Nurs Child Young People*. 2021:Online ahead of print. doi: 10.7748/ncyp.2021.e1331.

103. Einspieler C, Marschik PB, Pansy J, et al. The general movement optimality score: A detailed assessment of general movements during preterm and term age. *Dev Med Child Neurol*. 2016;**58**:361-368.
104. Qiu J, Jiang Y, Li F, Tong Q, Rong H, Cheng R. Effect of combined music and touch intervention on pain response and β-endorphin and cortisol concentrations in late preterm infants. *BMC Pediatr*. 2017;**17**:e38.
105. Chen W, Zheng J, Shen G, et al. Music therapy alleviates motor dysfunction in rats with focal cerebral ischemia-reperfusion injury by regulating BDNF expression. *Front Neurol*. 2021;**12**:e666311.
106. Lordier L, Meskaldji D, Grouiller F, et al. Music in premature infants enhances high-level cognitive brain networks. *Proc Natl Acad Sci U S A*. 2019;**116**:12103-12108.
107. Ren H, Jiang X, Meng L, et al. fNIRS-based dynamic functional connectivity reveals the innate musical sensing brain networks in preterm infants. *IEEE Trans Neural Syst Rehabil Eng*. 2022:Online ahead of print. doi: 10.1109/TNSRE.2022.3178078.
108. Perani D, Saccuman MC, Scifo P, et al. Functional specializations for music processing in the human newborn brain. *Proc Natl Acad Sci U S A*. 2010;**107**:4758-4763.
109. Partanen E, Mårtensson G, Hugoson P, Huotilainen M, Fellman V, Ådén U. Auditory processing of the brain is enhanced by parental singing for preterm infants. *Front Neurosci*. 2022;**16**:e772008.
110. Ferrari F, Cioni G, Einspieler C, et al. Cramped synchronized general movements in preterm infants as an early marker for cerebral palsy. *Arch Pediatr Adolesc Med*. 2002;**156**:460-467.
111. Bruggink JLM, Van Braeckel KNJA, Bos AF. The early motor repertoire of children born preterm is associated with intelligence at school age. *Pediatrics*. 2010;**125**:e1356-e1363.
112. Ullsten A, Eriksson M, Klässbo M, Volgsten U. Live music therapy with lullaby singing as affective support during painful procedures: A case study with microanalysis. *Nordic J Music Therapy*. 2016;**26**:142-166.
113. Chiera M, Cerritelli F, Casini A, et al. Heart rate variability in the perinatal period: A critical and conceptual review. *Front Neurosci*. 2020;**14**:e561186.
114. Fairchild KD, O'Shea TM. Heart rate characteristics: Physiomarkers for detection of late-onset neonatal sepsis. *Clin Perinatol*. 2010;**37**:581-598.
115. Standley J. Music therapy research in the NICU: An updated meta-analysis. *Neonatal Netw*. 2012;**31**:311-316.
116. Huang Y, Lei Y, Xie W, Cao H, Yu X, Chen Q. Effect of music therapy on infants who underwent mechanical ventilation after cardiac surgery. *J Card Surg*. 2021:Online ahead of print. doi:10.1111/jocs.15976.
117. Massimello F, Billeci L, Canu A, et al. Music modulates autonomic nervous system activity in human fetuses. *Front Med (Lausanne)*. 2022;**9**:e857591.
118. Sakatani K, Chen S, Lichty W, Zuo H, Wang YP. Cerebral blood oxygenation changes induced by auditory stimulation in newborn infants measured by near infrared spectroscopy. *Early Hum Dev*. 1999;**55**:229-236.
119. Anderson DE, Patel AD. Infants born preterm, stress, and neurodevelopment in the neonatal intensive care unit: Might music have an impact? *Dev Med Child Neurol*. 2018;**60**:256-266.
120. Antinmaa J, Salonen J, Jääskeläinen SK, Kaljonen A, Lapinleimu H. Continuous positive airway pressure treatment may negatively affect auditory maturation in preterm infants. *Acta Paediatr*. 2021:Online ahead of print. doi:10.1111/apa.16029.
121. Ettenberger M, Bieleninik Ł, Epstein S, Elefant C. Defining attachment and bonding: Overlaps, differences and implications for music therapy clinical practice and research in the neonatal intensive care unit (NICU). *Int J Environ Res Public Health*. 2021;**18**:e1733.
122. Kehl SM, La Marca-Ghaemmaghami P, Haller M, et al. Creative music therapy with premature infants and their parents: A mixed-method pilot study on parents' anxiety, stress and depressive symptoms and parent-infant attachment. *Int J Environ Res Public Health*. 2020;**18**:e265.

123. Loewy J, Stewart K, Dassler A, Telsey A, Homel P. The effects of music therapy on vital signs, feeding, and sleep in premature infants. *Pediatrics*. 2013;**131**:902-918.
124. Robertson AM, Detmer MR. The effects of contingent lullaby music on parent-infant interaction and amount of infant crying in the first six weeks of life. *J Pediatr Nurs*. 2019;**46**:33-38.
125. Hamm EL, Chorna OD, Flanery A, Maitre NL. A parent-infant music therapy intervention to improve neurodevelopment after neonatal intensive care. *Acta Paediatr*. 2017;**106**:1703-1704.
126. Prouhet PM, Gregory MR, Russell CL, Yaeger LH. Fathers' stress in the neonatal intensive care unit: A systematic review. *Adv Neonatal Care*. 2018;**18**:105-120.
127. Koliouli F, Gaudron CZ, Bourque CJ, Raynaud J. Parental sense of competence, paternal stress and perceived construction of the relationship with the premature newborn: A mixed method study. *Early Hum Dev*. 2022;**168**:e105576.
128. Almadhoob A, Ohlsson A. Sound reduction management in the neonatal intensive care unit for preterm or very low birth weight infants . *Cochrane Database Syst Rev*. 2020;**1**:CD010333.
129. Haslbeck FB, Bassler D. Music from the very beginning-A neuroscience-based framework for music as therapy for preterm infants and their parents. *Front Behav Neurosci*. 2018;**12**:e112.
130. Wachman EM, Lahav A. The effects of noise on preterm infants in the NICU. *Arch Dis Child Fetal Neonatal Ed*. 2011;**96**:F305-F309.
131. Chifa M, Hadar T, Politimou N, Reynolds G, Franco F. The soundscape of neonatal intensive care: A mixed-methods study of the parents' experience. *Children (Basel)*. 2021;**8**:e644.
132. Rossetti A. Environmental music therapy (EMT): Music's contribution to changing hospital atmospheres and
perceptions of environments. *Music and Medicine*. 2020;**12**:130-141.
133. Loewy J. NICU music therapy: Song of kin as critical lullaby in research and practice. *Ann N Y Acad Sci*. 2015;**1337**:178-185.
134. Casavant SG, Cong X, Moore J, Starkweather A. Associations between preterm infant stress, epigenetic alteration, telomere length and neurodevelopmental outcomes: A systematic review. *Early Hum Dev*. 2019;**131**:63-74.
135. Bembich S, Risso FM, Stan N, et al. Preterm newborn adaptive responses to daily nursing during neonatal intensive care unit stay, associate with neurodevelopment, 2 years later. *Am J Perinatol*. 2021:Online ahead of print. doi: 10.1055/a-2077.
136. Pineda R, Raney M, Smith J. Supporting and enhancing NICU sensory experiences (SENSE): Defining developmentally-appropriate sensory exposures for high-risk infants. *Early Hum Dev*. 2019;**133**:29-35.