Early-life endocrine regulation and neurodevelopmental outcomes

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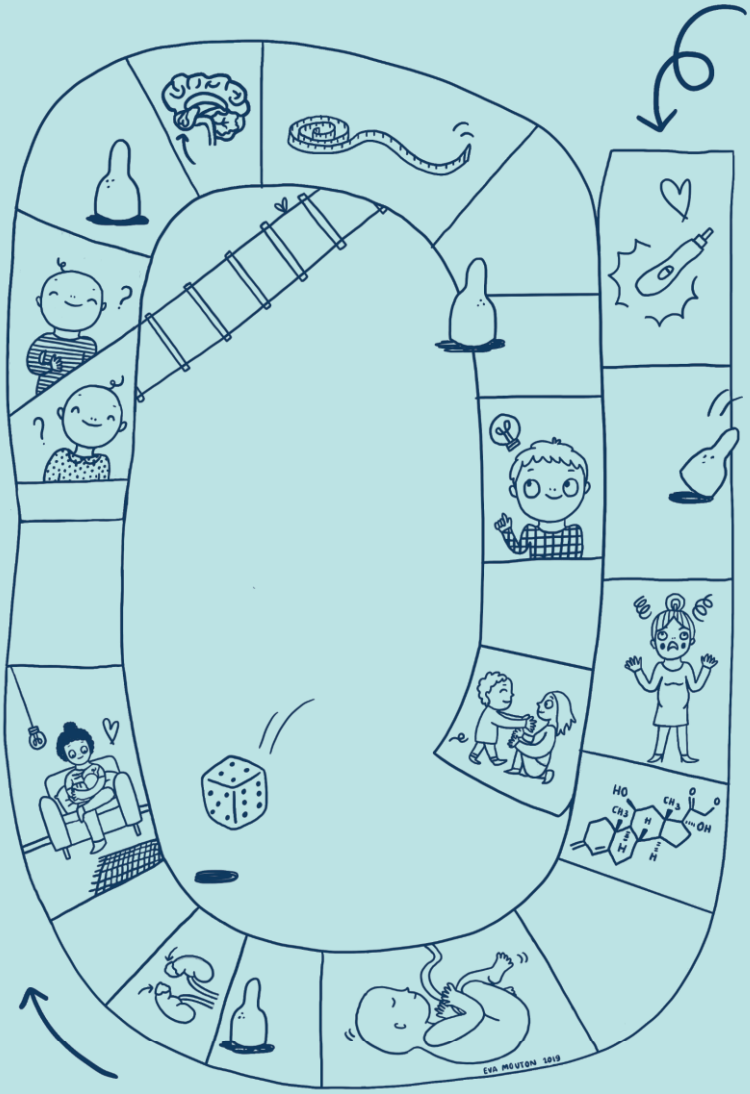
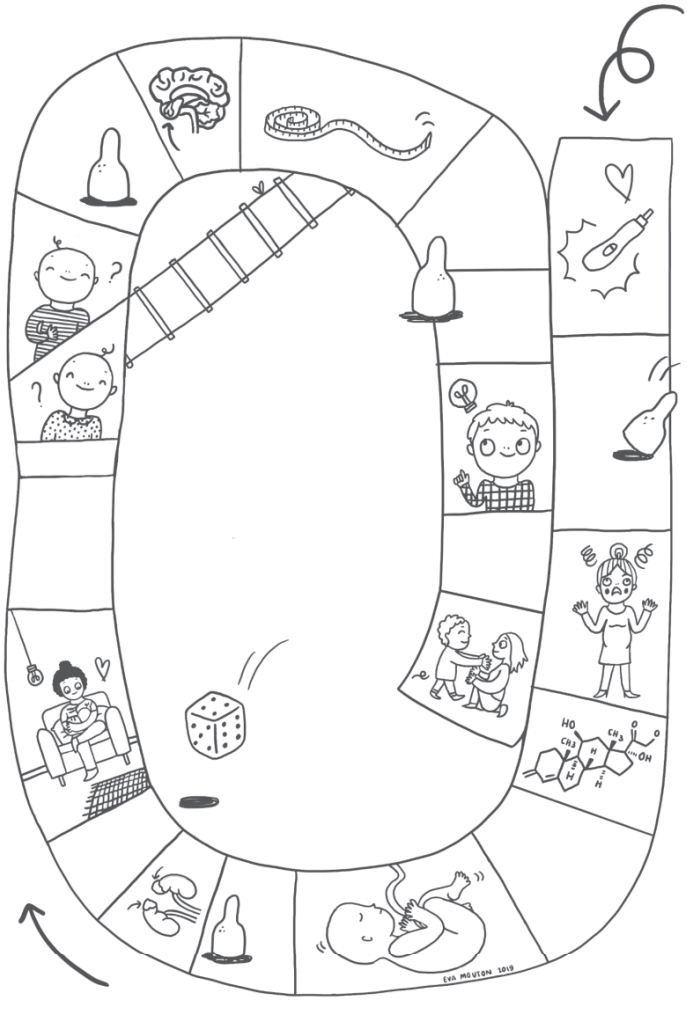
Discussion

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General discussion

GENERAL DISCUSSION

In this chapter, the main findings of this thesis are summarized, some strengths and limitations are reflected on, and implications for future research are discussed.

METHODOLOGICAL CONSIDERATIONS

Composition of study population

In order to measure the correct determinants, composing an appropriate study population is crucial. When studying preterm infants, many inclusion criteria have previously been used, ranging from a gestational age (GA) of <37 weeks to <28 weeks. Additionally, infants have also been included based on birth weight (BW), from <2,500 grams to <1,000 grams. In **chapters 13 and 14** we have compared the differences in outcomes when infants were included based on being born very preterm (VP; i.e., GA<32 weeks) and/or with a very low birth weight (VLBW; i.e., BW<1,500 grams) by using a cohort which was established in 1983, the Project On Preterm and Small-for-gestational-age infants (POPS cohort). Not surprisingly, VP infants significantly differed from VLBW infants in growth pattern, final height and neurodevelopmental outcomes. For future studies it is desirable to use inclusion criteria that result in representative samples of preterm infants. Since the definition of prematurity is based on pregnancy duration and not because of a low birth weight, we recommend that future studies in preterm infants should include subjects based on gestational age rather than birth weight, at least in countries where gestational age can be reliably assessed in utero. This is in line with previous recommendations which were based on short-term outcomes.[1-4] In addition, the inclusion criteria of the study population should be taken into account when interpreting results from previous studies, since results in populations included based on BW cannot necessarily be applied to preterm populations, and vice versa. In meta-analyses it is especially important to include a uniform group of infants, as only in this way valid conclusions can be reached.

Influence of sex on (endocrine) outcomes

Aside from including the appropriate study population, statistical analyses should also be performed correctly, including adjustment for potential confounders and/or effect modifiers. With regard to hypothalamus-pituitary-adrenal (HPA) axis activity, it was previously thought that sex differences arose during puberty, under the influence of sex hormones.[5,6] However, as shown in **chapters 9 and 10**, basal HPA-axis activity as well as HPA-axis reactivity already appear to be different between sexes in early childhood.

Consequently, it is important to take sex into account when analyzing HPA-axis (re)activity, regardless of age.

Standardization of protocols

Lastly, comparing sex differences in HPA-axis reactivity in **chapter 10** was hampered by the lack of standardization, both with regard to the testing protocol as well as when presenting results. We recommend that future studies assessing HPA-axis reactivity take the following considerations into account: 1) the type of stress that is to be assessed and which level of the HPA-axis is to be tested, 2) whether a standardized stress protocol is available for the desired type of stress to be tested, and 3) the use of a standardized presentation of results, by reporting both absolute glucocorticoid (GC) concentrations as well as derived variables, which will allow for a full overview of HPA-axis reactivity. This would enable comparisons between studies, which consequently will lead to improved conclusions with regard to stress reactivity in general and sex differences in HPA-axis reactivity specifically.

EARLY-LIFE CORTISOL REGULATION

Intra-uterine glucocorticoid regulation

By using GC concentrations measured in hair cut directly postpartum, we attempted to describe intra-uterine GC regulation in **chapters 2 and 3**.

In **chapter 2**, neonatal hair GCs were found to be >5 times higher compared to maternal GC levels directly postpartum, with a sharp decrease within ±6 weeks after birth. However, GC concentrations remained significantly higher than maternal GC levels, and it therefore appears that hair GC levels in the infant reflect both the intra- and extrauterine period at that age. Additionally, a strong positive association was found between neonatal hair GC concentrations and gestational age. Weaker, but nonetheless significant, associations were found with other perinatal factors, such as birth weight (but only in kgs, not when expressed as SDS), perinatal infection, and delivery via caesarian section. The association with gestational age is thought to reflect the positive feedback loop, a placenta-driven phenomenon, which causes an increase in GCs at the end of the third trimester.[7] This positive feedback loop might be a part of the mechanism behind the induction of labor, and appears to promote fetal organ maturation.[8,9]

Next, the associations between maternal distress during pregnancy and neonatal hair GC levels were assessed in **chapter 3**. Elevated stress scores pre- and perinatally were associated with decreased neonatal hair GC levels, with the largest decrease seen in infants who were exposed to persistent maternal stress. In contrast, maternal hair GCs were found to be increased when mothers experienced excessive anxiety symptoms

around birth. We speculate that exposure to increased maternal stress and/or maternal GCs in utero is associated with suppression of fetal HPA-axis activity. This in turn might underlie the associations between fetal exposure to maternal stress and/or excessive maternal GCs in utero and neurodevelopmental problems as well as altered HPA-axis settings in the offspring.[10] Lastly, maternal use of selective antidepressants did not affect neonatal hair GC levels, and antidepressants are therefore unlikely to explain the associations between maternal stress and neonatal hair GC levels.

Extra-uterine glucocorticoid regulation

After birth, infants are still exposed to small amounts of maternal GCs through breastmilk. Previously, our group has shown that breastmilk GCs follow the maternal HPA-axis activity, but the effect of this diurnal rhythm on the infant is unknown. In **chapter 4** we have summarized the existing knowledge on the effects of breastmilk GCs on offspring. Both in vitro and in vivo studies have shown that breastmilk GCs could promote intestinal maturation. Additionally, systemic effects have also been found, and breastmilk GCs might also affect the intestinal microbiome. However, both the laboratory analysis of GCs, as well as the sample collection in most studies leave a lot to be desired. Many studies use immunoassays, which show cross-reactivity with other hormones,[11] and none of the studies took the diurnal rhythm of breastmilk GCs into account.

We therefore conducted the Cortisol in Mother's Milk (CosMos) study, of which the results are presented in **chapters 5 to 8**. In order to ascertain that any associations found between breastmilk GCs and outcomes in the offspring were not due to other components in breastmilk, the association between breastmilk GC levels and macronutrient concentrations was studied in **chapter 5.** No association between breastmilk GCs and macronutrients were found, probably due to differences in excretion mechanisms: whereas breastmilk GCs are presumably excreted into breastmilk via passive diffusion,[12] macronutrients are subject to active secretion.[13]

Next, the effect of the diurnal rhythm of breastmilk GCs was assessed. In **chapter 6**, the diurnal rhythm of GCs in infants' saliva was determined, and several possible influencing factors were analyzed. At the age of one month, infants displayed a diurnal GC rhythm at a group level, which was strikingly found to be biphasic, possibly reflecting HPA-axis development. In utero, fetuses appear to already display a diurnal HPA-axis activity, but with a peak in the afternoon.[14] Our results might reflect the transitional period between fetal-type and adult-type HPA-axis activity. Of the studied influences, only breastmilk GC exposure and variability were weakly associated with the time of peak in the infants. These associations might be due to a signaling effect of breastmilk GCs, or due to the increased mother-infant synchrony seen in breastfed compared to formula-fed infants. Lastly, the associations between breastmilk GC rhythmicity and the infants' behavior, sleep and body composition were assessed in **chapters 7 and 8**. No associations were

found, although some results seemed to suggest that outcomes with regard to infant behavior and sleep could be modified by infant sex and/or experienced maternal stress. The lack of associations might be due to methodological shortcomings, such as a small sample size and a short follow-up, but it could also mean that breastmilk GC rhythmicity does not significantly influence the infants.

Sex differences in basal cortisol concentrations and HPA-axis reactivity in children aged 0-18 years were assessed in **chapters 9 and 10**. The meta-analysis performed in **chapter 9** showed that basal cortisol levels were already different between boys and girls prepubertally, with higher serum, saliva and urine cortisol levels in boys <8 years. During and after puberty, cortisol levels also showed sex differences, but now cortisol levels in serum and saliva were found to be lower in boys compared to girls >8 years, while urine cortisol levels remained higher in boys. However, the sex differences in serum cortisol levels were not very robust. In **chapter 10**, sex differences in HPA-axis reactivity were analyzed in a systematic review. Due to the heterogeneity of methods and presentation of results of all the included studies, age-stratified analyses could not be performed. Additionally, due to this methodological heterogeneity, although it appeared that girls had a more variable diurnal rhythm, a higher cortisol awakening response and a stronger response to social stress tests than boys, definitive conclusions could not be drawn. However, although the sex differences found in these studies were of a small magnitude and not very conclusive, they might contribute to the sex-specific origins of health and disease in the long-term.

NEURODEVELOPMENTAL OUTCOMES IN PRETERMS

Preterm thyroid regulation

Due to the immature hypothalamus-pituitary-thyroid (HPT) axis after preterm birth, preterm infants are at a risk of developing transient hypothyroxinaemia of prematurity (THoP). THoP is characterized by low T4 but normal TSH levels, and has previously been associated with adverse neurodevelopmental outcomes in childhood.[15-18] We used the POPS cohort to assess the effects of THoP on neurodevelopment at age 19 years in **chapters 11 and 12**. No associations were found between THoP and IQ or neuromotor development. THoP was associated with more internalizing and total problem behavior. However, it is unclear whether these associations are due to causality, especially since problem behavior has a multifactorial etiology. Since no effects were found on IQ and neuromotor development, and only small effects were found on behavior, our results do not support screening preterm infants for THoP. As a recent review stresses,[19] it is important to distinguish THoP from congenital hypothyroidism, so repeated screening for this purpose seems necessary. Recent findings also point towards a role of the placenta

in the availability of thyroid hormones in preterm infants,[20,21] suggesting that thyroid regulation is complex, especially in preterm infants.

Intra- and extrauterine growth

Significant medical advances have been made with regard to the treatment of preterm infants in the past decades. This has shifted attention towards long-term outcomes in these infants, especially since only a modest decrease in morbidity has been observed, despite decreasing mortality. Growth has previously been shown to be a significant contributor to neurodevelopmental outcomes.[22-25] In **chapter 15**, growth patterns and the impact of these growth patterns on neurodevelopment are compared between cohorts 20 years apart. While the occurrence of adverse growth patterns (i.e., small-for-gestational-age (SGA) without catch-up growth or appropriate-for-gestational-age with postnatal growth retardation) has decreased, which might be a reflection of improved care over time, the associations between adverse growth and neurodevelopmental outcomes did not significantly differ. Therefore, it is important to remain focused on achieving optimal growth in preterm infants.

This conclusion is strengthened by the results of **chapters 13 and 14**, in which the effects of being born VP and/or with a VLBW were compared. While these studies were not strictly performed to assess the effect of intra-uterine growth, the presence of SGA infants was highest in the VP-/VLBW+ group, and lowest in the VP+/VLBW- group. Concomitantly, growth trajectories and final height, as well as neurodevelopmental and functional outcomes were worst in the VP-/VLBW+ group, and best in VP+/VLBW- infants. Whether growth directly impacts neurodevelopment, or whether a common denominator is associated with both worse growth and worse neurodevelopmental outcomes is unclear. Additionally, the POPS cohort, in which these analyses were performed, was established in 1983. It is therefore likely that the distribution of infants between the VP and/or VLBW groups has changed. Moreover, due to improved care, which has resulted in better growth as seen in **chapter 15**, the magnitude of associations might have changed. However, the results of **chapters 13 and 14** do support the notion that continued attention should be paid to growth, both in utero and after birth.

STRENGTHS AND LIMITATIONS

One of the strengths of this thesis is the use of novel laboratory techniques to measure GCs. Our group previously developed an LC-MS/MS method to measure GCs in breast-milk, which enabled us to assess the associations between the GC diurnal rhythm in milk and (neurodevelopmental) outcomes in offspring. We also used GCs measured in hair for some of our analyses, which offers a retrospective view of HPA-axis activity,[26] thereby

enabling us to assess intra-uterine HPA-axis regulation. Additionally, a wide variety of statistical methods were used, among them the use of specialized rhythm software, as well as the comparison of two cohorts. Lastly, several of these studies were performed using the POPS cohort, which encompasses 94% of the children born VP and/or VLBW in 1983. It is therefore a large and comprehensive cohort, with a long follow-up period.

This thesis also has its limitations. All of these studies are observational, and it is therefore not possible to assess the causality of any associations found. Observational studies are also subject to several sources of bias and uncontrollable confounding factors. For instance, in this thesis, research was hampered by inclusion of a selected population (**Chapters 2, 3, 5-8 and 15**), losses to follow-up (**Chapters 3, 11-15**), unstandardized timing of collection of samples (**Chapters 5-9**), use of non-specific laboratory methods (**chapters 9 and 10**), selection by survival (**Chapters 11 and 12**), and lack of standardized protocols and data presentation (**Chapter 10**). Additionally, the studies which used the POPS cohort (**Chapters 11-14**) might not be externally valid, since this cohort was established in 1983, and the associations found could be subject to changes in time. The most obvious change between the POPS cohort and the current NICU population, is the distribution of infants among the three VP and/or VLBW groups, as studied in **chapters 13 and 14**. Due to increased surveillance during pregnancy, severe growth retardation is less common, and it is therefore likely that the number of children born VP-/VLBW+ has decreased in comparison with the VP+ groups.

Lastly, newly gained knowledge cannot retroactively be applied to past research. The effect of THoP on neurodevelopment and behavior was therefore studied in the entire POPS cohort (**chapters 11 and 12**), rather than only the VP infants, as recommended in **chapter 13 and 14**. However, stratified analyses according to gestational age (< and ≥ 29 weeks) were performed, which did not change the results.

FUTURE PERSPECTIVES AND IMPLICATIONS

Future research

As was seen in several of the chapters in this thesis, research in children and in general would benefit from an increase in standardization. When studying preterm infants it is best to include infants based on gestational age rather than birth weight, at least in countries where gestational age can be reliably assessed in utero. Simultaneously, the application of results to clinical practice should be limited to the researched population in the study, and not extrapolated to infants with different characteristics. Moreover, systematic reviews and meta-analyses should only use studies with uniform inclusion criteria, as only then valid conclusions can be drawn. Methodology, especially for stress tests, should also be standardized as best as possible, which will facilitate the comparison

of results between studies. Lastly, correcting for sex is recommended when analyzing results of studies into HPA axis activity.

Glucocorticoid regulation

Although this thesis has already shed some light on intra-uterine HPA-axis activity, future research should further elucidate this. Additionally, HPA-axis development in early life should be further investigated through a longitudinal study. This way, the origins of the biphasic diurnal rhythm found in this thesis can be further researched, while other aspects of HPA-axis development, such as the possible influence of breastmilk GCs, can also be studied in more detail.

Knowledge of physiological HPA-axis development and activity in healthy newborns enables the subsequent study of pathological HPA-axis development and activity. In preterm infants, HPA-axis activity is often inadequate, manifested as adrenal insufficiently.[27] Future research should investigate HPA-axis development in preterm infants, as well as the associations between HPA-axis activity and clinical correlates including refractive hypotension and bronchopulmonary dysplasia (BPD). Additionally, prematurity has been associated with an upregulated HPA-axis in childhood,[28] and it should be further investigated whether this is associated with early-life HPA-axis activity.

Neurodevelopment in preterms

Neurodevelopment in preterm infants is a difficult and multifactorial issue. This thesis once again confirms the importance of adequate growth, both in utero as well as after birth, on neurodevelopmental outcomes. It is therefore important to pay continued attention to promoting healthy growth in preterm infants. THoP does not appear to influence neurodevelopment at a later age, although selection by survival cannot be ruled out, since T4 concentrations were significantly lower in the deceased group. Additionally, it is well known that thyroid hormones are crucial for the developing brain,[29] and the etiology of THoP appears to be more complex than previously thought.[20,21] It is therefore recommendable to further study the effect of THoP on neurodevelopment in a more recent cohort, in which the prevalence of morbidity and mortality is different compared to the POPS cohort. However, the influence of hormones might not be noticeable due to the bigger influences of general preterm illnesses. For example, a recent study showed that BPD explained 65% of the variance in intelligence.[30] The prevention of BPD is therefore of paramount importance. The HPA-axis has been implicated as a contributor to the development and/or prevention of BPD, and should therefore also be studied.

CONCLUSIONS

In this thesis, we have explored HPA-axis activity and development. We found that hair GCs reflect intra-uterine HPA-axis activity and were greatly affected by gestational age, while maternal distress during pregnancy also has its influences. After birth, HPA-axis activity in 1-month-old infants showed a biphasic diurnal rhythm, possibly reflecting HPA-axis development. Breastmilk GC rhythmicity might influence the infant's HPA-axis activity at age 1 month, but it was not associated with other neurodevelopmental and growth outcomes in the infants at age 3 months. Neurodevelopment at age 19 was associated with intra- and extra-uterine growth, but not with thyroid functioning in preterm infants. Lastly, in order to improve research and the comparison of studies, we concluded that methodological standardization with regard to inclusion criteria as well as testing protocols should be encouraged. These new insights form a foundation and a framework for future studies, particularly with regard to HPA-axis functioning in preterm infants and its effects on long-term neurodevelopmental outcomes.

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