Title: Salivary and hair glucocorticoids and sleep in very preterm children during school age

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Running title: Salivary and hair glucocorticoids in very preterm children

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Summary

Very preterm birth involves increased stress for the child, which may lead to programming of the hypothalamic-pituitary-adrenal (HPA) axis activity and poor sleep in later life. Moreover, there is evidence for a relationship between HPA axis activity and sleep. However, research with objective sleep measures in very preterm children during school-age is rare. Eighty-five healthy children born very preterm (<32nd gestational week) and 91 full-term children aged 7-12 years were recruited for the present study. To assess HPA axis activity, salivary cortisol was measured at awakening, 10, 20, and 30 min later. In addition, hair cortisol and cortisone concentrations were quantified using liquid chromatography tandem mass spectrometry to assess cumulative endocrine activity over the preceding months. One night of in-home polysomnographic sleep assessment was conducted to assess sleep duration, sleep continuity, and sleep architecture. Children born very preterm showed significantly lower levels of cortisol at awakening and lower overall post-awakening cortisol secretion, lower cortisone in hair, and earlier sleep onset than full-term children. Across the whole sample, overall post-awakening cortisol secretion was positively related to sleep onset time and negatively to sleep duration. The association between prematurity status and post-awakening cortisol secretion was partially mediated by earlier sleep onset time. In conclusion, this study provides evidence for a possible down-regulation of the HPA axis activity and slightly earlier sleep phase in very preterm children during school age.

1. Introduction

The hypothalamic-pituitary-adrenal (HPA) axis controls adaptive reactions of the organism to stressors by managing the secretion of glucocorticoids including cortisol (Clements, 2013). Early life events may be involved in long-term programming of the HPA axis during fetal and early postnatal development (Kajantie and Räikkönen, 2010). One such early life event is very preterm birth, defined as live birth before 32 weeks of gestation are completed, which occurs in approximately 1 to 2% of all births world-wide (Child Trends, 2015) and involves increased risk for cognitive and psychosocial impairments across the life span (Aarnoudse-Moens, et al., 2009; Lemola, 2015). Very preterm birth involves several adversities that could induce HPA axis programming: First, pregnancy-related aspects, such as maternal infections, inflammations, and prenatal stress, may influence both the risk of preterm birth and children's brain development (Buss et al., 2012; Goldenberg et al., 2008; Monk et al., 2016). Second, children born very preterm suffer from the immature functioning of the lungs, often leading to hypoxia (Saigal and Doyle, 2008) and the adrenal gland leading to potential adrenal insufficiency (Fernandez and Watterberg, 2009). Third, children born very preterm are exposed to many distressing medical procedures including blood draws and mechanical ventilation (Anand, 2001; Brummelte et al., 2015; Grunau et al., 2007).

Studies with children born very preterm and animal models of early life adversities show similar HPA axis alterations – often involving persistent down-regulation of HPA axis activity (Feng et al., 2011; Kaseva et al., 2014). School aged children born very preterm or with very-low-birth-weight (VLBW; < 1500 g) show lower diurnal cortisol profiles (Wadsby et al., 2014), faster decreasing cortisol levels in the evening (Perkinson-Gloor et al., 2015), and decreased salivary cortisol responses to social stress (Buske-Kirschbaum et al., 2007), which is also consistent with findings in adults (Kaseva et al., 2014). Relatedly, boys born very preterm exposed to more distressing medical procedures had lower diurnal cortisol levels at age 7 (Brummelte et al., 2015). Moreover, Grunau et al. (2013) reported lower hair cortisol levels in very preterm compared to full-term children. However, there are also conflicting data. For example, preterm born children were found to exhibit no differences regarding morning (Brummelte et al., 2015; Perkinson-Gloor et al., 2015), evening (Quesada et al., 2014), and diurnal cortisol profiles (Buske-Kirschbaum et al., 2007; Kaseva et al., 2014) and salivary cortisol responses to

social stress (Brummelte et al., 2015; Buske-Kirschbaum et al., 2007), or even higher salivary cortisol levels at awakening (Buske-Kirschbaum et al., 2007; Quesada et al., 2014).

Beside trait factors, there are also more transient state factors that affect HPA axis function (Stalder et al., 2016). An important state factor affecting cortisol secretion after morning awakening is the duration and quality of sleep the preceding night (Elder et al., 2014; Lemola et al., 2015). Children with short and poor sleep had increased HPA axis activity after awakening (Fernandez-Mendoza et al., 2014; Pesonen et al., 2014; Räikkönen et al., 2010). Two studies examining the relationship between sleep architecture assessed by sleep-electroencephalography (EEG) and morning cortisol secretion showed increased morning cortisol secretion in children with short sleep duration, shorter relative amounts of slow wave sleep (SWS), longer relative amounts of light sleep (including stage 1 sleep and stage 2 sleep), and rapid-eye-movement (REM) sleep (Hatzinger et al., 2013; Lemola et al., 2015). Importantly, there is also evidence that sleep regulation is altered after preterm birth (Brooks and Canal, 2013) possibly leading to differences compared to term born peers during adolescence and young adulthood involving earlier bedtimes and circadian preference (Björkqvist et al., 2014; Hibbs et al., 2014; Strang-Karlsson et al., 2010). Moreover, poor sleep is also more prevalent in children born very preterm compared to full term peers including more sleep disordered breathing (Rosen et al., 2003), nocturnal awakenings, light sleep, and less SWS (Perkinson-Gloor et al., 2015).

Taken together, children born very preterm are at an increased risk for HPA axis alterations and poor sleep, which, in turn, are also likely to be interrelated. However, there are important gaps in knowledge and existing research is characterized by heterogeneous findings. Part of this may be due to methodological factors related to saliva sampling, which may be addressed by adhering to recent methodological recommendations (see Stalder et al., 2016). Applying a multi-method assessment strategy by additionally using the recently introduced method of hair steroid analysis, providing a stable and trait-like measure of integrated long-term cortisol secretion (Stalder and Kirschbaum, 2012) may further allow to examine whether findings from saliva measures can be corroborated. In addition, research examining the complex links between very preterm birth, morning cortisol secretion, and sleep alterations during the preceding night is important for resolving the apparent paradox that poor sleep was related to increased HPA axis activity (e.g. Fernandez-Mendoza et al., 2014), while children born very preterm often showed both poor sleep and decreased HPA axis activity (e.g. Wadsby et al., 2014).

The present study thus tested the following hypotheses: First, we hypothesized that HPA axis activity, assessed through post-awakening cortisol and hair cortisol and cortisone, is decreased in children born very preterm compared to full-term children. Moderation of these associations by sex was also examined, given previous findings on sex differences in HPA axis activity. Further, additional analyses tested associations of birth weight and gestational age with HPA axis activity within the group of very preterm children expecting decreasing HPA axis activity the earlier gestation children were born. Second, we hypothesized that earlier sleep times and poorer sleep (more nocturnal awakenings, more light sleep, and less SWS) would be found in children born very preterm compared to full-term children. Third, we hypothesized that post-awakening cortisol would be negatively associated with sleep duration, sleep continuity (including higher sleep efficiency and less nocturnal awakenings), and SWS and positively associated with light sleep and REM-sleep. Finally, we examined whether differences in sleep of the preceding night accounted for differences in post-awakening cortisol secretion between children born very preterm and full-term.

2. Methods

2.1. Study population

Between May 2013 and August 2014, 85 healthy very preterm children (<32nd gestational week; age: $M = 9.5$ years, $SD = 1.4$; range: 7.4 to 12.4) and 91 full-term children (age: $M = 9.6$, $SD = 1.4$; range: 6.9 to 13.0) were recruited for the present study, which was the second wave of a longitudinal study on very preterm birth, HPA axis activity, and sleep (see e.g. Perkinson-Gloor et al., 2015; Lemola et al., 2015 for reports on the first study wave). Due to dropout from and resampling after the first study wave the present sample was only partially overlapping with the sample of the first wave. In the present sample 46 (54.1%) of the very preterm children and 43 (47.3%) of the full-term children already participated in the first study wave, while 39 (45.9%) of the very preterm and 48 (52.7%) of the full-term children were newly recruited for the second wave.

Very preterm children were recruited from an initial cohort of 260 prematurely born children treated at the University Children's Hospital Basel (Switzerland) between June 2001 and December 2006. Fifty-two (20.0%) of the 260 very preterm children met the exclusion criteria; no information on neurobehavioral development until age 2 years ($n = 39$), severe developmental delay ($n = 7$), insufficient

German language skills of parents to give informed consent (n = 6). Further 38 (14.6%) very preterm children lived outside of Switzerland or too distant from the study center (> 100 km). Of the remaining 170 eligible very preterm children, 22 (12.9% of eligible children) could not be traced. Thus, 148 (87.1% of the eligible children, 56.9% of the initial sample) were contacted by phone and 85 (50.0% of eligible children, 32.7% of initial cohort) agreed to participate in the current study. Participating preterm children did not differ from non-participants with regard to birth weight (1325 g vs. 1260 g, $F(1,257)$ = 1.52, p = .219), gestational age (29.7 weeks vs. 29.5 weeks, $F(1,258)$ = .98, p = .323), and length of hospital stay (51.8 days vs. 53.3 days, $F(1,224)$ = .21, p = .649). Four (4.7%) out of 85 children born very preterm were born small for gestational age (SGA). The sample characteristics are presented in Table 1.

The 91 full-term children (>37 weeks of gestation) were recruited from official birth notifications. The samples were comparable regarding age and sex. All children attended primary or secondary school in Switzerland. Five very preterm children and one full-term child received additional support at school (e.g. by a remedial teacher) or visited small group classes.

Post-hoc power analysis using G*Power was performed to evaluate the statistical power given the sample size of the study (Faul et al., 2007). Regarding mean differences between very preterm and full-term children the chance of detecting effects of medium size (d =.50) was 91% at a .05 alpha level (two-sided). Regarding correlations between two variables, the power analyses indicated a 99% chance of detecting effects of medium size (r = .30) at a .05 alpha level (two-sided; based on the total sample size). Therefore, we considered the study to be sufficiently powered to detect medium size effects (Cohen, 1988).

(Insert Figure 1 and Table 1 about here)

2.2. Procedure

Trained study personnel visited the children at home on a regular school day to administer in-home sleep-EEG, collect hair samples, and instruct parents on how to collect saliva samples the following morning. Parents completed questionnaires to assess demographic data. From the medical files of the University Children's Hospital Basel information on neonatal health of the very preterm children was

obtained. Assent was obtained from the children and parents gave written informed consent for the children to participate. The study was approved by the Ethics Committee of Basel and performed in accordance with the ethical standards laid down in the Declaration of Helsinki.

2.3. Variables

2.3.1. Assessment of post-awakening cortisol secretion

To assess post-awakening cortisol secretion, parents were instructed to collect four saliva samples from their children at 0, 10, 20, and 30 min after the child's awakening. Parents were instructed that their children were not allowed to eat or drink and brush their teeth until saliva sampling was completed. Awakening times ranged from 0501 h to 0730 h ($M = 0635$ h, $SD = 22$ min). Due to home visits being conducted on a Friday in four children, these had later awakening times on the following Saturday morning. However, analyses examining salivary cortisol and sleep did not differ when these children were excluded, therefore they were included in the sample used for analyses. Saliva samples were collected using the "Salivette" device (Sarstedt, Nümbrecht/Germany). Free salivary cortisol concentrations were analyzed using a time-resolved immunoassay with fluorometric detection ''Coat-A-Count'' Cortisol RIA from DPC (Diagnostics Products Corporation; obtained through H. Biermann GmbH, Bad Nauheim, Germany).

The statistical analyses strived to follow published guidelines on post-awakening cortisol assessment (Stalder et al., 2016). Specifically, three measures were computed to quantify different aspects of post-awakening cortisol secretion: the level of cortisol on awakening (S1), the area-under-the-curve with respect to increase (AUC_I) and with respect to ground (AUC_G; Pruessner et al., 2003). To deal with outliers, salivary variables were log-transformed before building AUC_G and AUC_I. S1 was interpreted as the endpoint of the pre-awakening cortisol increase and the AUC_I was interpreted as reflecting the cortisol awakening response (CAR; the CAR is viewed as a normal part of the human circadian rhythm in which cortisol levels increase across the first 30 to 40 min after awakening; Stalder et al., 2016). The AUC_G was viewed as an additional estimate of overall post-awakening cortisol secretion (Stalder et al., 2016). Cortisol values were skewed and data were thus log-transformed before computing composite measures. Finally, as suggested by the guidelines, additional analyses were run in which available PSG data were utilized to compare parent-reported light-on-times to objectively

measured awakening times. In these additional analyses, the main analytical strategy (see below) was repeated after exclusion of children with a discrepancy between parent-reported cortisol sampling time and objective PSG-based awakening time of more than 5 min ('parent reported light-on-time' – ' in-home PSG awakening time > 5 min; Stalder et al., 2016). After exclusion of these children, the findings of the additional analyses were equal regarding significance and effect size (data not shown).

2.3.2. Assessment of hair glucocorticoids

Trained study personnel cut hair strands (3 mm diameter, minimum: 3 cm length) with fine scissors at the base of the hair shaft from a *posterior vertex* position from 2 small spots. Hair samples were labeled, secured on aluminum foil and stored in a dark and dry cupboard at room temperature until analyses. A first part of 38 hair samples (23.3% of the cohort) were sent to and analyzed in the laboratory of TU Dresden, Germany in autumn 2013, the other 125 hair samples (76.7% of the cohort) in autumn 2014. Hair cortisol and cortisone concentrations were determined from scalp-near 3 cm hair segments using liquid chromatography tandem mass spectrometry (LC-MS/MS), which is considered the current gold standard method for hair steroid analysis (Gao et al., 2016). Wash and steroid extraction procedures followed the protocol described in Stalder et al. (2012; study II) with minor adaptations. Briefly, samples were washed twice in 3 ml isopropanol for 3 minutes. For glucocorticoid extraction 7.5 mg of hair were incubated with 1.8 ml of methanol for 18 hours at room temperature. The methanol was evaporated at 50 °C under a constant stream of nitrogen until the samples were completely dried. The dry residue was resuspended using 175 μl double-distilled water. 100 μl were used for analysis by a Shimadzu HPLC-tandem mass spectrometry system (Shimadzu, Canby, Oregon, USA) coupled to an ABSciex API 5000 Turboion-spray triple quadrupole tandem mass spectrometer (AB Sciex, Foster City, California) with purification by on-line solid-phase extraction. Intra and interassay coefficients of variance for hair cortisol and cortisone have been shown to be between 3.7–8.8 % (Gao et al., 2013). Outliers in hair glucocorticoid variables were truncated to a value of 2 interquartile ranges above the median.

2.3.3. Sleep assessment

Sleep was assessed using in-home PSG during a single night at the children's home. In-home EEG was used to minimize the differences between the child's usual sleep conditions and the conditions during the study night. Using the Compumedics Somté PSG, polysomnogram signals C3/A2 and C4/A1 EEG, right and left electrooculogram and bipolar submental electromyogram were obtained. Two experienced raters visually analyzed the EEG reports according to the standard procedures (Rechtschaffen and Kales, 1968). The following sleep indices were evaluated: Sleep continuity: Sleep duration (the total sleep time, i.e., the time in bed minus time spent awake in hours), sleep efficiency (sleep duration/time in bed × 100), and nocturnal awakenings (number of arousals from sleep). Sleep architecture (%): Stage 1 sleep, stage 2 sleep, SWS (SWS: Stages 3 and 4 sleep), REM sleep, and REM latency (min). In addition parents completed a short sleep questionnaire for the night of the EEG assessment and reported the child's awakening time. After one outlier of bedtime was winsorized to a value of 2 interquartile ranges above the median, bedtime ranged from 1940 h to 2310 h ($M = 2119$ h, $SD = 39$ min). Polysomnographic sleep data was available for 58 (68.2%) very preterm and 85 (93.4%) full-term children. As there were no differences in salivary cortisol measures between participants and non-participants of the PSG-sleep assessment (all p-values > 0.10) participation on the PSG should not have strongly affected our findings. For 126 (71.6%) children both sleep and post-awakening cortisol was available (very preterm: 51 (60.0%); full-term: 75 (82.4%)).

2.4. Control variables

All analyses were controlled for first language, maternal education, children's age, and sex if not stated

otherwise. Analyses involving salivary cortisol were additionally controlled for awakening time as

measured by polysomnography and analyses involving hair cortisol variables were additionally

controlled for the time point of the lab analyses (i.e., if the analysis took place in autumn 2013 or in

autumn 2014). Due to the extended time period of the study assessment, analyses with HPA axis activity

were additionally controlled for season (Stalder et al., 2016). All statistical analyses were performed

with IBM® SPSS® Statistics 22 (IBM Corporation, Armonk NY, USA) for Apple Mac®.

3. Results

3.1. Preliminary analyses

First, preliminary analyses were conducted to describe the CAR and to assess associations of salivary

and hair glucocorticoids and sleep measures with trait and state factors that possibly affect HPA axis

activity and sleep including age, sex, familial demographic background, season of assessment, and pre-

and postnatal treatments.

An increase in cortisol secretion over the post-awakening period (i.e. defined as 'fourth salivary

sample' – 'first salivary sample' > 1.5 nmol/L; Miller et al., 2013), indicating a cortisol awakening

response, was observed in 107 (69.9%) children. The first sample on awakening (S1) was positively

related to child age ($r = .17$, $p = .037$) and female sex ($F(1,138) = 4.52$, $p = .035$, $d = 0.36$). Moreover,

the post-awakening AUC_G was positively related to child age ($r = .19$, $p = .020$). The examination of

associations with hair glucocorticoid concentrations revealed a negative association between hair

cortisone and child age ($r = -.16$, $p = .041$) and boys had significantly higher hair cortisol ($F(1,149) = $

5.14, $p = .025$, $d = 0.37$) and cortisone levels ($F(1,149) = 11.29$, $p < .001$, $d = 0.54$) than girls. No

associations between familial demographic background (i.e., maternal education and language) and

HPA axis variables were found. Regarding season of assessment, a higher CAR (AUC_I) was found in

children tested during the winter season compared to the other seasons ($F(1,120) = 8.28$, $p = .005$, $d = $

0.83). Regarding pre- and postnatal treatments, 57 out of 85 (67.1%) very preterm infants had received

prenatal steroids (e.g. dexamethasone, betamethasone, prednisone) and only 7 out of 85 (8.2 %) very

preterm infants had received postnatal steroids (budesonide). We did not observe significant correlations

of prenatal steroid treatments, ventilation, intubation, and continuous positive airway pressure (CPAP) with any salivary or hair glucocorticoid measure (all p-values > 0.10). As only 7 children were exposed to postnatal steroid treatment, this variable was not analyzed.

Sleep duration was negatively associated with child age ($r = -.47$, $p < .001$) and boys showed longer REM sleep latency than girls ($t(141) = 2.43$, $p = .016$, $d = 0.41$). No other significant relations between sleep measures and child age and sex and familial demographic background were observed (all p-values > 0.10).

3.2. Differences in salivary and hair glucocorticoids between very preterm and full-term children and associations with birth weight and gestational age

To test our first hypothesis that children born very preterm show decreased HPA axis activity compared to full-term children, we performed analyses of covariance (ANCOVA) with salivary morning cortisol and hair cortisol and cortisone as dependent variables. Table 2 shows the results of these ANCOVAs. Children born very preterm showed a lower S1 ($F(1,140) = 3.93$, $p = .049$, $d = 0.35$) and AUC_G ($F(1,138) = 6.58$, $p = .011$, $d = 0.45$) than full-term children. There were no mean differences in the CAR (AUC_I) between very preterm and full-term children. Figure 2 depicts the patterns of post-awakening cortisol secretion for the two groups. Very preterm children showed lower hair cortisone concentrations ($F(1,149) = 9.52$, $p = .002$, $d = 0.52$) than full-term children. There were no mean differences in hair cortisol between very preterm and full-term children. Moreover, there was no evidence for moderation of the relationship between prematurity status and HPA axis variables by sex (data not shown).

In additional analyses we tested whether birth weight and gestational age were positively related to salivary and hair glucocorticoids within the group of very preterm children applying multiple regression analyses. Neither birth weight nor gestational age were related to salivary cortisol (all p-values > 0.10). Higher birth weight was at trend level related to higher hair cortisol ($\beta = 0.21$, $t = 1.95$, $p = .055$) and hair cortisone concentrations ($\beta = 0.20$, $t = 1.96$, $p = .055$), while higher gestational age was at trend level associated with higher cortisone levels in hair ($\beta = .18$, $t = 1.79$, $p = .078$).

(Insert Figure 2 about here)

3.3. Differences in sleep measures between very preterm and full-term children and associations with birth weight and gestational age

To test our second hypothesis that very preterm children show earlier sleep onset and poorer sleep compared to full-term children, we performed ANCOVA with objective sleep measures assessed by PSG as dependent variables. Table 2 shows the results of these ANCOVAs. Very preterm children had a significantly earlier sleep onset time compared to full-term children (very preterm children: M = 2111 h; full-term children: M = 2124 h; $F(1,134)$ = 6.29, p = .013, d = 0.44), while there were no significant differences in awakening times (very preterm children: M = 0634 h; full-term children: M = 0635 h; $F(1,134)$ = 0.03, p =.872, d = 0.03) and a trend towards longer sleep duration ($F(1,134)$ = 3.44, p = .066, d = 0.33). There were no mean differences in sleep efficiency and sleep architecture between very preterm and full-term children (all p-values > 0.10). Moreover, we tested if sex moderated the relationship between prematurity status and sleep variables. More nocturnal awakenings were found in very preterm girls compared to full-term girls but not between very preterm and full-term boys (girls: $F(1,51)$ = 4.09, p = .048, d = 0.56; boys: $F(1,76)$ = 0.06, p = .801, d = 0.06; prematurity status × sex interaction: ($F(1,133)$ = 4.49, p = .036).

In additional analyses we tested whether birth weight and gestational age were related to sleep variables within the group of very preterm children applying multiple regression analyses. Within the subsample of very preterm children, neither birth weight nor gestational age were significantly related to sleep variables, although higher birth weight and gestational age tended to be related to higher sleep efficiency (birth weight: β = .266, t = 1.80, p = .078; gestational age: β = .255, t = 1.75, p = .087).

(Insert Table 2 about here)

3.4. Associations of post-awakening cortisol with objective sleep measures

To test our third hypothesis that salivary post-awakening cortisol secretion is associated with objective sleep measures, hierarchical regression analyses were conducted with post-awakening cortisol secretion measures as dependent variables. Table 3 shows the results of these analyses. The post-awakening

AUC_G was positively associated with sleep onset time ($\beta = .36$, $t = 3.57$, $p < .001$) and negatively associated with sleep duration ($\beta = -.34$, $t = -3.42$, $p < .001$) and REM latency ($\beta = -.20$, $t = -2.30$, $p = .023$). Sleep efficiency, nocturnal awakenings, stage 2 sleep, SWS, and REM sleep were unrelated with post-awakening AUC_G. In addition, none of the sleep variables were significantly associated with S1 and the post-awakening CAR (AUC_I). Moreover, there was no evidence for moderation of the relationship between objective sleep measures and indices of post-awakening cortisol secretion by prematurity status or sex (data not shown).

(Insert Table 3 about here)

3.5. Mediation of the relationship between prematurity status and post-awakening cortisol secretion by sleep

Finally, we examined whether differences in sleep of the preceding night accounted for differences in post-awakening cortisol secretion between children born very preterm and full-term. Mediation was tested applying a bootstrapping approach using the INDIRECT procedure (Preacher & Hayes, 2008). Sleep onset time was the only sleep variable that was significantly associated with the independent variable (prematurity status: B = -.40, *SE* = .18, $t = -2.25$, $p = .027$) and an outcome variable (with post-awakening cortisol secretion AUC_G: B = .36, *SE* = .10, $t = 3.57$, $p < .001$ controlling children's age, sex, awakening-time, and season of assessment; both independent and outcome variable were z-standardized prior to analyses to improve interpretability). The indirect path via sleep onset time on AUC_G was significant (B = -.14; 95% bootstrap bias-corrected confidence interval from -.35 to -.03). Moreover, the association of prematurity status with AUC_G was partially attenuated when sleep onset time was additionally controlled which is consistent with partial mediation (from B = -.52, *SE* = .18, $t = -2.95$, $p = .004$ to B = -.36, *SE* = .18 $t = -2.01$, $p = .047$).

4. Discussion

This is the first study that focused on differences in HPA axis activity between very preterm and full-term children examining the role of sleep measured with in-home PSG. Our key findings are that very preterm birth was related to lower post-awakening cortisol levels and decreased cortisone in hair,

suggesting decreased HPA axis activity. In addition, earlier sleep onset time and longer sleep duration were associated with lower post-awakening cortisol secretion. Earlier sleep onset time partially mediated the association between prematurity status and post-awakening cortisol secretion.

4.1. Differences in salivary and hair glucocorticoids between very preterm and full-term children

First, we expected decreased HPA axis activity among very preterm compared to full-term children, which was partially supported by the data. Compared to full-term children, very preterm children showed lower post-awakening S1 and AUC_G, which is consistent with prior studies reporting down-regulation of HPA axis activity in very preterm children (Kaseva et al., 2014; Perkinson-Gloor et al., 2015; Wadsby et al., 2014). Also consistent with down-regulation of the HPA axis in very preterm children, we found lower hair cortisone, but not hair cortisol, levels in very preterm compared to full-term children, which is similar to a study reporting lower hair cortisol in preterm children (Grunau et al., 2013). The finding that only hair cortisone, but not cortisol, was related to preterm birth in the present study is difficult to interpret. It has been proposed that cortisone concentrations in hair may arise through local cortisol-to-cortisone conversion by the enzyme 11-beta-hydroxysteroid dehydrogenase type 2 and thus may reflect systemically circulating cortisol concentrations. Moreover, hair cortisone might even yield more reliable estimates of cumulative HPA axis activity than hair cortisol, as often highly outlying hair cortisol concentrations are found in a minority of the samples, which decreases reliability (see Stalder et al., 2013, for a more detailed discussion).

A first mechanism which may contribute to down-regulation of HPA axis activity in very preterm children may be long-term habituation related to prolonged exposure to stress during the postnatal phase involving medical complications and distressing treatments related to premature birth (Kaseva et al., 2014; Wadsby et al., 2014). Consistent with these findings, one study reported lower cortisol levels in school-age children who had more neonatal pain and stress related to a higher number of skin-breaking procedures after birth compared to children with less skin-breaking procedures (Brummelte et al., 2015). A second explanation may be pre- and neonatal therapeutic exposure to glucocorticoids, especially dexamethasone, which may possibly lead to down-regulation of the HPA axis activity in very preterm children (Karemaker et al., 2008). Decreased cortisol secretion after postnatal treatment with dexamethasone could however not be confirmed by Grunau et al. (2007) who studied basal salivary

cortisol in preterm children aged 6, 8, and 18 months. Similarly, we found no significant association between pre- and neonatal treatments (including prenatal glucocorticoids, ventilation, intubation, and CPAP) and later HPA axis activity in the present study. Further possible mechanisms contributing to alteration of HPA axis activity include immaturity of the adrenal gland in very preterm children leading to adrenal insufficiency (Fernandez and Watterberg, 2009) as well as pregnancy related aspects, such as maternal infections, inflammations, and prenatal stress, which may increase the risk of preterm birth and neurodevelopmental alterations (Buss et al., 2012; Goldenberg et al., 2008).

However, there are also studies with contrasting results. Two prior studies for instance found higher cortisol levels right at awakening in preterm compared to full-term children aged 8 to 12 years (Buske-Kirschbaum et al., 2007), particularly in girls (Quesada et al., 2014). There are three possibly relevant factors for these inconsistent findings.

A first possibly relevant factor is related to differences in birth weight/gestational age. Buske-Kirschbaum et al. (2007) and Quesada et al. (2014) also included moderately preterm children (gestational age between 32nd and 36th week) while the present study only included very preterm children. Though speculative, it is possible that there is a negative relationship between birth weight(/gestational age) and HPA axis activity above a certain level of birth weight(/gestational age) — as it was for instance found for boys with a birth weight between 2600g-4200g (Jones et al., 2006) — while this relationship becomes inverted below this level of birth weight(/gestational age), such that in children below a birth weight of around 2000g(/32nd gestational week) there is a positive relationship between birth weight(/gestational age) and HPA axis activity indicating down-regulation of the HPA axis with increasing degree of prematurity. As there were no children born moderately preterm in the current study we could not test this assumption. In a similar vein, the inconsistency in previous research might be related to the number of SGA children in the sample. While for instance in our study only 4 out of 85 very preterm children were born SGA, this ratio was considerably higher in Quesada et al. (2014) with 9 out of 30 preterm children.

A second possibly relevant factor for these inconsistent findings is related to sex differences. In general, sex differences in HPA axis activity have often been reported. Consistent with our study, Grunau et al. (2013) for instance found higher hair cortisol in boys. Important for differences between preterm and full-term children, Quesada et al. (2014) found moderation of the association between

prematurity status and HPA axis activity by sex – preterm girls had higher awakening cortisol levels than full-term girls but no such differences were found in boys. In our study there were somewhat more boys than girls (58% vs 42%) which might bias our overall findings towards effects in boys. As we found no moderation of the effects of prematurity status by sex, we however do not favor this explanation.

A third possibly relevant factor for these inconsistent findings is related to differences in stress exposure and medical treatments for preterm children. Different treatment regimens at different study sites may lead to differential effects on HPA axis activity. Taken together, divergent findings regarding HPA axis activity in children of prior studies may be due to differences in gestational age and birth weight, sex, and different treatment regimens in the pre- and early postnatal phase in different samples.

4.2. Differences in sleep between very preterm and full-term children

Second, we expected earlier sleep onset times and poorer sleep patterns in very preterm compared to full-term children. We could confirm that very preterm children had earlier sleep onset times compared to term-born children. This finding is consistent with results from Björkqvist et al. (2014), Hibbs et al. (2014), and Strang-Karlsson et al. (2010), who also found earlier sleep onset times and more morningness (i.e., the characteristic of being most active and alert during the morning) in adolescents and adults born very preterm or with very low birth weight. In contrast to the findings from the prior wave of our study when children were approximately two years younger (Perkinson-Gloor et al., 2015), we could not confirm more stage 2 sleep and less SWS in very preterm compared to full-term children in the present study wave. Between middle childhood and early adolescence sleep architecture greatly develops involving decreases in SWS and REM sleep and increases in stage 1 and stage 2 sleep (Ohayon et al., 2004). It is possible that these changes in sleep architecture during late childhood may have led to the convergence of the sleep architecture between very preterm and full-term children. However, we found that very preterm girls had more nocturnal awakenings than full-term girls while no such difference was found in boys. In the prior study wave, very preterm children also showed more nocturnal awakenings but no sex differences were found (Perkinson-Gloor et al., 2015; Lemola et al., 2015).

4.3. Associations of post awakening cortisol with objective sleep

Third, we hypothesized post-awakening cortisol to be negatively associated with sleep duration, sleep continuity (as represented by higher sleep efficiency and less nocturnal awakenings), and SWS and to be positively associated with light sleep and REM-sleep. Data did partially support this assumption in that increased post-awakening AUC_G was associated with shorter sleep duration. This is consistent with studies showing increased HPA axis activity to be associated with short sleep (Fernandez-Mendoza et al., 2014; Lemola et al., 2015; Räikkönen et al., 2010). In addition, we found that post-awakening AUC_G was positively related to sleep onset time and negatively associated with REM latency. However, no relationship of post-awakening cortisol with sleep continuity, SWS, and REM-sleep was found. Again, it is possible that the prior findings (Hatzinger et al., 2013; Lemola et al., 2015) could not be replicated due to older age of the children in the present study.

4.4. Mediation of the relationship between prematurity status and post-awakening cortisol secretion by sleep

We further explored the research question if differences in sleep of the preceding night accounted for differences in post-awakening cortisol secretion between very preterm and full-term children. Partial mediation of the effect of prematurity status on decreased post-awakening cortisol secretion by earlier sleep onset time was found. It is therefore possible that decreased HPA axis activity and early sleep onset time (i.e. morningness) – phenomena which both have often been reported in very preterm children (e.g. Hibbs et al., 2014; Kaseva et al., 2014) – share a common etiology. Having said that, one has to bear in mind that the difference in sleep onset time between very preterm and full-term children was only 13 min in the present study which reflects a modest to moderate effect size ($d = 0.44$). Moreover, based on our data it remains impossible to settle the question regarding the direction of causality.

4.5. Strengths and limitations

Our study has some limitations. First, we cannot draw conclusions regarding causal relations due to the correlative study design. Second, salivary cortisol was only measured on a single day, which may reduce reliability and decrease the power to find positive results. As single day measurement does not

introduce systematic error, this may be regarded as less problematic for the interpretation of positive findings than confounding by systematically covarying state factors introducing systematic error (see Stalder et al., 2016, for a detailed discussion). Third, we have no objective information on participants' compliance regarding the instructions of saliva collection, which may also reduce reliability of the morning cortisol secretion indices (Stalder et al., 2016). Future research may avoid this limitation by saliva sampling with electronic time stamp. Fourth, pubertal stage and menstrual cycle, which can both affect post-awakening cortisol secretion, were not assessed. Finally, there are several other factors that may have a long-term influence on children's HPA axis activity that could not be controlled such as maternal stress and socioeconomic status of the family during pregnancy and children's concurrent psychosocial stress.

Among the strengths of our study, sleep assessment was conducted at the children's home by PSG which allowed studying the relationship between HPA axis activity and the sleep pattern of the preceding night as well as controlling for objectively measured awakening times. Moreover, we also consider it a strength of the study that HPA axis activity was measured in both saliva, which informs on the acute HPA axis activity, and in hair by which cumulative HPA axis activity across the preceding months can be assessed.

5. Conclusions

In conclusion, our study suggests that children born very preterm have lower levels of cortisol at awakening and lower overall post-awakening cortisol secretion, lower cortisone in hair, and slightly earlier sleep onset than full-term children. The results are consistent with previous research indicating that very preterm birth is related to possible down-regulation of HPA axis activity during late childhood and early adolescence. Moreover, the results indicate less post-awakening cortisol secretion in children with longer sleep duration, earlier sleep onset, and longer REM latency. In particular, earlier sleep onset co-occurred with decreased HPA axis activity in very preterm children. Thus, our results support the notion that HPA axis activity and sleep are associated and that in accordance with previous studies on animals and humans a down-regulation of the HPA axis activity may be programmed early in life. Future research may examine the possible role of down-regulation of the HPA axis activity in very

preterm children for their psychosocial adjustment, cognitive and academic functioning, and

psychological wellbeing.

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Table 1 Sample characteristics of very preterm and full-term children.

	Very preterm (n = 85)		Full-term (n = 91)		P
	M/N	(SD/%)	M/N	(SD/%)	
Age, years	9.5	(1.4)	9.6	(1.4)	.755
Sex, male	51	(60.0)	51	(56.0)	.595
Gestational age, weeks	29.7	(2.0)	39.5	(1.5)	<.001
Birth weight, grams	1325.1	(407.4)	3307.5	(443.9)	<.001
Prenatal treatment with glucocorticoids	57	(67.1)	0	(0.0)	<.001
Postnatal treatment with glucocorticoids	7	(8.2)	0	(0.0)	.005
Ventilation	33	(38.8)	0	(0.0)	<.001
Intubation	25	(29.4)	0	(0.0)	<.001
Continuous positive airway pressure	63	(74.1)	0	(0.0)	<.001
Infant respiratory distress syndrome	69	(81.2)	0	(0.0)	<.001
Apnea of Prematurity	66	(77.6)	0	(0.0)	<.001
Bronchopulmonary Dysplasia	7	(8.2)	0	(0.0)	.005
First language (German)	56	(72.7)	78	(86.7)	.024
Maternal education					<.001
No vocational training	9	(10.6)	1	(1.1)	
Vocational training	52	(62.4)	40	(44.0)	
University	19	(22.4)	47	(51.6)	

Note: P-values of the χ^2 test, Fisher's exact test, or analyses of variance.

Table 2 Salivary cortisol, hair glucocorticoids, and sleep in very preterm and full-term children.

	Very preterm		Full-term		*d*	*P*
	M	(SD)	M	(SD)		
Salivary morning cortisol secretion[a]						
AUC$_G$	0.5	(0.1)	0.5	(0.1)	0.45	.011
AUC$_I$	0.6	(0.1)	0.6	(0.1)	0.02	.927
First sample (S1)	0.8	(0.2)	0.9	(0.2)	0.35	.049
Second sample	0.9	(0.2)	1.0	(0.2)	0.38	.031
Third sample	1.0	(0.2)	1.1	(0.2)	0.48	.008
Fourth sample	1.0	(0.4)	1.1	(0.2)	0.21	.228
Hair variables[b]						
Cortisol	2.0	(2.1)	1.9	(1.8)	0.07	.672
Cortisone	8.7	(6.7)	10.9	(7.7)	0.52	.002
Sleep variables[c]						
Sleep onset time	21:11	(0:40)	21:24	(0:38)	0.44	.013
Awakening time	6:34	(0:26)	6:35	(0:21)	0.03	.872
Sleep duration (h)	9.0	(0.7)	8.9	(0.7)	0.33	.066
Sleep efficiency (%)	93.1	(3.0)	93.6	(2.8)	0.03	.849
Nocturnal awakenings (number)	17.2	(7.7)	15.0	(6.2)	0.23	.194
Stage 1 sleep (%)	3.9	(2.4)	3.3	(2.3)	0.17	.327
Stage 2 sleep (%)	46.8	(5.1)	48.0	(4.9)	0.19	.275
Slow wave sleep (%)	21.3	(5.1)	21.5	(4.6)	0.05	.781
REM sleep (%)	25.5	(4.1)	24.7	(3.7)	0.20	.257
REM latency (min)	116.7	(45.0)	108.1	(40.7)	0.25	.159

Note: AUC$_G$ = area-under-the-concentration-time-curve with respect to the ground, AUC$_I$ = area-under-the-concentration-time-curve with respect to the increase, REM = rapid eye movement.

[a] log-transformed before building AUC$_G$/AUC$_I$; adjusted for first language, maternal education, children's age, sex, season, and awakening time.

[b] truncated to a value of 2 interquartile ranges above the median; adjusted for first language, maternal education, children's age, sex, season, and time point of the lab analyses.

[c] Cohen's *d, P*-values: z-standardized data; adjusted for first language, maternal education, children's age, and sex.

Table 3 Multiple regression results with sleep parameters predicting post-awakening cortisol measures.

Sleep variables[a]	Post-awakening cortisol secretion		
	First sample (S1)	AUC_I	AUC_G
Sleep onset time	.18†	.13	.36***
Awakening time[b]	-.13	.15†	-.03
Sleep duration (h)	-.18†	-.11	-.34***
Sleep efficiency	-.02	.05	.03
Nocturnal awakenings	-.01	.00	-.01
Stage 1 sleep (%)	.16†	-.11	.11
Stage 2 sleep (%)	-.04	.07	.01
Slow wave sleep (%)	-.05	.00	-.07
REM sleep (%)	.04	.00	.04
REM latency (min)	-.10	-.08	-.20*

Note: Data are standardized regression coefficients.

AUC_G = area-under-the-concentration-time-curve with respect to the ground, AUC_I = area-under-the-concentration-time-curve with respect to the increase, REM = rapid eye movement.

[a] adjusted for children's age, sex, season, prematurity status, and awakening time.

[b] adjusted for children's age, sex, season, and prematurity status.
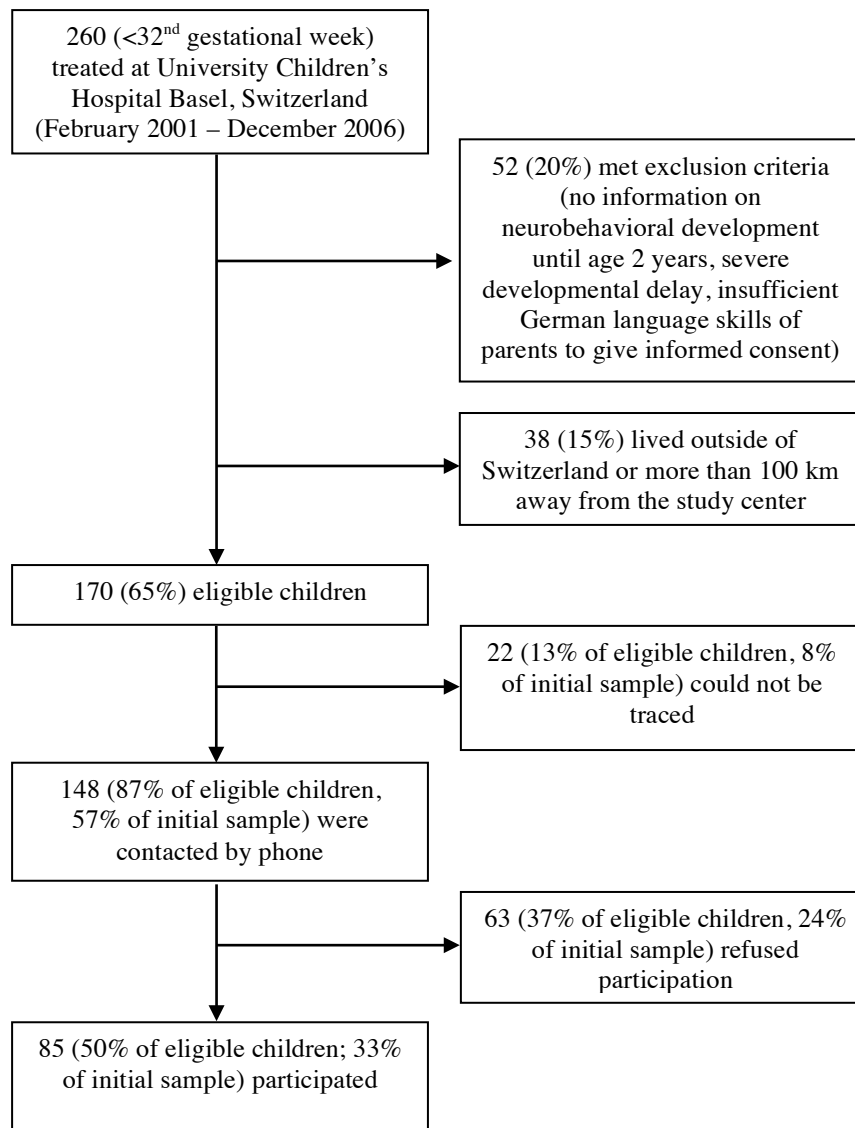
†$p < .10$, *$p < .05$, ***$p < .001$ (two-tailed).

260 (<32nd gestational week) treated at University Children's Hospital Basel, Switzerland (February 2001 – December 2006)

52 (20%) met exclusion criteria (no information on neurobehavioral development until age 2 years, severe developmental delay, insufficient German language skills of parents to give informed consent)

38 (15%) lived outside of Switzerland or more than 100 km away from the study center

170 (65%) eligible children

22 (13% of eligible children, 8% of initial sample) could not be traced

148 (87% of eligible children, 57% of initial sample) were contacted by phone

63 (37% of eligible children, 24% of initial sample) refused participation

85 (50% of eligible children; 33% of initial sample) participated

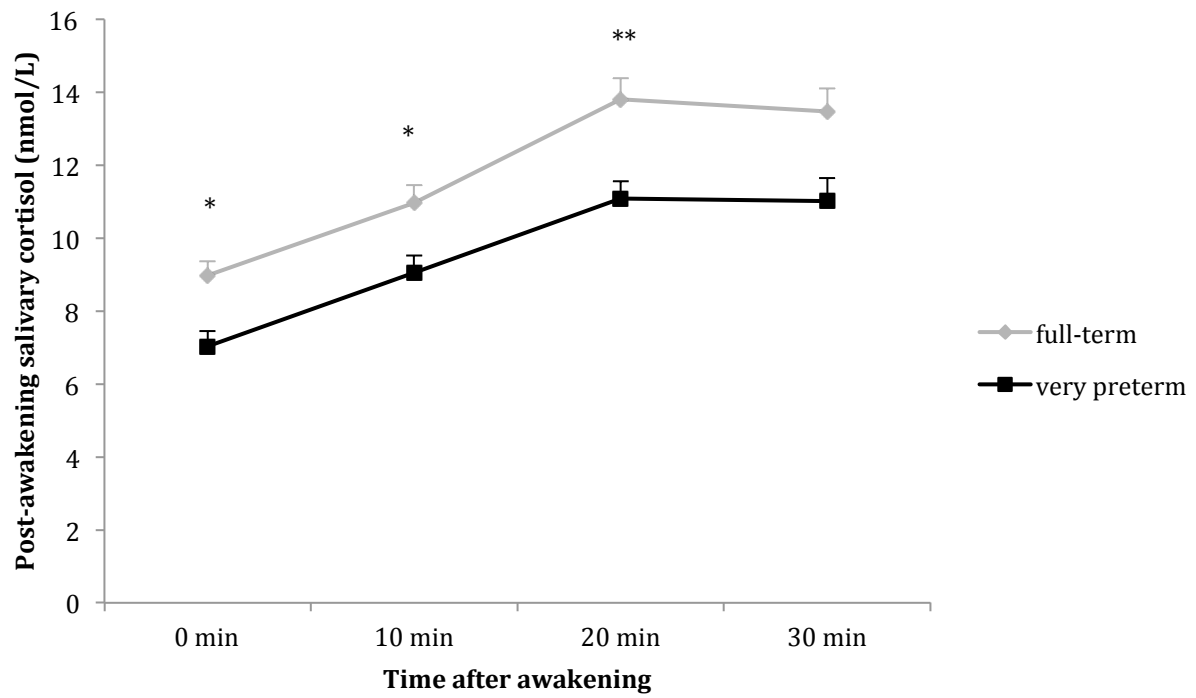**Fig. 1.** Inclusion procedure of very preterm children.

Fig. 2. Post-awakening salivary cortisol secretion of very preterm and full-term children.

*p < .05, **p < .01 (two-tailed).