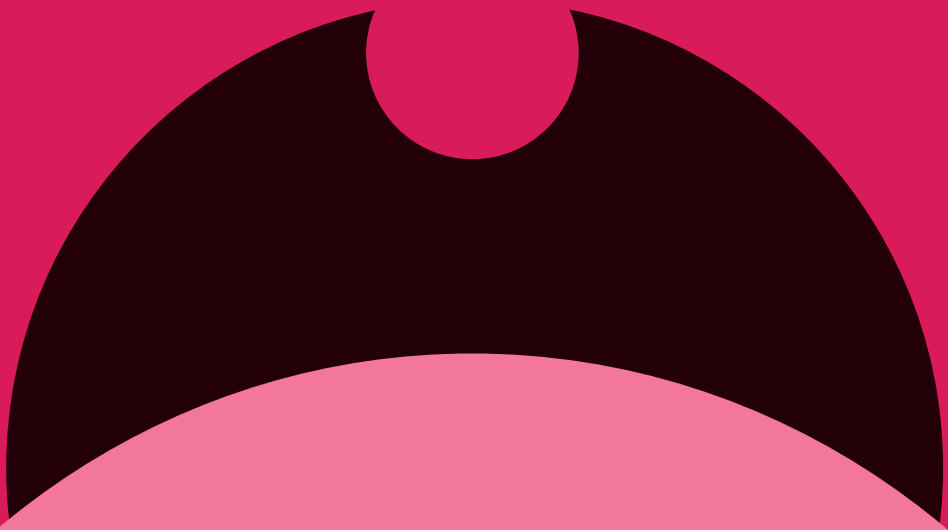
Nathalie S. Saridjan



Of Cortisol and Children

Hypothalamic-pituitary-adrenal (HPA) axis activity and the development of pre-schoolers in the general population

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Nathalie Siti Saridjan

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Hypothalamic-pituitary-adrenal (HPA) axis activity and the development of pre-schoolers in the general population

Over cortisol en kinderen

Hypothalamus-hypofyse-bijnier (HPA) as activiteit en de ontwikkeling van peuters en kleuters in de algemene bevolking

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CHAPTER 1

General introduction

GENERAL INTRODUCTION

Child psychiatric disorders are among the most significant public health problems affecting a large part of the population. Consistently, prevalence rates between 15-20% are reported in different countries across the world (WHO, 2005). In pre-school children both externalizing problems, such as aggressive behaviour and hyperactivity, and internalizing problems, such as anxiety, are the most common problems (Tick et al., 2007). These problems can place a huge burden on affected children, their families, and society. Often, these childhood disorders affect the individual lastingly, most adult mental health problems have their onset or a precursor in childhood (Reef et al., 2010). Overall, mental health disorders are estimated to account for 7.4% of the global burden of disease (Whiteford et al., 2013). In order to decrease this burden, it is imperative to better understand the etiology of childhood mental health disorders.

The development of internalizing and externalizing problems is assumed to be the result of multiple causes, influenced by genetic and environmental factors and their interaction(s) (Cummings, Davies, & Campbell, 2000). One of the factors associated with both internalizing and externalizing problems is stress, in the broadest sense of the term. Although it has been shown that more stressful events such as early social adversity or neglect leads to a higher risk of both internalizing and externalizing problems, the psychobiological mechanisms underlying these associations are poorly understood.

One of the most important stress systems in the human body, and the primary focus of this thesis, is the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1). This HPA axis is the main neuroendocrine system that is activated in response to physical or psychosocial stress. In reaction to a stressful situation, the hypothalamus secretes corticotropin-releasing hormone (CRH), which in turn triggers the release of adrenocorticotropin hormone from the anterior pituitary. ACTH then induces the secretion of cortisol from the adrenal glands (de Kloet, 2003).

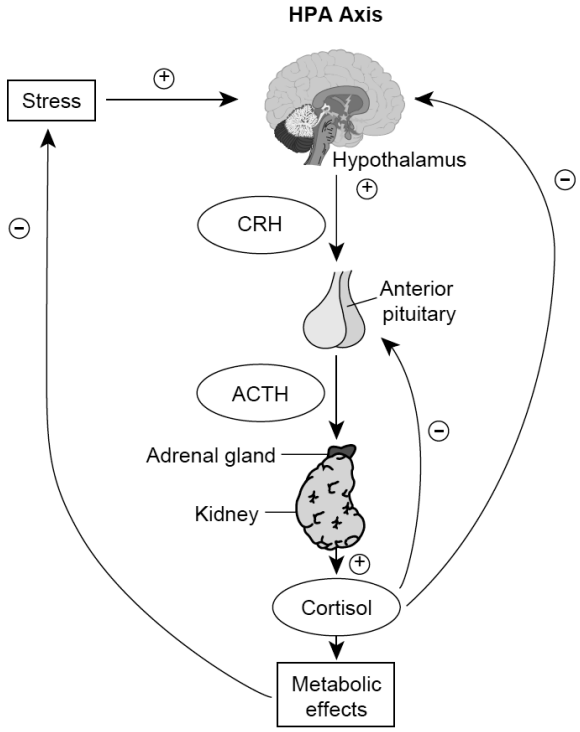

Figure 1: Schematic presentation of the HPA axis.
Abbreviations: HPA = hypothalamic-pituitary-adrenal, CRH = corticotropin-releasing hormone, ACTH = adrenocorticotropic hormone. From: S Hiller-Sturmhöfel, A Bartke. The endocrine system: an overview. Alcohol Health Res World: 1998, 22(3); 153-64.

Research on HPA axis activity in humans dates back to the 1950s. As only cortisol, the hormonal end product of the HPA axis, was relatively easily measurable in blood and urine, cortisol levels have since then been used as a proxy for HPA axis activity. Since the late 1980s cortisol is also measurable in saliva. This allowed for a more uncomplicated and less troublesome sample collection, which could also be used in larger observational studies but also in studies with children. Over the past 25 years, salivary cortisol has frequently been used as a biomarker of psychological stress in stress studies (Kirschbaum & Hellhammer, 1994), not only in adults but also in children (Doom & Gunnar, 2013). Cortisol research initially focused on salivary cortisol responses, that is HPA axis reactivity, following physical and psychological stress (Kirschbaum & Hellhammer, 1989).

Another characteristic of the HPA axis is its diurnal rhythm. The diurnal secretion of cortisol is characterized by high levels in the morning due to a steep rise in cortisol shortly after awakening, followed by a decline in cortisol levels toward the evening. The HPA axis is a very

delicate system and it is involved in regulating a range of bodily functions such as energy metabolism and immune system functioning (Clow et al., 2004). Also important, the HPA axis develops and matures in early childhood. Infants are born without a diurnal cortisol rhythm and this rhythm probably emerges in the first years of life (Watamura et al., 2004). Dysregulation of the diurnal cortisol rhythm have also been associated with internalizing and externalizing problems (Ruttle et al., 2011).

Epidemiological studies of the HPA-axis in early life are lacking. Few studies have addressed the question whether early alterations in the HPA-axis precede child developmental problems such as internalizing, externalizing or cognitive problems. Most of these studies focussed on cortisol reactivity but have not addressed the diurnal rhythm. The present thesis is an attempt to close this gap.

The aim of this thesis is to extend the knowledge on determinants of the developing diurnal cortisol rhythm in infants and the associations between the infant's diurnal cortisol rhythm and child development in a general population study.

The research is embedded within the Generation R Study, a large population-based cohort study from fetal life onwards in Rotterdam, the Netherlands (Tiemeier et al., 2012). It was designed to identify early biological and environmental determinants of growth, development and health. Pregnant women living in the study area with an expected delivery date between April 2002 and January 2006 were invited to participate. Most of the research was conducted within in a randomly selected subgroup, referred to as the Generation R Focus Cohort. The Focus Cohort consists only of Dutch children of Caucasian origin and their parents, to exclude potential confounding or effect modification by ethnicity. Studies conducted in the Focus Cohort were able to utilise more in-depth assessments of postnatal growth and development that could not be applied in the whole cohort due to time, financial or logistical constraints. HPA axis activity was measured in a subsample of participating children at the age of 14 months. Information on children's psychosocial functioning and sleep were measured by questionnaires at various ages.

OUTLINE

In chapter 2, we examine determinants of variations of the diurnal cortisol rhythm in the second year of life. In chapter 3, we explore the associations between the diurnal cortisol rhythm and cognitive functioning, internalizing and externalizing problems, and abdominal pain and constipation. In chapter 4, we study the associations between sleep problems and

internalizing problems early in life, as well as the relationship between the diurnal cortisol rhythm with sleep duration and sleep problems in pre-schoolers. In chapter 5, the main findings of these studies are discussed, together with their methodological considerations, and both research and clinical implications.

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CHAPTER 2

Determinants of the diurnal cortisol rhythm

Saridjan NS, Huizink AC, Koetsier JA, Jaddoe VW, Mackenbach JP, Hofman A, Kirschbaum C, Verhulst FC, Tiemeier H. Do social disadvantage and early family adversity affect the diurnal cortisol rhythm in infants? The Generation R Study.

Horm Behav. 2010 Feb;57(2):247-54.

ABSTRACT

Dysregulation of diurnal cortisol secretion patterns may explain the link between adversities early in life and later mental health problems. However, few studies have investigated the influence of social disadvantage and family adversity on the hypothalamic–pituitary–adrenal (HPA) axis early in life. In 366 infants aged 12–20 months from the Generation R Study, a population-based cohort from fetal life onwards, parents collected saliva samples from their infant at 5 moments over the course of 1 day. The area under the curve (AUC), the cortisol awakening response (CAR) and the diurnal cortisol slope were calculated as different composite measures of the diurnal cortisol rhythm. Information about social disadvantage and early adversity was collected using prenatal and postnatal questionnaires.

We found that older infants showed lower AUC levels; moreover, infants with a positive CAR were significantly older. Both the AUC and the CAR were related to indicators of social disadvantage and early adversity. Infants of low income families, in comparison to high income families, showed higher AUC levels and a positive CAR. Infants of mothers who smoked during pregnancy were also significantly more likely to show a positive CAR. Furthermore, infants of mothers experiencing parenting stress showed higher AUC levels. The results of our study show that effects of social disadvantage and early adversity on the diurnal cortisol rhythm are already observable in infants. This may reflect the influence of early negative life events on early maturation of the HPA axis.

INTRODUCTION

Early life adversity has been associated with a broad range of negative mental health outcomes in adulthood, like post-traumatic stress disorder (Koenen et al., 2007), depression and anxiety (Levitan et al., 2003). Moreover, early adversity can already lead to behavioral problems in childhood that continue through adolescence and adult life (Appleyard et al., 2005). Indeed, there is strong evidence from studies in children that early environmental risk factors increase the risk of behavioral problems, such as symptoms of attention-deficit/hyperactivity and conduct disorder (Counts et al., 2005). Flaherty et al. (2006) also found that early adversity is associated with poor physical health in children.

Early adversity, however, involves a broad range of disadvantages to which a child can be exposed to. Socio-economic disadvantage has been associated, for instance, with developmental delays in 3-year-old children (Emerson et al., 2009) and with poorer child behavioral outcomes in 5-year-olds (Kohen et al., 2008). Likewise, family adversities have consistently been associated with poor childhood outcomes. Maternal depression, poor parenting and parenting stress are major family risk factors for psychosocial problems in the offspring (Petterson and Albers, 2001 and Crnic et al., 2005). In addition, other possible risk factors early in life such as maternal smoking during pregnancy and low birth weight are also known to have an impact on behavioral problems in children (Robinson et al., 2009 and Alati et al., 2009).

In response to physical or psychosocial stress, the hypothalamic–pituitary–adrenal (HPA) axis, one of the body's primary stress systems, is activated resulting in short term release of cortisol (Orth and Kovacs, 1998). Chronic exposure to stress early in life may lead to re-adaptations of the HPA axis activity. One of these alterations is dysregulation of the diurnal cortisol rhythm. In healthy adults the normal diurnal rhythm is characterised by post-waking peak cortisol levels (cortisol awakening response) and subsequent declining cortisol levels throughout the day (Edwards et al., 2001). Altered diurnal rhythms have been associated with different kinds of stressors, e.g. work-related stress is related to elevated evening cortisol levels (Rydstedt et al., 2008).

Early childhood adversity has been associated with altered diurnal cortisol rhythms in both adolescence and adulthood. In general, findings indicate that early adversity is associated with higher basal cortisol levels throughout the day or a flatter diurnal slope (Nicolson, 2004 and van der Vegt et al., 2009). Furthermore, associations with altered morning cortisol levels have also been found. Both lower morning levels in adults (Meinlschmidt and Heim, 2005) and higher morning levels in adolescents (Halligan et al., 2004) were reported. However, it

remains unclear whether these altered diurnal rhythms are a consequence of early adversity, since most studies of childhood adversity were retrospectively reported by adults.

Severe early adversity has also been associated with altered diurnal cortisol rhythms in children. Romanian orphan-reared toddlers exhibited low morning cortisol levels and blunted diurnal cortisol patterns (Carlson and Earls, 1997). In contrast, when Romanian orphans were examined six and a half years after adoption, those adopted after more than 8 months of institutionalization showed higher levels of cortisol across the day (Gunnar et al., 2001). Dozier et al. (2006) found both patterns of low and high cortisol production in foster children aged 20 to 60 months, compared with children who were never in foster care. More recently, Bruce et al. (2009) found that different early adverse experiences can lead to both low and high morning cortisol levels in 3- to 6-year-old foster children. However, these studies in high risk samples cannot be translated easily to the general population.

The inconsistencies of these studies of early adversity in children make interpretation of its influence on the HPA axis activity difficult. Firstly, the HPA axis develops and matures in early childhood. Infants are born without a diurnal cortisol rhythm; this rhythm probably emerges during the first 18 months of life (Gunnar and Donzella, 2002 and Herbert et al., 2006). Watamura and colleagues (2004) even posited that the maturation of the HPA axis continues to the third year of life. Moreover, sampling salivary cortisol in infants is difficult (Egliston et al., 2007). Subsequently, only few studies have focused on the influence of early adversity on the diurnal cortisol rhythm in infants and toddlers. Recently, Ouellet-Morin and colleagues (2009) examined the influence of early family adversity on diurnal cortisol activity in 6-month-old twins. They found that early family adversity modulates the heritability of morning cortisol levels in infants. Family adversity was defined by seven risk factors (prenatal maternal smoking, low birth weight, low family income, low maternal education, single parenthood, young motherhood, and maternal hostile behaviors). However, by studying a cumulative index of different family adversity factors, the effect of individual indicators of early family adversity on diurnal cortisol secretion in infants cannot be understood.

The objective of the present study is to study the effects of indicators of social disadvantage and other early adversities on the diurnal cortisol rhythm of infants in a population-based cohort. Studying risk factors occurring early in life in relation to infant cortisol rhythms may help further understand the pathway through which dysregulation of the HPA axis and psychopathology occurs. We hypothesize that infants in the second year of life show a diurnal rhythm of cortisol secretion and that this rhythm, even at a young age, is influenced by several risk indicators of early adversity.

SUBJECTS AND METHODS

Setting

This study was embedded within the Generation R Focus Study, a cohort study investigating growth, development and health from fetal life onwards in Rotterdam, the Netherlands, which has been described in detail elsewhere (Jaddoe et al., 2008). The Generation R Focus Study, a subgroup within the Generation R Study, was conducted to obtain detailed measurements of the child's development in an ethnically homogeneous group of indigenous Dutch children to exclude confounding or effect modification by ethnicity. The participating children were born between February 2003 and August 2005. The children visited the research center regularly for various somatic and behavioral assessments. Written informed consent was obtained from all participants. The study has been approved by the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam.

Study population

For the current study, infants who visited the research center for the Focus Study around 14 months were eligible for assessment of the diurnal cortisol profile. Of the 882 infants who attended the Focus Cohort examination, parents of 602 infants (68%) returned one or more saliva samples. Of the 280 non-responders, 3 (1%) were too busy, 90 (32%) tried but failed to obtain saliva samples and 187 (67%) gave no reason for non-response. To compute a cortisol composite measure, two morning samples or at least three saliva samples per infant had to be obtained. In total, 236 infants had to be excluded because they did not meet these criteria. The area under the curve was calculated in 277 infants, the diurnal cortisol slope in 297 infants and the cortisol awakening response in 314 infants. A total of 366 infants (41% of 882) were included in one or more analyses of a composite measure.

Social disadvantage and early adversity

Primarily, we followed the approach of Ouellet-Morin et al. (2009) and studied the following indicators of socio-economic disadvantage and early family adversity: young maternal age, single parenthood, low maternal education, low family income, maternal smoking during pregnancy, distress and hostility during pregnancy, poor family functioning during pregnancy, and low birth weight. We also studied the influence of parenting stress as a marker of postnatal family adversity.

Maternal age, maternal educational level, and family income were determined at enrolment using self-report. Educational level was categorized in three levels: low, middle and high education. Family income was dichotomized in net income less than 2000 euro and more than 2000 euro a month.

Indicators of family adversity were measured using prenatal and postnatal questionnaires. Information on maternal distress, hostility and family functioning was obtained by postal questionnaires at 20 weeks of pregnancy. Distress levels and hostile behavior during pregnancy were assessed using the Brief Symptom Inventory (BSI), a validated 53-item (5-point scale) self-report symptom inventory outlined to ascertain the psychological state of individuals (Derogatis and Melisaratos, 1983). The mean total score of the BSI, the Global Severity Index (GSI) and indicator of current psychological distress levels, was obtained by dividing the sum score by the numbers of completed items. The internal consistency of the GSI in this sample was $\alpha = 0.92$. For dichotomization, we used 0.365 as a cut-off, as recommended in the manual to differentiate between low to average range scores (< 0.365) and above average range scores (≥ 0.365) in the general Dutch population (De Beurs, 2006). The 5-item hostility scale of the BSI, e.g. "uncontrollable bursts of anger," was used to assess hostile behavior. Family functioning was measured with the General Functioning subscale (GF) of the Family Assessment Device (FAD) (Byles et al., 1988). GF is a validated overall self-report measure of health or pathology of the family, which consists of 12 items. We used a cut-off of 2.00, which categorized 10% of our sample as having poor family functioning. The internal consistency of GF in this sample was $\alpha = 0.89$. Parenting stress was measured at 18 months by the Nijmeegse Ouderlijke Stress Index-Kort (NOSIK; De Brock et al., 1992), the Dutch version of the Parenting Stress Index-Short Form (Abidin, 1983). The NOSIK comprises 25 questions on two domains: parenting stress due to parental factors and parenting stress due to child factors. Only the 11 items of the parental domain were used in the present analyses. Examples of questions are: "Parenthood of this child is harder than I thought" or "I often do not understand my child." Items were assessed on a four-point Likert scale. Analogous to the manual (De Brock et al., 1992), scores were summed and divided by the number of completed items. We used a cut-off of 0.54, which categorized 10% of our sample as having parenting stress, in concordance to Van der Pal et al. (2008). Higher scores indicate greater levels of stress. The NOSIK has good reliability (Cronbach's alpha = 0.95) and validity (De Brock et al., 1992). Internal reliability for the 11 items in the current study, measured by Cronbach's alpha, was 0.96.

Other early adversity indicators were low birth weight and maternal smoking during pregnancy. Birth weight was taken from community midwife and hospital registries. Information about maternal smoking was obtained by (postal) questionnaires at enrolment and 20 and 30 weeks of pregnancy. Mothers were classified as smokers or non-smokers during pregnancy.

Covariates

Age of cortisol sampling and gender of the infant were entered as covariates in all models. We also considered several perinatal and obstetric complications as potential confounders: parity, pregnancy complications (gestational diabetes, hypertension, preeclampsia), mode of delivery, gestational age at birth and Apgar-score 5 min after birth.

Date of birth, gender of the infant, and information on perinatal and obstetric complications were obtained from community midwife and hospital registries at birth.

Nap duration during the day of cortisol sampling was also considered as confounder. In an accompanying questionnaire, all parents were asked to report duration of each nap during the day of cortisol sampling. Total duration and number of naps was calculated from this information.

Cortisol sampling

Prior to the Focus Study visit, parents were asked to collect five saliva samples at home using Salivette sampling devices (Sarstedt, Rommelsdorf, Germany). Parents received detailed written instructions with pictures concerning the saliva sampling. They were told to collect the five saliva samples during one single weekday: immediately after awakening, 30 min later, around noon, between 1500 h and 1600 h, and at bedtime. For the noon saliva sample collection, parents reported a mean deviation in sampling time of 0.42 h. Parents were asked not to let their infants eat or drink 30 min before saliva sampling to avoid disturbances of the cortisol levels. Besides these restrictions, the infants were free to follow their normal daily routines on the sampling day. Parents were asked to keep the samples stored in their freezer until they visited the research centre. If parents forgot to bring the samples, they were asked to mail the Salivettes to the laboratory of the Department of Epidemiology at the Erasmus MC (Jaddoe et al., 2007). Here, the samples were centrifuged and frozen at − 80°C. After completion of the data collection, all frozen samples were sent on dry ice in one batch by courier to the laboratory of the Department of Biological Psychology laboratory at the Technical University of Dresden for analysis. Salivary cortisol concentrations were measured using a commercial immunoassay with chemiluminescence detection (CLIA; IBL Hamburg, Germany). Intra- and interassay coefficients of variation were below 7% and 9%, respectively.

Analyses of cortisol

We excluded two infants because they were older than 20 months. None of the infants used systemic corticosteroid medication. Nine infants used corticosteroid-containing medication locally. Excluding the infants using corticosteroid-containing medication did not change the results. Thus we only report results of the analyses including these infants.

For each time point, cortisol values that were above the 99th percentile (> 200 nmol/L) were excluded (n = 18, equalling n = 12 children) from the analysis to reduce the impact of outliers.

No analyses using single cortisol time point measures were performed, since individual cortisol levels show a high day-to-day variability (Hucklebridge et al., 2005). Moreover, such an approach increases the risk of type II error due to multiple testing. We calculated three

composite variables of the separate cortisol measures within a day: the area under the curve (AUC), the diurnal cortisol slope and the cortisol awakening response (CAR).

The AUC was used as a measure of total cortisol secretion during the day (from awaking in the morning until bedtime in the evening). It was determined by the total area under the curve given by the cortisol measurements in nmol/L on the y-axis and the time between the cortisol measurements on the x-axis (Pruessner et al., 2003). To correct for differences in length of total sampling interval time, the AUC was divided by number of hours between the first cortisol measurement at awakening and the last cortisol measurement before going to bed. The AUC was computed only for those who collected at least three saliva samples.
The diurnal cortisol slope was used as a measure of the diurnal cortisol decline. It was calculated by fitting a linear regression line for each infant, which predicted the cortisol values from time since awakening. The slope was computed by using the first saliva sample and at least two other cortisol time point measures. To avoid any effect of the CAR on the slope (Adam et al., 2006 and Cohen et al., 2006), the second cortisol sample (30 min after awakening) was not included in this measure of the slope.

The CAR was calculated as the difference between the cortisol value at awakening and the value 30 min after awakening (Kunz-Ebrecht et al., 2004). The difference was only calculated if the cortisol value 30 min after awakening was taken between 15 and 60 min after awakening. 95% of the parents reported to have sampled the first saliva sample immediately or within 15 min after awakening.

Statistical analysis
Although the computed variables AUC, slope and CAR showed a slightly skewed distribution, we decided not to transform these variables since regression residuals were normally distributed. Furthermore, this makes interpretation of the results more straight-forward.

We used linear regression models to test the associations between indicators of family adversity and the composite variables of cortisol. All associations were adjusted for gender and age of sampling, if applicable. We considered several perinatal and obstetric complications as potential confounders, as well as nap duration during the day of cortisol sampling. However, since these covariates did not change the effect estimates by more than 10%, we did not include these in our final models.

For ease of interpretation, analyses of covariance (ANCOVAs) were used to give estimates of the mean composite cortisol outcome and the differences between the categories of each determinant. Due to small group numbers (< 15) we were unable to study the effects

of young maternal age (< 20 years), single parenthood and low birth weight (< 2500 g) on diurnal cortisol secretions.

The effect of maternal age, birth weight and sampling age on the composite cortisol variables was also tested using these determinants continuously in a linear regression model.

For the relationship between birth weight and the cortisol outcomes we expected a U-shaped relationship. To test this, we also added birth weight squared to the linear regression model. Finally, we added possible antecedent or confounding factors of the association between birth weight and cortisol outcomes to the model, such as smoking during pregnancy, gestational age and family income.

Non-response analysis

First, we compared characteristics–such as socio-economic status, prenatal maternal smoking and birth weight–of the 602 infants and mothers of whom we received at least one saliva sample to the 280 non-responders who returned no samples. There were no significant differences between these groups. Second, we compared the characteristics of our study population of 366 infants to the 236 infants who did not return enough saliva samples. In this analysis we found a significant difference in gender of the infant. The infants of the study population were more often boys (57%) compared with the infants who returned not enough saliva samples (47% boys, chi-square = 5.70, df = 1, p = 0.02). The groups showed no significant differences in other sample characteristics.

RESULTS

Table 1 shows the characteristics of our study population. Mean maternal age at enrolment was 31.9 years; only one mother (0.3%) was younger than 20 years. Furthermore, only 14 single mothers (3.8%) were present in our sample. 38.5% of the mothers had a high educational level, i.e. university level or higher, indicating higher socio-economic status. Parents sampled the saliva for cortisol of their children between the ages of 11.7 to 19.3 months.

Maternal age was not associated with the AUC (β per year: − 0.06 nmol/L, 95% CI: − 0.20; 0.09, p = 0.44), the slope (β per year: 0.01 nmol/L/h, 95% CI: − 0.01; 0.04, p = 0.31), or the CAR (β per year: 0.08 nmol/L, 95% CI: − 0.20; 0.35, p = 0.60) of her infant. Infant diurnal cortisol rhythms of boys and girls did not differ significantly as assessed by AUC (boys: 8.0 nmol/L, girls: 8.6 nmol/L, difference: − 0.6, 95% CI: − 1.6; 0.5), diurnal cortisol slope (boys: − 1.1 nmol/L/h, girls: − 1.0 nmol/L/h, difference: − 0.1, 95% CI: − 0.2; 0.1) and CAR (boys: − 2.1 nmol/L, girls: − 1.5 nmol/L, difference: − 0.6, 95% CI: − 2.6; 1.5).

The age of the infant was negatively related to the total cortisol level during the day; older infants displayed lower AUC levels (β per month: − 0.73 nmol/L, 95% CI: − 1.21; − 0.26, p = 0.003). Contrary, a positive association between sampling age and the slope was found (β per month: 0.14 nmol/L/h, 95% CI: 0.05; 0.22, p = 0.002), indicating that older infants showed flatter diurnal cortisol slopes. Almost all the infants showed a decline in cortisol levels during the day, only 19 infants (6.4%) had no diurnal decline.

Fig. 1 shows the mean diurnal cortisol rhythms by categories of age. Infants below 16 months had a significantly steeper slope compared with older infants (mean: − 1.07 vs. − 1.49 nmol/L/h, 95% CI of difference: − 0.77; − 0.07, p = 0.02). The figure illustrates that, on average, infants under 16 months did not show a positive CAR. 119 infants (37.9%) showed a rise of cortisol after awakening; these infants were significantly older than those without (mean difference: 0.3 months, p = 0.01). However, the positive association between sampling age and the CAR was not found when age was entered into the analyses as a continuous variable (β per month: 0.63 nmol/L, 95% CI: − 0.33; 1.59, p = 0.20).

Table 2 shows the associations of social disadvantage and early adversity indicators with the composite cortisol measures AUC and slope. Socio-economic status was significantly associated with the AUC. Infants of low income families had significant higher diurnal cortisol levels (mean AUC: 10.25 nmol/L) compared with infants of high income families (mean AUC: 8.10 nmol/L, difference: 2.16 nmol/L, 95% CI: 0.34; 3.98, p = 0.02). Parenting stress was significantly associated with the AUC. Infants of families with high levels of parenting stress had significant higher diurnal cortisol levels (mean AUC: 9.81 nmol/L) compared with infants of families without parenting stress (mean AUC: 8.12 nmol/L, difference: 1.70 nmol/L, 95% CI: 0.11; 3.28, p = 0.036).

Table 1. Maternal and infant characteristics (n = 366)

	N	Percentage (%)[a]	Mean (SD) or Median (100% range)
Maternal characteristics			
Age (years)	366		31.9 (3.8)
Marital status			
married/living together	340	92.9	
no partner	14	3.8	
Educational level			
low	26	7.1	
middle	193	52.7	
high	141	38.5	
Family income (net per month)			
low income, < 2000 euro	38	10.4	
Smoking during pregnancy (yes)	40	10.9	
Distress during pregnancy (GSI-score)	343		0.10 (0.00-1.67)
Family functioning during pregnancy	342		1.25 (1.00-2.75)
Parity (nulliparous)	222	60.8	
Pregnancy complications (yes)	21	5.8	
Mode of delivery (spontaneous)	234	67.4	
Infant characteristics			
Gender (boys)	207	56.6	
Gestational age at birth (weeks)	366		40.3 (32.9; 42.9)
Birth weight (grams)	366		3517 (517)
Apgar-score 5 minutes after birth	357		10 (5-10)
Age of cortisol sampling (months)	366		14.3 (11.7 –19.3)
Cortisol values (nmol/L)			
1 (awake)	366		15.33 (0.08; 51.03)
2 (30 min)	339		13.05 (0.07; 55.56)
3 (noon)	336		5.41 (0.05; 47.30)
4 (16:00h)	304		4.88 (0.21; 40.48)
5 (bedtime)	304		2.03 (0.09; 58.50)
AUC (nmol/L)	277		7.92 (0.21; 27.83)
Slope (nmol/L/h)	297		-0.95 (-3.82; 2.91)
CAR (nmol/L)	314		-2.83 (-22.1; 37.6)
Nap duration during the day (h)	338		2.5 (0.93)

SD: standard deviation; GSI: Global Severity Index of the Brief Symptom Inventory; nmol/L: nanomoles per liter; AUC: area under the curve; Slope: diurnal cortisol decline during the day (a more negative value indicates a steeper slope); CAR: cortisol awakening response (a positive value indicates a rise of cortisol after awakening and a negative value indicates no rise of cortisol).

[a] Because of missing information on marital status and educational level, not all of the percentages add up to 100%.

Table 2. Effects of social disadvantage and early adversity on diurnal cortisol rhythm[a].

	AUC (nmol/L) *N* = 277	Difference to reference (95% CI)	Slope (nmol/L/h) *N* = 297	Difference to reference (95% CI)
Socio-economic status				
Maternal education				
Low	9.64	1.75 (– 0.61; 4.11)	– 1.02	– 0.00 (– 0.43; 0.43)
Medium	8.39	0.50 (– 0.59; 1.59)	– 1.04	– 0.02 (– 0.21; 0.17)
High	7.89	Reference	– 1.02	Reference
		P for trend: 0.15		*P* for trend: 0.90
Family income (net/month)				
Low, < 2000 euro	10.25	2.16 (0.34; 3.98) *	– 0.89	0.14 (– 0.17; 0.44)
High, > 2000 euro	8.10	Reference	– 1.03	Reference
Early family adversity				
Distress during pregnancy[b]				
Above average range	8.21	– 0.23 (– 2.02; 1.57)	– 1.03	– 0.00 (– 0.31; 0.31)
Below to average range	8.43	Reference	– 1.03	Reference
Hostile behaviour during pregnancy[b]				
Yes	7.55	– 0.93 (– 2.95; 1.09)	– 0.71	0.35 (– 0.01; 0.70)
No	8.48	Reference	– 1.06	Reference
Family functioning during pregnancy[b]				
Poor	8.53	0.15 (– 1.52; 1.81)	– 0.81	0.26 (– 0.03; 0.56)
Healthy	8.38	Reference	– 1.07	Reference
Parenting stress[b]				
Yes	9.81	1.70 (0.11; 3.28) *	– 1.09	– 0.05 (– 0.24; 0.34)
No	8.12	Reference	– 1.04	Reference
Other early adversities				
Maternal smoking during pregnancy				
Yes	8.16	– 0.15 (– 1.91; 1.60)	– 0.99	0.06 (– 0.25; 0.37)
No	8.31	Reference	– 1.05	Reference
Birth weight[c]				
< 3000 g	8.50	0.17 (– 1.41; 1.74)	– 1.12	– 0.06 (– 0.33; 0.21)
3000–4000 g	8.33	Reference	– 1.06	Reference
> 4000 g	7.99	– 0.34 (– 1.79; 1.11)	– 0.91	0.15 (– 0.11; 0.40)

Values are estimated means of CAR, and the differences with 95% confidence intervals compared to the reference category, derived from ANCOVAs. CAR: cortisol awakening response (a positive value indicates a rise of cortisol after awakening and a negative value indicates no rise of cortisol); nmol/L: nanomoles per liter.

Parity, pregnancy complications, mode of delivery, gestational age at birth and Apgar-score 5 min after birth were considered as potential confounders, but were not included in the final models since they did not change the effect estimates by more than 10%.

[a] Models adjusted for gender and age of sampling continuously.

[b] See methods section for definition of cut-offs and measures used.

[c] Models not adjusted for age of sampling continuously. * $p < 0.05$.

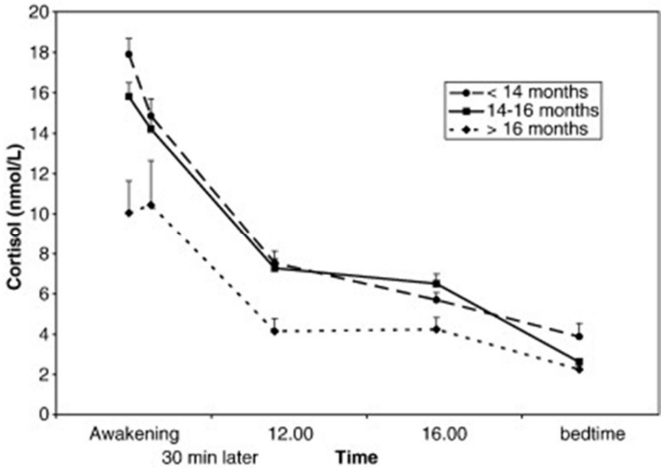

Figure 1. Mean salivary cortisol concentrations (in nmol/L) during the day by age category.
Both the total cortisol secretion during the day (AUC) and the diurnal cortisol slope differ significantly between the depicted age categories.
AUC: > 16 months: 5.85 nmol/L (reference), 14-16 months: 8.43 nmol/L, difference: 2.58 nmol/L, 95% CI: 0.63; 4.53, p=0.01. < 14 months: 8.63 nmol/L, difference: 2.78 nmol/L, 95% CI: 0.80; 4.76, p=0.006. P for trend=0.04.
Slope:> 16 months: -0.66 nmol/L/h (reference), 14-16 months: -0.99 nmol/L/h, difference: -0.33 nmol/L/h, 95% CI: -0.69; 0.02, p=0.07. < 14 months: -1.17 nmol/L/h, difference: -0.52 nmol/L/h, 95% CI: -0.88; -0.16, p=0.005. P for trend=0.003.
Number of infants: < 14 months, N=153; 14-16 months, N=184; > 16 months, N=28.

Table 3 shows the associations of social disadvantage and early adversity indicators with the cortisol awakening response. Infants of low income families also showed, on average, a positive CAR (mean: 1.94 nmol/L), whereas infants of high income families showed a CAR (mean: − 2.26 nmol/L, difference: 4.21 nmol/L, 95% CI: 0.87; 7.55, p = 0.01). Likewise, we observed, a positive CAR in infants of mothers who smoked during pregnancy (mean: 1.54 nmol/L), whereas infants of mothers who did not smoke during pregnancy generally had a negative CAR (mean: − 2.29 nmol/L, difference: 3.83 nmol/L, 95% CI: 0.62; 7.04, p = 0.02). Adjusting for time of awakening did not change the results meaningfully.

Infants of mothers with and without distress during pregnancy showed no significant differences in their levels of AUC, slope or CAR (see Table 2 and Table 3 for details). Also, no differences in cortisol measures were found between infants of mothers with hostile behavior during pregnancy as opposed to infants of non-hostile mothers, although there was a trend towards a difference in diurnal slopes (p = 0.06) and CAR (p = 0.07) between the two groups (see also Table 2).

Infants with a birth weight above 4000 g showed a positive cortisol response after awakening (mean CAR: 0.77 nmol/L), but not those with a birth weight between 3000 and 4000 g (mean

CAR: − 2.36 nmol/L, difference: 3.14 nmol/L, 95% CI: 0.35; 5.93, p = 0.03). Adjusting for gestational age did not change the results.

Table 4 shows the relation between continuous measure of birth weight and the CAR. There was a trend towards a non-linear relationship, as birth weight squared just failed to reach significance (p = 0.057). Adjusting for gestational age, family income, and infant gender slightly attenuated the quadratic term (p = 0.08). The curvilinear relation disappeared after adjusting for maternal smoking during pregnancy (p = 0.18). However, in the fully adjusted model without the quadratic term, birth weight was significantly linearly associated with the CAR (β: 2.77, 95% CI: 0.26; 5.29, p = 0.03).

Table 3. Effects of social disadvantage and early adversity on cortisol awakening response[a].

	CAR (nmol/L) *N* = 314	Difference to reference (95% CI)
Socio-economic status		
Maternal education		
Low	0.89	3.63 (– 0.38; 7.63)
Medium	– 1.72	1.01 (– 1.19; 3.22)
High	– 2.73	Reference
		P for trend: 0.09
Family income (net/month)		
Low, < 2000 euro	1.94	4.21 (0.87; 7.55) *
High, > 2000 euro	– 2.26	Reference
Early family adversity		
Distress during pregnancy[b]		
Above average range	0.44	2.32 (– 1.16; 5.79)
Below to average range	– 1.88	Reference
Hostile behaviour during pregnancy[b]		
Yes	1.43	3.37 (– 0.32; 7.06)
No	– 1.94	Reference
Family functioning during pregnancy[b]		
Poor	– 0.29	1.57 (– 2.03; 5.16)
Healthy	– 1.85	Reference
Parenting stress[b]		
Yes	– 0.89	1.25 (– 1.96; 4.47)
No	– 2.14	Reference
Other early adversities		
Maternal smoking during pregnancy		
Yes	1.54	3.83 (0.62; 7.04) *
No	– 2.29	Reference
Birth weight[c]		
< 3000 g	– 2.36	0.00 (– 2.99; 3.00)
3000–4000 g	– 2.36	Reference
> 4000 g	0.77	3.14 (0.35; 5.93) *

Values are estimated means of CAR, and the differences with 95% confidence intervals compared to the reference category, derived from ANCOVAs. CAR: cortisol awakening response (a positive value indicates a rise of cortisol after awakening and a negative value indicates no rise of cortisol); nmol/L: nanomoles per liter.
Parity, pregnancy complications, mode of delivery, gestational age at birth and Apgar-score 5 min after birth were considered as potential confounders, but were not included in the final models since they did not change the effect estimates by more than 10%.

[a] Models adjusted for gender and age of sampling continuously.

[b] See methods section for definition of cut-offs and measures used.

[c] Models not adjusted for age of sampling continuously.

* $p < 0.05$.

Table 4. Influence of birth weight on the cortisol awakening response (CAR).

	Model 1 Unadjusted		Model 2 Adjusted for basic confounders		Model 3 Additionally adjusted for maternal smoking	
	Beta (95% CI)	*p*-value	Beta (95% CI)	*p*-value	Beta (95% CI)	*p*-value
Linear model						
Birth weight	1.50 (-0.52; 3.52)	0.15	1.99 (-0.48; 4.46)	0.12	2.77 (0.26; 5.29)	0.031
Non-linear model						
Birth weight	-15.3 (-32.8; 2.12)	0.085	-14.5 (-33.2; 4.13)	0.13	-9.98 (-28.9; 8.92)	0.30
Birth weight squared	2.47 (-0.07; 5.02)	0.057	2.37 (-0.28; 5.03)	0.080	1.82 (-0.85; 4.49)	0.18

Values are regression coefficients (95% confidence intervals) from linear regression models and reflect the difference in cortisol awakening response (in nanomoles per liter) per kilogram change in birth weight.
Model 1 = unadjusted model.
Model 2 = as model 1, additionally adjusted for gestational age at birth, family income, and infant gender.
Model 3 = as model 2, additionally adjusted for maternal smoking during pregnancy.

DISCUSSION

The present study showed that socio-economic disadvantage and postnatal parenting stress were associated with the diurnal cortisol rhythms of infants aged 12 to 20 months. Low family income showed the clearest relationship with the diurnal cortisol rhythm; infants of low income families showed higher cortisol levels during the day and a more positive cortisol awakening response (CAR), compared with infants of high income families. Also maternal smoking during pregnancy, another indicator of early adversity, was positively related to the CAR. Interestingly, infants with higher birth weight were also more likely to have a positive CAR.

We found that infants of low income families had higher diurnal cortisol levels as measured by the AUC. Only few studies on cortisol used the AUC to characterize total diurnal cortisol levels in young children. Watamura et al. (2004) found higher cortisol levels during the day, as measured by the AUC, among 12-, 18- and 24-month children than among 30- and 36-month children. These results are in line with the findings in our study, were older infants displayed lower levels of AUC. However, the study of Watamura and colleagues was designed to examine the development of the diurnal cortisol rhythm and did not investigate whether family adversity affects HPA axis activity early in life. Nonetheless, our results are compatible with the results of Lupien et al. (2000). They found that children, aged 6 to 10 years old, with low socio-economic status show higher morning basal cortisol levels than children with high socio-economic status. Educational level of the mother, another indicator for socio-economic status, had a similar albeit non-significant relation with this cortisol composite measure in our study.

Postnatal parenting stress was also related to higher diurnal cortisol levels as measured by the AUC. Parenting stress has been associated with a range of negative outcomes for children including insecure attachment and behavioral problems (Crnic et al., 2005). Although the mechanism that underlies the association between parenting stress and child functioning is not completely clear, it is believed that parenting behavior is the indirect link between parenting stress and child adjustment. Our results suggest that stress hormone levels may be one of the biological pathways behind this link between parenting and child adjustment or behavior. It must be pointed out that parenting stress, in contrast to the other determinants in our study, was assessed cross-sectionally. Thus reversed causality cannot be ruled out. Behavioral or developmental problems in children could lead to higher cortisol levels during the day and, independently, could have precipitated high levels of parenting stress.

Most studies assessing the relationship between early adversity and altered diurnal cortisol rhythms in children examined only morning cortisol levels (e.g. Bruce et al., 2009 and Kertes et al., 2008). Ouellet-Morin et al. (2009) found an effect of family adversity on morning cortisol levels in a laboratory setting only and not in the home setting. The development of the CAR early in life is rarely investigated, even though Bartels et al. (2003) suggested that the cortisol awakening response could be a useful endophenotype of HPA axis functioning in children. Data from studies on healthy adults showed that the CAR is a discrete and distinctive part of the adult diurnal cortisol rhythm (Clow et al., 2004), although approximately 25% of healthy adults do not show a response of cortisol after awakening (Wust et al., 2000). Fries et al. (2009) speculate that the anticipation of the upcoming day is of major relevance for the CAR. Several other correlates of the CAR, such as gonadal steroids, have been also described (Oskis et al., 2009).

Our results show that more often than not there was no positive CAR in this very young population. It is a challenge for parents to sample saliva from infants, in particular, directly after awakening. Stable circadian rhythms with clear 24 h variation in endocrine secretion may be less established in infants than in adults. Moreover, not all infants may have a mature HPA response prior to awakening. Infants awake several times per day after naps, which encompass deep sleep. It is unlikely that a clear CAR follows each awakening. Yet, adjusting our analyses for total nap duration during the day of sampling did not change the results.

We observed that certain indicators of socio-economic disadvantage and other early adversities were related to the CAR. Infants of low income families displayed a more positive CAR, compared with infants of higher income families. We also found that infants of mothers who smoked during pregnancy showed a more positive CAR than infants from non-smoking mothers. Literature on tobacco smoke exposure in utero in relation to the developing HPA

axis of the offspring is scarce. A study of McDonald et al. (2006) is one of the few to report the effect of in utero exposure to tobacco smoking on alterations of hormone secretion in newborns. They found that umbilical adrenocorticotropin hormone levels were significantly elevated in smoke-exposed babies, while umbilical cortisol levels were similar. However, newborns do not have a diurnal cortisol rhythm yet, thus results cannot be compared easily.

In the present study, infants with a higher birth weight displayed a more positive response of cortisol after awakening than infants with lower birth weight. If not fully adjusted, the relationship between birth weight and the CAR was curvilinear, but correction for maternal smoking during pregnancy explained the effect of low birth weight on the CAR.

Several explanations for the observed relationship of social disadvantage and other early adversities with diurnal cortisol rhythms are conceivable. First, the associations between indicators of early adversity and the CAR could be chance findings. Second, the greater likelihood of a positive CAR in older infants could be due to more difficulties with saliva sampling in younger infants. However, if sampling problems underlie our results, we would expect a relation between low socio-economic background and indicators of poor compliance, namely the absence of a positive response of CAR in this group but the reverse was observed. Moreover, the inverse relationship between socio-economic status and the CAR has been described previously albeit in the elderly (Wright and Steptoe, 2005).

At first sight, the pattern emerging from the relations of indicators of early family adversity with the CAR seems inconsistent. Infants of lower income families, those who experienced maternal smoking during pregnancy, and those with a higher birth weight were all more likely to have a positive CAR. However, birth weight and socio-economic status are inversely related to each other.

On the other hand, our findings suggest that the CAR could indicate maturation of the HPA axis. Firstly, in our study a positive CAR was more frequent in older infants and in those with mild early adversity, even if adjusted for age of cortisol sampling. Secondly, offspring of lower socio-economic status are known to mature faster, e.g. show earlier pubertal development, than offspring of higher socio-economic status (Belsky et al., 1991 and Ellis and Essex, 2007). Likewise, maternal smoking during pregnancy accelerates neuro-endocrine maturation. Windham et al. (2004) found that menses onset, which is under control of the hypothalamic–pituitary–gonadal system, occurs 4 months earlier in girls exposed to prenatal smoking. Fried et al. (2001) showed a similar trend in boys, in whom pubertal milestones occurred at an earlier age when prenatally exposed to smoking. The relationship between birth weight within the normal range and endocrine maturation is less clear. Birth weight is not only the

result of endocrine regulation, but also the outcome of prenatal environment and genetic predisposition. However, a higher birth weight in combination with higher weight gain early in life leads to overweight in childhood (Dubois and Girard, 2006) and a higher body mass index or obesity leads to earlier puberty in girls (Kaplowitz, 2008). The possibility that the CAR signals maturation of the endocrine system in infants needs to be investigated.

Although our results indicate that socio-economic disadvantage and exposure to prenatal smoking may accelerate maturation of the HPA axis as measured by the CAR, this pattern corresponding to maturation was not observed for the diurnal cortisol levels as measured by the AUC. Both infants of low income families and infants of families with high levels of parenting stress showed higher diurnal cortisol levels (after adjusting for age), as did the younger infants in our study. This can be interpreted as inconsistency, but higher diurnal cortisol levels could also indicate higher stress levels of the child. This effect would thus be superimposed upon the effect of maturation on the HPA axis.

The strengths of this study are the large population based sample and its prospective design; it is a challenge to study diurnal cortisol rhythms in infants in a sample size of this magnitude. However, the study also has some limitations. Families with higher incomes and mothers who were higher educated were overrepresented by design in our study population if compared to the general Dutch population (CBS StatLine, Statistics Netherlands, 2009 and Jaddoe et al., 2008). This may make our study less generalizable and may have limited the variations of the indicators of early adversity. Most importantly, this reduces the power to find associations between these indicators of early adversity and the diurnal cortisol rhythm. Indeed, young maternal age and single parenthood could not be investigated in the current sample, so only indicators of mild early adversity were studied.

Furthermore, conducting this type of research in a large community-cohort of young children entails difficulties in sampling. In infants sampling depends both on cooperation of the child and the parent (Egliston et al., 2007). To enhance parental cooperation, we asked parents to sample saliva on just 1 single day and not on more (consecutive) days. Asking too much from parents in studies with other assessments raises the dropout risk. Even the present design might have led to selection of healthy, non-fussing infants and their cooperating parents. However, the non-response analyses showed no relevant differences in maternal and infant characteristics between our study population and the non-responders.

Finally, adherence to the sampling protocol is a potential problem as the CAR is especially sensitive to deviation from instructions (Kudielka et al., 2003). Unfortunately, we did not have the opportunity to make use of any compliance devices. We tried to take compliance into

account by the definition of our cortisol composite measures. Although 95% of the parents reported to have taken the first saliva sample at or within 15 min after awakening, we cannot rule out that some infants were earlier awake than their parents. Also, no information about sleep quality during the preceding night or during the sampling day was available. As noted above, it is hard to conceive how this phenomenon could explain our findings. Poor compliance most probably introduced noise, i.e. misclassification of the CAR, making it more likely that we underestimated the effects of family adversity on the HPA axis activity.

In conclusion, this study shows that infants aged between 12 and 20 months show age-related changes in HPA axis activity. Perhaps most importantly, we showed that certain effects of socio-economic disadvantage and early adversity on the diurnal cortisol rhythm are already observable in very young children. Our study also suggests that the CAR is an indicator of the maturation of the HPA axis. The results of this study may help understand the mechanism by which the HPA axis is involved in the pathway to behavioral and emotional problems, and may partly explain the vulnerability of children from lower socio-economic background to psychopathology.

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CHAPTER 3

The diurnal cortisol rhythm and psychosocial functioning

Chapter 3.1

Diurnal cortisol rhythm and cognitive functioning in toddlers

Saridjan NS, Henrichs J, Schenk JJ, Jaddoe VW, Hofman A, Kirschbaum C, Verhulst FC, Tiemeier H.
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ABSTRACT

Little is known about the relationship between diurnal cortisol secretion patterns and cognitive function early in life. This population-based study examined whether diurnal cortisol rhythms and cognitive functioning in toddlers are related. Within the Generation R Study, parents of 364 infants (median age: 14.2 months) collected saliva samples at five moments during one day. We assessed the diurnal cortisol rhythm by calculating the area under the curve (AUC), the cortisol awakening response (CAR), and the diurnal slope. Verbal cognitive functioning and fine motor development was determined at age 18 months. Nonverbal cognitive functioning was assessed at age 30 months. A more positive CAR was associated with a lower risk of delay in language comprehension (OR per 1-SD CAR: 0.62, 95%CI: 0.40–0.98, p = .04), a lower risk of nonoptimal fine motor development (OR per 1-SD slope: 0.74, 95%CI: 0.57–0.96, p = .03), and a lower risk of delay in nonverbal cognitive development (OR per 1-SD CAR: 0.58, 95%CI: 0.38–0.90, p = .02). Also, children with flatter slopes had a lower risk of delay in nonverbal cognitive development (OR per 1-SD slope: 0.51, 95%CI: 0.34–0.76, p = .001). Higher AUC levels were associated with a higher risk of delay in language production. These results show that variations in diurnal cortisol rhythms are already associated with variations in cognitive functioning at a young age. Infants with a diurnal cortisol pattern indicative of less stress and more cortisol reactivity, that is, lower AUC levels and a more positive CAR, show a lower risk of delay in cognitive functioning as toddlers.

INTRODUCTION

The relationship between cortisol and cognition has intrigued many researchers ever since the reported side effects of decreased cognitive abilities following the therapeutic use of glucocorticoids as anti-inflammatory drugs (Lupien, Maheu, Tu, Fiocco, & Schramek, 2007). Cortisol is the most important human glucocorticoid and is produced as the hormonal end-product of the hypothalamic-pituitary-adrenal (HPA) axis. This hormone is not only released by the adrenal cortex in response to a wide range of stressors but also regulates energy metabolism. More importantly for cognition, cortisol modulates neurotransmitter systems and regulates the plasticity and circuitry of many brain regions, thereby affecting numerous cognitive domains (e.g., Erickson, Drevets, & Schulkin, 2003).

Glucocorticoids exert their effect through the mineralocorticoid and glucocorticoid receptor, both abundantly expressed in the brain (Oitzl, Champagne, van der Veen, & de Kloet, 2010). Importantly, corticosteroids influence neural plasticity (Radley et al., 2011). Several investigators demonstrated an effect of glucocorticoids on medial temporal lobe structures, where corticosteroids have been shown to also affect neuronal excitability, synaptic development, and memory (Henckens et al., 2011). Another brain region affected by cortisol is the prefrontal cortex (PFC), which is critical for working memory, executive functioning, and extinction processes in learning (MacDonald, 2008). In adults both high glucocorticoid levels and inhibited cortisol synthesis have been associated with memory impairments (Lupien et al., 2005), and higher levels have also been related to smaller volumes of the hippocampus (Knoops, Gerritsen, van der Graaf, Mali, & Geerlings, 2010). Similarly, an effect of glucocorticoid administration on memory functions has been shown in younger adults (Lupien et al., 2002). Research investigating the effect of cortisol on children's cognitive development is rare. Moreover, so far, specific effects of either elevated or blunted glucocorticoid levels on language production or development have — to the best of our knowledge — not been reported. Therefore, the aim of the current study was to examine the influence of cortisol on cognitive development in early childhood.

In humans, cortisol levels show a distinct pattern throughout the day. This diurnal cortisol rhythm in adults is characterized by postwaking peak cortisol levels and subsequent declining cortisol levels throughout the day (Edwards, Clow, Evans, & Hucklebridge, 2001). Dysregulation of the normal diurnal rhythm may impact cognitive functioning. Diurnal cortisol secretion patterns have, however, been studied mostly in the context of cognitive decline in the elderly. As Evans et al. (2011) pointed out, studies investigating an HPA axis in relation to both ageing and cognitive functioning are complicated by the dynamic nature of the diurnal cortisol pattern. Diverse computations of cortisol measures resembling different aspects of

the diurnal cortisol rhythm have been used to capture both basal levels and dynamics of change over the day, such as the diurnal cortisol slope (Beluche, Carrière, Ritchie, & Ancelin, 2010), the area under the curve (AUC), and the cortisol awakening response (CAR; Heaney, Phillips, & Carroll, 2010).

Although the precise role and importance of the changes of cortisol in the highly dynamic period immediately after awakening are still under current investigation, Fries, Dettenborn, and Kirschbaum (2009) showed that the hippocampus may play an important role in the regulation of the cortisol awakening response. As the hippocampus is central to long-term memory consolidation and is involved in other cognitive processes (Sweatt, 2004), the relationship between the cortisol awakening response and cognitive functioning is of particular interest in adults and in children.

The diurnal rhythm of cortisol in children is still under development. Infants are born without a diurnal cortisol rhythm and this rhythm emerges during the first 18 months of life. Watamura, Donzella, Kertes, and Gunnar (2004) even posited that there is an ongoing maturation of the HPA axis up to the third year of life. Studies of HPA axis activity in infants and toddlers have focused on various aspects of psychological development, such as temperament, behavior, and attachment but less on cognitive development (van Bakel & Riksen-Walraven, 2004; Watamura, Donzella, Alwin, & Gunnar, 2003).

Recent studies suggest that the relationship between basal cortisol levels and cognition not only evolves over time but is also intimately linked to the relation with socioeconomic status (SES). SES is an important predictor of cognitive functioning (Hackman & Farah, 2009). Moreover, in the present cohort, we previously found that SES was associated with variations in diurnal cortisol patterns in infants (Saridjan et al., 2010). This suggests that SES must be carefully controlled for when investigating the association between diurnal cortisol secretion patterns and cognitive function early in life. Also, in our sample, older infants showed a more positive cortisol awakening response, indicating that this may reflect a maturing diurnal cortisol pattern. If this interpretation is correct, that a better developed CAR is a sign of maturation, one might assume that a more positive cortisol awakening response may also be associated with better cognitive development.

To our knowledge, there are no existing studies examining the diurnal cortisol rhythm, including the cortisol awakening response, in relation to cognitive development early in life. In healthy older people, changes in the cortisol awakening response have been associated with older age and changes in cognitive performance. An inverse relationship was found between age and cognitive performance with an attenuated cortisol awakening response (Evans et al.,

2011). Interestingly, these researchers found that individuals who performed better on the cognitive tests tended to have a more dynamic CAR, that is, the rise in morning cortisol was followed by a more dynamic (steeper) average fall from that peak. However, these results cannot be extrapolated straightforwardly to children. Heaney et al. (2010) demonstrated that the diurnal cortisol rhythm and the cortisol awakening response differ with age even in healthy adults.

We examined cortisol patterns early in life in relation to cognitive functioning in toddlers. As opposed to most other studies assessing cortisol reactivity to a stressor, we focused on diurnal cortisol secretion patterns. Moreover, we also determined the cortisol awakening response, a distinct feature of the HPA axis superimposing the diurnal rhythm and representing the response of the HPA axis to awakening (Wilhelm, Born, Kudielka, Schlotz, & Wust, 2007). We used both language functioning at age 18 months and nonverbal cognitive functioning at age 30 months as outcome measures, but by design these are essentially cross-sectional analyses with a single assessment of cortisol and cognitive function. As the interrelation between cognitive and motor development in typically developing children is underpinned by fine motor control (Davis, Pitchford, & Limback, 2011), we also examined fine motor development at age 18 months.

Our overall aim was to examine the relationship between variations in diurnal cortisol rhythm and cognitive functioning in toddlers. We tested two hypotheses. First, we examined whether levels of cortisol, as indicated by the AUC, are related to less optimal verbal and nonverbal development. Second, we postulated that a more reactive HPA axis, as indicated by a higher cortisol awakening response, is related to a more optimal verbal and nonverbal cognitive development. Furthermore, the different outcomes, that is, language, nonverbal, and fine motor development, allowed us to test whether any of the observed associations were consistent across different domains of cognitive performance.

METHODS

Setting

This study was embedded within the Generation R Focus Study, a cohort study investigating growth, development, and health from fetal life into young adulthood in Rotterdam, the Netherlands. The cohort has been described in detail elsewhere (Jaddoe et al., 2008). The Generation R Focus Study, a subgroup within the Generation R Study, is conducted to obtain detailed measurements of the child's development in an ethnically homogeneous subgroup to exclude confounding or effect modification by ethnicity. Only children of Dutch national

origin were included in this group, that is, the children, their parents, and their grandparents were all born in the Netherlands. The participating children were born between February 2003 and August 2005. The children visited the research center regularly for various somatic and behavioral assessments. Written informed consent was obtained from all participants. The study has been approved by the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam.

Study Population

For the current study, children who visited the research center for the focus study around age 14 months were eligible for assessment of the diurnal cortisol profile. Parents of 602 children who attended the focus cohort examination returned one or more saliva samples. Of these, 236 children had to be excluded, because two morning samples or at least three saliva samples per child had to be obtained to compute a cortisol composite measure. The area under the curve was calculated in 277 children, the diurnal cortisol slope in 297 children, and the cortisol awakening response in 314 children. For 366 children, at least one of these cortisol composite measures could be computed.

At age 18 months, information on language development was available in 354 children (97% of 366) and information on fine motor development was available in 339 children (93% of 366). Information on nonverbal cognitive development at age 30 months was available in 332 children (91% of 366). A total of 364 children (99% of 366) were included in one or more analyses of the relation between cortisol and cognitive functioning.

Salivary Cortisol Measurements

An extensive description of the cortisol measurement and analysis was presented previously (Saridjan et al., 2010). Prior to the focus study visit at 14 months, parents were instructed to collect five saliva samples at home using Salivette sampling devices (Sarstedt, Rommelsdorf, Germany). Parents received detailed written instructions with pictures concerning the saliva sampling. These saliva samples were collected during one single weekday: immediately after awakening, 30 minutes later, around noon, between 3 and 4 pm, and at bedtime. For the noon salvia sample collection, parents reported a mean deviation time of 0.42 h (equalling 26 minutes). Parents were asked not to let their infant eat or drink 30 minutes before saliva sampling to avoid disturbances of the cortisol levels. Besides these restrictions, the infants were free to follow their normal daily routines on the sampling day. Parents were asked to record information about sampling times on the Salivette tubes as well as on an enclosed schematic form. Here, information about napping time and food intake also had to be added. The Salivettes were gathered at the laboratory of the Department of Epidemiology at the Erasmus MC. Here, the samples were centrifuged and frozen at −80°C. After completion of

the data collection, all frozen samples were sent on dry ice in one batch by courier to the laboratory of the Department of Biological Psychology laboratory at the Technical University of Dresden for analysis. Salivary cortisol concentrations were measured using a commercial immunoassay with chemiluminescence detection (CLIA; IBL Hamburg, Germany). Intra- and interassay coefficients of variation were below 7% and 9%, respectively.

For each time point, cortisol values that were above the 99th percentile (>200 nmol/L) were excluded (n = 18, equalling n = 12 children) from the analysis to reduce impact of outliers.

We calculated three composite variables of the separate cortisol measurements within a day: the area under the curve (AUC), the diurnal cortisol slope, and the cortisol awakening response (CAR). These independent variables characterize different aspects of the HPA axis activity. The AUC was used as a measure of total cortisol secretion during the day (from awakening in the morning until bedtime in the evening). It was determined by the total area under the curve given by the cortisol measurements in nmol/L on the y-axis and the time between the cortisol measurements on the x-axis, in the same way as previously described by Pruessner, Kirschbaum, Meinlschmid, and Hellhammer (2003) using the formula for calculating the area under the curve with respect to the ground. To correct for differences in length of total sampling interval time, the AUC was divided by number of hours between the first cortisol measurement at awakening and the last cortisol measurement before going to bed. The AUC was computed only for those who collected at least three saliva samples. Sleeping hours during the day were not associated with the AUC.

The diurnal cortisol slope was used as a measure of the diurnal cortisol decline. It was calculated by fitting a linear regression line for each child, which predicted the cortisol values from time since awakening. The slope was computed by using the first saliva sample and at least two other cortisol time point measures. To avoid any effect of the CAR (Adam, Hawkley, Kudielka, & Cacioppo, 2006; Cohen et al., 2006), the second cortisol sample (30 minutes after awakening) was not included in this measure of the slope. Flatter slopes, as indexed by less negative betas, imply a slower cortisol decline during the day. This can be due to relatively lower morning cortisol levels or relatively higher levels in the afternoon or evening. To determine the influence of the first and last cortisol levels on the slope, the correlation between these cortisol levels and the slope was analyzed.

The CAR was also used as an index of the HPA axis activity. It was calculated as the difference between the cortisol value at awakening and the value 30 minutes after awakening (Kunz-Ebrecht, Kirschbaum, Marmot, & Steptoe, 2004). The CAR was only calculated if the cortisol value 30 minutes after awakening was taken between 15 minutes and 60 minutes after

awakening. Ninety-five percent of the parents reported to have sampled the first saliva sample immediately or within 15 minutes after awakening.

All the composite cortisol measures were eventually z standardized across our sample to obtain meaningful effect estimates in our statistical models.

Assessment of Covariates

The choice of potential confounders was determined a priori and based on earlier literature. Socioeconomic-status-related variables (maternal educational level, family income, maternal smoking during pregnancy), obstetric and neonatal variables (parity, gestational age at birth, birth weight), and other known determinants of (mother-reported) infant cognitive abilities (infant gender, infant age, duration of breastfeeding) were considered as possible confounders, in line with studies of growth and cognition (Belfort et al., 2008). Since birth hypoxia has been shown to influence cognitive development in preterm-born infants (Hopkins-Golightly, Raz, & Sander, 2003), we also considered Apgar score 5 minutes after birth as a possible confounder.

Information about maternal age, maternal educational level, parity, and family income were obtained at enrollment using self-report. Educational level was categorized in three levels: low (no or primary education and lower vocational training), middle (intermediate and higher vocational training), and high education (university or higher). Family income was dichotomized in net income less than 2000 euro and more than 2000 euro a month. Maternal smoking was determined by postal questionnaires during pregnancy. Mothers were classified as smokers or nonsmokers during pregnancy. Date of birth, gestational age at birth, birth weight, Apgar score 5 minutes after birth, and gender of the infant were obtained from community midwife and hospital registries at birth. Data on duration of breastfeeding were collected from postal questionnaires after birth, with items on breastfeeding at the child's age of 2 months, 6 months and 12 months.

Assessment of Cognitive Outcome Measures

Verbal Cognitive Development

To assess language development at 18 months of age, we used the Dutch version of the MacArthur Short Form Vocabulary Checklists (N-CDI 2A), which measures the word production and comprehension of children aged 16 to 30 months (Zink & Lejaegere, 2003). This short-form version, which contains a list of 112 words, is based on the original MacArthur Communicative Development Inventory (MCDI) consisting of 680 words (Fenson et al., 1994, 2000). For the vocabulary measure reported here, parents check the words they think their child understands (receptive language development) and the words they have heard their child say (expressive language development). The number of positive responses was summed

for both receptive and expressive language development. We converted the sum scores into age- and gender-specific percentile scores based on the norms of the validation study of the Dutch short form of the MCDI (Zink & Lejaegere, 2003). As the percentile scores of word production and comprehension were not normally distributed, they were dichotomized. A delay in word production or comprehension was defined as scores below the 10th percentile for each scale in line with previous research (Dale, 1996; Henrichs et al., 2011). A less stringent cutoff score, defined as scores below the 15th percentile, has also been used (Daniels, Longnecker, Rowland, & Golding, 2004; Henrichs et al., 2010), this was also tested to check consistency. The Dutch short form of the MCDI has excellent internal consistency and test-retest reliability, as well as concurrent validity (Zink & Lejaegere, 2003). Internal consistencies of word production and comprehension were very high, that is, $\alpha > .97$ and $\alpha > .98$, respectively. Furthermore, validity results revealed that both language production and comprehension scores on the short form predicted the respective scores on the original form of the MCDI with very high accuracy, that is, $r = .97$ and $r = .99$, respectively (Zink & Lejaegere, 2003). Fenson et al. (1994) reported a correlation of .73 between the original MCDI and a standard tester-administered measure of expressive vocabulary.

Nonverbal Cognitive Development

Nonverbal cognitive development at 30 months of age was assessed using the Dutch version of the Parent Report of Children's Abilities (PARCA; see Saudino et al., 1998). The PARCA is an hour-long test consisting of a parent-report part and a parent-administered part. The parent-report part comprises 26 questions, which assess the areas of quantitative skills, spatial abilities, symbolic play, planning and organizing, adaptive behaviors, and memory. The parent-administered part consisted of 22 items within three categories of tasks: (a) matching-to-sample, (b) block building, and (c) imitation. Parents were asked to follow the instructions to administer each item and to indicate the child's response. A total score for the parent-administered part was obtained by summing across the child's scores on each task. The total PARCA score was computed by summing the scores from the parent-report and the parent-administered parts. In line with the definitions of verbal cognitive delay, nonverbal cognitive delay was defined as scores below the 10th age- and gender-specific percentile, as well as a less stringent cutoff of scores below the 15th percentile.

In 2-year-old children internal consistency of the parent-report component was estimated .74 and the internal consistency for the parent-administered part .83 (Saudino et al., 1998). Age-corrected scores on the parent-report part of the PARCA significantly predicted performance on the Mental Development Index (MDI) of the Bayley Scales of Infant Development-II ($r = .49$). The validity of parental measures is supported by a review of 23 studies investigating the relation between parental ratings and standard administered measures (Dinnebeil & Rule, 1994).

Fine Motor Development

The fine motor development scale of the Dutch translation of the Minnesota Infant Development Inventory (MIDI) was used to assess this developmental milestone attainment of 18-month-old children by maternal report (Ireton & Thwing, 1980). We used seven age-appropriate items, according to the MIDI manual instructions (Ireton, 1992; Reilly & Eaves, 2000). Parents were asked to indicate the milestones their child was able to perform. By totaling the "yes" responses, sum scores were obtained. Difference scores were then calculated by subtracting the fine motor development age from the child's calendar age at assessment of the MIDI. Because of a ceiling effect in our sample, a median split was performed by categorizing the children into those with an optimal fine motor development and those with a nonoptimal fine motor development.

Statistical Analysis

To examine whether nonresponse was selective, we compared the maternal and infant characteristics of our study population with the characteristics of the mothers and infants with no information on the cortisol composite measures and the cognitive measures. For continuous variables approaching a normal distribution, we used independent t-tests, for continuous non-normally distributed variables Mann-Whitney U tests, and for categorical variables chi-square statistics. Analyses of missing data showed that children with insufficient cortisol sampling or without information on their cognitive measures were more often girls (53.8% vs. 43.1%, chi-square = 6.55, df = 1, p = .01) and had lower Apgar scores 5 minutes after birth (Apgar score below 8: 10.2% vs. 4.5%, chi-square = 7.34, df = 1, p = .01) . These children did not differ on any other characteristics compared with the children included in the current study sample.

Although the computed variables AUC, slope, and CAR showed a slightly skewed distribution, we decided not to transform these variables since regression residuals of the association between the composite cortisol variables and the cognitive outcome measurements were normally distributed. Furthermore, this makes interpretation of the results more straight forward. The correlation between the different cortisol composite variables was tested, as well as the correlation between covariates, the cortisol composite measures, and the cognitive outcome measures using Spearman's rho, polyserial, and polychoric correlations where appropriate.

We used logistic regression models to test the associations between the composite variables of cortisol and a delay in verbal and nonverbal cognitive development (defined as scores below the 10th and 15th age- and gender-specific percentile). In addition, linear regression models were used to test the associations between the composite variables of cortisol and

the continuous nonverbal cognitive development measures. All associations were firstly adjusted for child's age at cortisol sampling. In line with the method of Mickey and Greenland (1989), other covariates were included in the analyses if the effect estimates of risk in delay of cognitive development changed meaningfully (> 5%). As socioeconomic status is an important confounder in our analyses, we carefully controlled for these variables in our models. In this study, we did not focus on the association between socioeconomic status and the cortisol composite measures as this has been published previously in Saridjan et al. (2010). In the final models we additionally adjusted for maternal educational level, family income, and maternal smoking during pregnancy. For the linear regression models, we also adjusted for gender of the child and age of PARCA assessment. As the percentile scores of verbal and nonverbal cognitive development were age and gender specific, the logistic regression models did not include child age and gender at cognitive assessment as covariates. We did not include parity, gestational age at birth, birth weight, Apgar score, and duration of breastfeeding in our models, since these covariates did not change the effect estimates meaningfully.

To study fine motor development at 18 months as an additional outcome measure, we first tested whether fine motor development was cross-sectionally and longitudinally related to a risk in delay of cognitive development using logistic regression. Next, the cortisol composite variables were associated to fine motor development to examine the specificity of any of the observed cognitive development findings.

All statistical analyses were performed with IBM SPSS Statistics 20 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Tables 1 and 2 show the maternal and child characteristics of our study population. Data on subject characteristics specifically divided by no delay versus delay in language production and optimal versus nonoptimal fine motor development is shown in supplementary tables S1 and S2. Mean maternal age at enrollment was 31.9 years and ranged from 16.2 to 43.3 years. The majority of the mothers initiated breastfeeding after birth; only 8.5% of the mothers did not start breastfeeding at all. The median cortisol values at different time points during the day were at awakening 15.33 nmol/L (range: 0.08–51.03), 30 minutes after awakening 13.04 nmol/L (range: 0.07–55.56), at noon 5.41 nmol/L (range: 0.05–47.29), around 16:00h 4.87 nmol/L (range: 0.21–40.48), and at bedtime 2.03 nmol/L (range: 0.09–58.50).

Table 1. Subject characteristics.

	Total *N*=364 #	No delay in nonverbal cognitive functioning *N*=267	Delay in nonverbal cognitive functioning *N*=44
Maternal characteristics			
Age, years	31.9 (3.7)	31.8 (3.8)	32.5 (2.6)
Educational level			
low (%)	6.9	6.1	4.5
middle (%)	53.0	56.1	36.4
high (%)	38.7	37.9	59.1 *
Family income (net per month)			
low, < 2000 euro (%)	10.2	12.6	7.5
Smoking during pregnancy (% yes)	10.7	9.0	18.2
Parity (% nulliparous)	60.7	58.4	68.2
Child characteristics			
Gender (% boys)	56.9	55.4	61.4
Gestational age at birth, weeks (range)	40.3 (32.7-42.7)	40.3 (34.7-42.7)	40.4 (34.4-42.9)
Birth weight, grams	3520 (515)	3568 (469)	3347 (543) *
Apgar-score 5 minutes after birth (range)	10 (5-10)	10 (7-10)	10 (5-10)
Duration of breast-feeding, months (range)	4 (0-12)	4 (0-12)	4.5 (0-12)
Age at cortisol sampling, months (range)	14.22 (11.7-19.3)	14.16 (12.6-19.3)	14.24 (13.0-17.2)
AUC, nmol/L (range)	7.92 (0.21; 27.8)	7.67 (0.21; 27.8)	8.73 (1.97; 23.5)
Slope, nmol/L/h (range)	-0.95 (-3.82; 2.91)	-0.92 (-3.82; 2.91)	-1.37 (-3.44; 0.39) *
CAR, nmol/L (range)	-2.85 (-22.1; 37.6)	-2.45 (-19.7; 37.6)	-6.23 (-18.1; 10.7) *

Notes. Values are means (SD) unless otherwise indicated. Independent t-tests were used for continuous normal distributed variables, Chi-square tests were used for categorical variables, and Mann-Whitney-U tests for continuous non-normal distributed variables; SD: standard deviation; AUC: area under the curve; Slope: diurnal cortisol decline during the day (a more negative value indicates a steeper slope); CAR: cortisol awakening response (a positive value indicates a rise of cortisol after awakening and a negative value indicates no rise of cortisol); nmol/L: nanomoles per liter. # N with information on verbal cognitive functioning = 354, N with information on nonverbal cognitive functioning = 311, N with information on both verbal and nonverbal cognitive functioning = 298. *p < .05.

Table 2. Subject characteristics.

	Total *N*=364 #	No delay in language comprehension *N*=316	Delay in language comprehension *N*=35
Maternal characteristics			
Age, years	31.9 (3.7)	31.9 (3.7)	32.1 (3.6)
Educational level			
low (%)	6.9	7.4	2.9
middle (%)	53.0	54.3	45.7
high (%)	38.7	38.3	51.4
Family income (net per month)			
low, < 2000 euro (%)	10.2	11.5	0
Smoking during pregnancy (% yes)	10.7	10.1	17.1
Parity (% nulliparous)	60.7	61.1	62.9
Child characteristics			
Gender (% boys)	56.9	55.4	68.6
Gestational age at birth, weeks (range)	40.3 (32.7-42.7)	40.4 (34.4-42.9)	39.6 (32.9-42.4) *
Birth weight, grams	3520 (515)	3525 (518)	3474 (519)
Apgar-score 5 minutes after birth (range)	10 (5-10)	10 (5-10)	10 (9-10)
Duration of breast-feeding, months (range)	4 (0-12)	4 (0-12)	4 (0-12)
Age at cortisol sampling, months (range)	14.22 (11.7-19.3)	14.19 (11.2-19.3)	14.35 (13.4-16.8)
AUC, nmol/L (range)	7.92 (0.21; 27.8)	7.82 (0.21; 27.8)	8.30 (1.97; 17.10)
Slope, nmol/L/h (range)	-0.95 (-3.82; 2.91)	-0.95 (-3.82; 2.05)	-0.92 (-3.44; 2.91)
CAR, nmol/L (range)	-2.85 (-22.1; 37.6)	-2.67 (-22.1; 37.6)	-6.23 (-18.9; 8.43) *

Notes. Values are means (SD) unless otherwise indicated. Independent t-tests were used for continuous normal distributed variables, Chi-square tests were used for categorical variables, and Mann-Whitney-U tests for continuous non-normal distributed variables. SD: standard deviation; AUC: area under the curve; Slope: diurnal cortisol decline during the day (a more negative value indicates a steeper slope); CAR: cortisol awakening response (a positive value indicates a rise of cortisol after awakening and a negative value indicates no rise of cortisol); nmol/L: nanomoles per liter. # N with information on verbal cognitive functioning = 354, N with information on fine motor development = 339, N with information on nonverbal cognitive functioning = 311, N with information on fine motor development, verbal and nonverbal cognitive functioning = 287. *p < .05.

Table 3 shows that the cortisol composite measures AUC and CAR were not correlated (Spearman's rho = .09, p = .16), the slope and AUC were negatively correlated (Spearman's rho = −.37, p < .01) whereas the slope and the CAR were positively correlated (Spearman's rho = .60, p < .01). The morning cortisol level was highly correlated with the slope (Spearman's rho = −.91, p < .01). In contrast, the evening cortisol level was not correlated to the slope (Spearman's rho = −.01, p = .90). This shows that in this sample of infants the slope largely depends on the early morning cortisol levels. The table also shows the significant correlations of the CAR with early delay in language comprehension and nonverbal cognition.

Table 3. Correlation between socio-economic status, cortisol composite measures and cognitive outcome measures.

	CAR	AUC	slope	Language comprehension at 18 months (delay vs. no delay)	Language production at 18 months (delay vs. no delay)	Fine motor development at 18 months (non-optimal vs. optimal)	Nonverbal cognitive functioning at 30 months (delay vs. no delay)
Maternal age (years)	0.07	-0.01	0.07	0.02	0.09	-0.03	0.11
Maternal educational level (low-middle-high)	-0.11	-0.08	-0.002	0.18	0.16	-0.05	0.24 *
Family income (low vs. high)	-0.21 *	-0.22 *	-0.09	0.85	-0.06	0.05	0.16
Maternal Smoking during pregnancy (yes/no)	0.18 *	-0.03	0.04	0.17	0.13	0.05	0.24
Parity	0.01	0.07	0.02	-0.01	0.23 *	-0.13	-0.09
Child's gender	0.03	0.09	0.05	-0.18	0.00	-0.02	-0.08
Gestational age (weeks)	0.03	-0.07	0.04	-0.18 *	-0.10	-0.15 *	-0.08
Birth weight (grams)	0.11 *	-0.09	0.09	-0.05	-0.05	-0.16 *	-0.23 **
Apgar score	-0.02	-0.02	0.07	0.13	0.11	-0.08	-0.05
Duration of breastfeeding (months)	-0.10	0.03	-0.12 *	-0.04	0.04	-0.08	0.02
CAR	-	0.09	0.60 **	-0.26 *	0.07	0.03	-0.27 *
AUC	0.09	-	-0.37 **	0.05	0.17 *	0.10	0.15
Slope	0.60 **	-0.37 **	-	-0.06	0.14	-0.17 *	-0.35 **

Notes. Values are correlation coefficients. Spearman's rho was used for correlations between continuous variables, polyserial correlations were calculated between continuous and categorical variables, and polychoric correlations were calculated between categorical variables. AUC: area under the curve; Slope: diurnal cortisol decline during the day (a more negative value indicates a steeper slope); CAR: cortisol awakening response (a positive value indicates a rise of cortisol after awakening and a negative value indicates no rise of cortisol); nmol/L: nanomoles per liter. *p < .05. **p < .01.

Although children with a delay in nonverbal cognitive functioning had significantly higher educated mothers, maternal educational level and the cortisol composite measures AUC, slope, and CAR were not correlated (results not shown). Children with a delay in nonverbal cognitive functioning also had a significantly lower birth weight. Interestingly, birth weight was positively correlated to the CAR (Spearman's rho = .11, p = .04) indicating that a higher birth weight is associated with a more positive CAR. The AUC and slope were not correlated with birth weight (results not shown).

Table 4 shows the cross-sectional association between infant HPA axis activity and the risk of a delay in language comprehension and production at 18 months. A more positive cortisol awakening response was associated with a lower risk of delay in language comprehension (OR per z-standardized score: 0.60, 95% CI: 0.39–0.94, p = .03), in an analysis adjusted for child's age at cortisol sampling. Correction for socioeconomic status and maternal smoking during pregnancy did not change the results (see Table 4). With a less stringent cutoff (delay in language comprehension defined as scores < 15th percentile), this association was nonsignificant (OR per z-standardized score: 0.70, 95% CI: 0.49–1.02, p = .06). Although the association between the CAR and language production appeared to be in the opposite direction, the effect estimate (OR per z-standardized score: 1.09, 95% CI: 0.81–1.47) was close to unity and clearly not significant. Higher levels of total cortisol secretion during the day, as measured by the AUC, were associated with a higher risk of delay in language production, independent of the child's age at cortisol sampling (OR per z-standardized score: 1.38, 95% CI: 1.03–1.84, p = .03). Correction for socioeconomic status and maternal smoking during pregnancy did not materially change the results (see Table 4).

We also found significant associations between infant HPA axis activity and the risk of a delay in nonverbal cognitive development at 30 months. Again, a more positive cortisol awakening response was associated with a lower risk of delay in nonverbal cognitive development (OR per z-standardized score: 0.58, 95% CI: 0.38–0.90, p = .02), in an analysis adjusted for child's age at cortisol sampling. Moreover, there was a significant association between the diurnal cortisol slope and a lower risk of delay in nonverbal cognitive development (OR per z-standardized score: 0.51, 95% CI: 0.34–0.76, p = .001). Correction for socioeconomic status and maternal smoking during pregnancy did not change these results (see Table 5). As the diurnal cortisol slope represents a decline in cortisol during the day, which is expressed as a negative number, this means that flatter slopes are associated with higher scores on nonverbal cognitive development. The associations between infant HPA axis activity and the risk of a delay in nonverbal cognitive development at 30 months were very similar if nonverbal cognitive delay was defined as nonverbal cognitive scores below the 15th age- and gender-specific percentile. A more positive CAR as well as flatter diurnal slopes were each

both significantly associated with a lower risk of delay in nonverbal cognitive development (OR per one SD CAR: 0.59, 95%CI: 0.40–0.86, p = .006; OR per one SD slope: 0.63, 95%CI: 0.45–0.89, p = .008).

In the linear regression models, we observed a trend for a positive association between a more positive cortisol awakening response and the z-standardized PARCA total score (β = 0.11, 95% CI: −0.01–0.23, p = .07). Moreover, there was a significant association between the diurnal cortisol slope and the z-standardized PARCA total score (β = 0.15, 95% CI: 0.02–0.27, p = .02) in the fully adjusted models.

Nonoptimal fine motor development at 18 months was related to a higher likelihood of a delay in language production at 18 months (OR 1.94, 95% CI: 1.11–4.81, p = .02) and a delay in nonverbal cognitive development at 30 months (OR 2.41, 95% CI: 1.21–4.81, p = .01). There was no significant relation between nonoptimal fine motor development and delayed language comprehension at 18 months (OR: 1.94, 95% CI: 0.86–3.96, p = .12).

Significant alterations in infant HPA axis activity were associated with a higher risk of nonoptimal fine motor development (OR per z-standardized score of the diurnal slope: 0.74, 95% CI: 0.57–0.96, p = .03) (see Table 6). This finding indicated that flatter slopes are associated with a lower risk of nonoptimal fine motor development. No associations were found between the AUC (OR per z-standardized AUC: 1.21, 95% CI: 0.94–1.56) or the CAR and nonoptimal fine motor development (OR per z-standardized CAR: 1.05, 95% CI: 0.83–1.34).

Table 4. Infant HPA axis activity and risk of delay in language comprehension and production at 18 months.

	Risk of Delay in Language Comprehension (*N* = 299)				Risk of Delay in Language Production (*N* = 302)			
	Model 1		Model 2		Model 1		Model 2	
z-standardized	OR (95% CI)	*p* value	OR (95% CI)	*p* value	OR (95% CI)	*p* value	OR (95% CI)	*p* value
CAR (nmol/L)	0.60 (0.39–0.94)	.03	0.62 (0.40–0.98)	.04	1.12 (0.85–1.48)	.42	1.09 (0.81–1.47)	.55
Slope (nmol/L/h)	0.89 (0.61–1.29)	.53	0.91 (0.62–1.35)	.65	1.30 (0.96–1.76)	.09	1.24 (0.91–1.70)	.18
AUC (nmol/L)	1.11 (0.76–1.61)	.59	1.24 (0.83–1.85)	.29	1.38 (1.03–1.84)	.03	1.37 (1.01–1.86)	.04

Notes. OR = odds ratio; CI = confidence interval; AUC: area under the curve; Slope: diurnal cortisol decline during the day (a more negative value indicates a steeper slope); CAR: cortisol awakening response (a positive value indicates a rise of cortisol after awakening and a negative value indicates no rise of cortisol); nmol/L: nanomoles per liter. Model 1: adjusted for child's age at cortisol sampling; Model 2: additionally adjusted for maternal educational level, family income and maternal smoking during pregnancy.

Table 5. Infant HPA axis activity and nonverbal cognitive functioning at 30 months.

		Risk of delay in nonverbal cognitive functioning			
		Model 1		Model 2	
z-standardized		OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
CAR (nmol/L)	N=269	0.59 (0.38-0.90)	0.01	0.58 (0.38-0.90)	0.02
Slope (nmol/L/h)	N=257	0.51 (0.35-0.75)	0.001	0.51 (0.34-0.76)	0.001
AUC (nmol/L)	N=242	1.29 (0.92-1.82)	0.14	1.41 (0.98-2.02)	0.06

Notes. OR = odds ratio, CI = confidence interval; AUC: area under the curve; Slope: diurnal cortisol decline during the day (a more negative value indicates a steeper slope); CAR: cortisol awakening response (a positive value indicates a rise of cortisol after awakening and a negative value indicates no rise of cortisol); nmol/L: nanomoles per liter. Model 1: adjusted for child's age at cortisol sampling; Model 2: additionally adjusted for maternal educational level, family income and maternal smoking during pregnancy.

Table 6. Infant HPA axis activity and fine motor development at 18 months.

		Risk of non-optimal fine motor development			
		Model 1		Model 2	
z-standardized		OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
CAR (nmol/L)	N=289	1.04 (0.83-1.32)	0.73	1.05 (0.83-1.34)	0.67
Slope (nmol/L/h)	N=274	0.75 (0.58-0.97)	0.03	0.74 (0.57-0.96)	0.03
AUC (nmol/L)	N=254	1.19 (0.93-1.53)	0.17	1.21 (0.94-1.56)	0.15

Notes. OR = odds ratio, CI = confidence interval; AUC: area under the curve; Slope: diurnal cortisol decline during the day (a more negative value indicates a steeper slope); CAR: cortisol awakening response (a positive value indicates a rise of cortisol after awakening and a negative value indicates no rise of cortisol); nmol/L: nanomoles per liter. Model 1: adjusted for child's age at cortisol sampling; Model 2: additionally adjusted for maternal educational level, family income and maternal smoking during pregnancy.

DISCUSSION

In this population-based study, we found that variations in diurnal cortisol patterns are associated with cognitive function early in life though not consistently across the different measures and ages. In line with our hypothesis, we found that children with a more positive cortisol awakening response showed a lower risk of delay in language comprehension as well as a lower risk of delay in nonverbal cognitive functioning. Higher levels of total cortisol secretion during the day were associated with a higher risk of delay in language production but not language comprehension. We also found that infants with flatter diurnal slopes showed a lower risk of delay in nonverbal cognitive functioning and fine motor development.

Thus, diurnal cortisol patterns characterized by a more positive awakening response and lower cortisol levels during the day, indicating higher cortisol reactivity and lower stress levels, were associated with better cognitive scores early in life.

Our results extend prior research focusing on the relationship between cortisol and cognition. Recently, Power, Li, and Hertzman (2008) addressed the associations among cortisol, cognitive development, and educational attainment in the general population over the life course. They found that higher cognitive test scores in childhood decreased the odds of flatter diurnal slopes and low morning cortisol levels in midadult life. However, their study lacked an early childhood measure of cortisol for a truly prospective analysis of cortisol effects on cognition. In contrast, in the current study, infant's diurnal cortisol patterns were assessed prior to the subsequent cognitive outcomes measured in toddlerhood. Nevertheless, our study essentially had a cross-sectional design.

Only a few studies have linked endogenous cortisol production early in life to cognitive development. Haley, Weinberg, and Grunau (2006), for example, found that 3-month old infants with higher cortisol responses had significantly better memory, regardless of prematurity. This study focused on cortisol responses, rather than on diurnal cortisol secretion patterns, as 3-month-old infants are not likely to have an established diurnal cortisol rhythm yet. Likewise, other studies measured cortisol reactivity in relation to cognitive development (Annett, Stansbury, Kelly, & Strunk, 2005; Blair, Granger, & Peters Razza, 2005; van Bakel & Riksen-Walraven, 2004). These studies mostly reported a positive relation between cortisol reactivity, that is, the response of cortisol after a stressor, and various assessments of cognitive development. However, so far, it has not been shown conclusively that a more reactive cortisol response parallels a more positive cortisol awakening response.

The results of our study suggest that a diurnal cortisol pattern characterized by a positive cortisol awakening response is associated with better cognitive development in toddlerhood. Since infants are born without a diurnal cortisol rhythm and this rhythm is still developing at 14 months, it is not surprising that most children in our study did not show a positive response of cortisol after awakening. The children with a more positive response of cortisol after awakening, resembling a pattern of older children and adults (Rosmalen et al., 2005; Wust et al., 2000), were at lower risk of delay in language comprehension and at lower risk of delay in nonverbal cognitive functioning.

Several possible explanations for the relationship between the cortisol awakening response and cognition must be discussed. A major concern when conducting cortisol research in a large community cohort of young children pertains to the difficulties in sampling. In infants

sampling depends both on cooperation of the child and the cooperation of the parent (Egliston, McMahon, & Austin, 2007). It is not unthinkable that more organized parents were more likely to correctly adhere to the sampling protocol and to return the saliva samples, thereby introducing selection bias. However, low family income correlated with a more positive cortisol awakening response (see detailed discussion in Saridjan et al., 2010). Furthermore, we found that a more positive cortisol awakening response is related to better cognitive scores early in life even if SES is taken into account.

Another explanation of our results regarding the relationship between the cortisol awakening response and cognitive functioning refers to maturation of the brain. Previous research has shown that the cortisol awakening response is regulated by the prefrontal cortex and structures of the limbic system; especially the hippocampus seems to play a central role in the regulation of the response of cortisol after awakening (Fries et al., 2009). It is well known that the hippocampus plays a central role in cognitive processing (Sweatt, 2004). Thus, our results might indicate that better cerebral functioning, in particular of the hippocampus, is reflected by a positive cortisol awakening response early in life. This, in turn, is related to better cognitive functioning. A plausible explanation could be that brain maturation underlies both the developmental pattern of effects on the diurnal cortisol rhythm and the better cognitive functioning. In our models, we corrected for age at cortisol sampling, thereby eliminating the influence of calendar age on the relationship between cortisol and cognition.

Alternatively, the development of the HPA axis and cognitive development could be independently regulated. Both could change and correlate with age and maturation, but there may not be a specific underlying biological mechanism regulating them in parallel. The individual variation in maturation or biological development might thus explain our findings. This would imply that the association between the diurnal cortisol rhythm and cognitive development may differ per age. Also, this relationship between cortisol and cognitive development could well be less obvious at older ages.

The developing HPA axis in young children makes the results of our study difficult to compare our results to those found in adults and the elderly. Even within adults, different associations between the cortisol awakening response and cognitive impairment have been observed. The results of our study, for example, are in line with the findings of Evans et al. (2011). Interestingly, they showed that poorer cognitive performance was associated with an attenuated CAR and a less steep cortisol fall across the day in healthy older people. Other studies with less comparable results used different methods of HPA axis assessment, such as the study of Lind, Edman, Nordlund, Olsson, and Wallin (2007), who investigated the cortisol awakening response after dexamethason administration. The study of Power et al. (2008)

assessed cognition in childhood in relation to cortisol levels in adult life, making these results hard to compare to ours.

We also found that flatter diurnal slopes in infants showed higher scores on nonverbal but not verbal cognitive development. The association found is in contrast to what has been found in adults, where more flattened diurnal slopes are associated with adverse mental health outcomes such as depressive and anxious symptoms (Bhattacharyya, Molloy, & Steptoe, 2008; Giese-Davis, Sephton, Abercrombie, Duran, & Spiegel, 2004). However, Kertes, Gunnar, Madsen, and Long (2008) showed that deprived care in children predicted growth delay in adopted children and that growth delay predicted steeper diurnal slopes, indicating that diurnal cortisol slopes in children may have to be interpreted differently in children than in adults. Flatter diurnal slopes in our study were mainly influenced by the levels of cortisol after awakening and not by the cortisol levels in the evening. Although, in the calculation of the diurnal slope, we omitted the second cortisol level to exclude the influence of the CAR on the slope, the slope and the CAR were positively correlated. Lower morning cortisol levels and morning cortisol rise determined both slope and a more positive cortisol awakening response. Thus, in our study, flatter diurnal slopes can be seen as a correlate of a more positive CAR, instead of a lack in diurnal variation or cortisol reactivity.

We found, in line with our hypothesis, that higher levels of AUC predicted a higher risk of delay in language production, even when taking age at cortisol sampling into account. However, we found no association between levels of AUC and language comprehension or the nonverbal measures of cognition. This may suggest a chance finding. Also, our results are in seeming contrast to previous reported findings that, similar to the study of Watamura and colleagues (2004), older infants had lower cortisol levels during the day as measured by the AUC (Saridjan et al., 2010). Although these results are compatible with the abovementioned theory of brain maturation as the underlying mechanism, another explanation must also be considered. Higher cortisol levels in children could be due to the experience of more stressful events, even if minor, that, if prolonged, result in lower cognitive scores. Although we tried to account for this by adjusting for maternal educational level and maternal smoking during pregnancy, we cannot exclude that other stressors underlie this association. These stressful events could explain the relationship between cortisol and cognition by influencing both HPA axis activity and cognition.

The structure of cognitive abilities in toddlerhood is far from clear, but the distinction between language and nonlanguage ("performance" in the Wechsler sense) emerges early (Lewis, 1983; Sattler, 1988). The use of both verbal and nonverbal cognitive measures enabled us to address distinctive features of cognitive ability in toddlerhood. Research showed that the

MCDI and PARCA not only made unique contributions to the prediction of general cognitive development at age 2 years but also overlapped in predicting general cognitive development (Saudino et al., 1998). Our results demonstrated that infants with cortisol secretion patterns indicative of an HPA axis resembling less stress and more reactivity have an advanced verbal as well as advanced nonverbal cognitive development. These findings suggest that the development of the diurnal cortisol rhythm parallels general cognitive development early in life possibly due to the underlying brain maturation. Another explanation not addressed in our study is the influence of genetics on brain maturation and therefore on the developing HPA axis and cognitive development.

One of the strengths of this study is the large population-based sample. Also, to our knowledge, this is the first study to relate cortisol diurnal patterns early in life to cognitive functioning measured at age 18 and 30 months. Nevertheless, the current study also has some limitations. The first concerns the nature of the study design. Due to the lack of repeated measures and the short interval between assessments, this was considered a cross-sectional study. An advantage is that nonverbal cognitive assessment at 30 months is more informative; a disadvantage is that additional selection bias could occur during assessments. Further, all temporal, that is, causal, inferences must be made very carefully as in any other cross-sectional study.

To enhance cooperation of the parents, in our study, we asked parents to sample saliva on just one single day and not on more (consecutive) days. This prevents taking day-to-day variability into account. However, asking more sampling from parents in a large multimeasure cohort increases the risk of dropout. Another limitation of our study was that compliance of the saliva sampling was not assessed by an objective measurement such as a timing device, for this we solely relied on parental report. Furthermore, we relied on parental report for the cognitive measures. We tried to correct for this by including educational level of the mother to our models. Even though parental report may not be an objective measure of a child's cognitive abilities, at a young age, parents spend a lot of time with their child and know their child best. Moreover, both parent-report measures of cognitive development, that is, MCDI and PARCA, have been shown to be reliable and valid measures of cognitive functioning in early childhood (Fenson et al., 1994; Saudino et al., 1998). These instruments have also been shown to predict tester-administered language problems later in childhood (Oliver, Dale, & Plomin, 2004).

Our analyses of missing data indicate that attrition was not random. There was a selective dropout of children with lower Apgar scores after birth. These children are at increased risk of delay in language development (Casiro et al., 1990). Marschik, Einspieler, Garzarolli, and

Prechtl (2007) found that lower Apgar scores were associated with a delay in word production. Even though this selection could have limited the variations in the cognitive scores, we had enough power to find an effect of cortisol on both verbal and nonverbal cognitive functioning. However, due to selection effects, it is unclear to what extent our results are representative of the general population of Dutch indigenous infants.

In conclusion, we found that variations in diurnal cortisol rhythms are associated with variations in cognitive functioning. Infants with cortisol secretion patterns indicative of an HPA axis resembling less stress and more reactivity have higher cognitive scores —independent of age — as toddlers. The results of this study can be a further step towards understanding the relationship between cortisol and human cognitive development. We tentatively speculate that the development of the diurnal cortisol rhythm parallels cognitive development early in life and that brain maturation might precede these developmental effects. However, more longitudinal investigations into long-term effects on cognitive functioning are needed.

Table S1. Subject characteristics.

	Total *N*=364 #	No delay in language production *N*=285	Delay in language production *N*=69
Maternal characteristics			
Age, years	31.9 (3.7)	31.8 (3.7)	32.4 (3.7)
Educational level			
low (%)	6.9	7.1	5.9
middle (%)	53.0	55.9	44.1
high (%)	38.7	37.0	50
Family income (net per month)			
low, < 2000 euro (%)	10.2	9.9	11.9
Smoking during pregnancy (% yes)	10.7	9.8	14.5
Parity (% nulliparous)	60.7	65.3	46.4 *
Child characteristics			
Gender (% boys)	56.9	56.5	56.5
Gestational age at birth, weeks (range)	40.3 (32.7-42.7)	40.3 (35.6-42.7)	40.1 (32.7-42.7)
Birth weight, grams	3520 (515)	3527 (506)	3479 (567)
Apgar-score 5 minutes after birth (range)	10 (5-10)	10 (5-10)	10 (8-10)
Duration of breast-feeding, months (range)	4 (0-12)	4 (0-12)	4 (0-12)
Age at cortisol sampling, months (range)	14.22 (11.7-19.3)	14.15 (11.7-19.3)	14.35 (12.7-18.1)
AUC, nmol/L (range)	7.92 (0.21; 27.8)	7.73 (0.71; 23.5)	8.69 (0.21; 27.8)
Slope, nmol/L/h (range)	-0.95 (-3.82; 2.91)	-1.00 (-3.82; 2.05)	-0.85 (-3.44; 2.91)
CAR, nmol/L (range)	-2.85 (-22.1; 37.6)	-2.96 (-22.1; 30.8)	-1.63 (-18.9; 37.6)

Notes. Values are means (SD) unless otherwise indicated. Independent t-tests were used for continuous normal distributed variables, Chi-square tests were used for categorical variables and Mann-Whitney-U tests for continuous non-normal distributed variables. SD: standard deviation; AUC: area under the curve; Slope: diurnal cortisol decline during the day (a more negative value indicates a steeper slope); CAR: cortisol awakening response (a positive value indicates a rise of cortisol after awakening and a negative value indicates no rise of cortisol); nmol/L: nanomoles per liter. #N with information on verbal cognitive functioning = 354, N with information on fine motor development = 339, N with information on nonverbal cognitive functioning = 311, N with information on fine motor development, verbal and nonverbal cognitive functioning=287. $*p < .05$.

Table S2. Subject characteristics.

	Total *N*=364 #	Optimal fine motor development *N*=191	Non-optimal fine motor development *N*=148
Maternal characteristics			
Age, years	31.9 (3.7)	32.0 (3.8)	31.8 (3.7)
Educational level			
low (%)	6.9	6.4	7.5
middle (%)	53.0	52.7	54.1
high (%)	38.7	41.0	38.4
Family income (net per month)			
low, < 2000 euro (%)	10.2	11.0	9.5
Smoking during pregnancy (% yes)	10.7	9.9	11.5
Parity (% nulliparous)	60.7	59.2	68.2
Child characteristics			
Gender (% boys)	56.9	56.0	57.4
Gestational age at birth, weeks (range)	40.3 (32.7-42.7)	40.4 (35.6-42.9)	40.1 (32.9-42.6) *
Birth weight, grams	3520 (515)	3572 (504)	3441 (529) *
Apgar-score 5 minutes after birth (range)	10 (5-10)	10 (7-10)	10 (5-10)
Duration of breast-feeding, months (range)	4.0 (0-12)	4.0 (0-12)	4.0 (0-12)
Age at cortisol sampling, months (range)	14.22 (11.7-19.3)	14.15 (12.6-18.9)	14.29 (11.7-19.0)
AUC, nmol/L (range)	7.92 (0.21; 27.8)	7.68 (0.82; 27.8)	8.51 (1.41; 23.2)
Slope, nmol/L/h (range)	-0.95 (-3.82; 2.91)	-0.91 (-3.82; 2.91)	-1.14 (-3.44;-0.05) *
CAR, nmol/L (range)	-2.85 (-22.1; 37.6)	-3.51 (-17.6; 37.6)	-2.63 (-22.1; 30.8)

Notes. Values are means (SD) unless otherwise indicated. Independent t-tests were used for continuous normal distributed variables, Chi-square tests were used for categorical variables, and Mann-Whitney-U tests for continuous non-normal distributed variables. SD: standard deviation; AUC: area under the curve; Slope: diurnal cortisol decline during the day (a more negative value indicates a steeper slope); CAR: cortisol awakening response (a positive value indicates a rise of cortisol after awakening and a negative value indicates no rise of cortisol); nmol/L: nanomoles per liter. #N with information on verbal cognitive functioning = 354, N with information on fine motor development = 339, N with information on nonverbal cognitive functioning = 311, N with information on fine motor development, verbal and nonverbal cognitive functioning=287. ∗p < .05.

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Chapter 3.2

The longitudinal association of the diurnal cortisol rhythm with internalizing and externalizing problems in pre-schoolers

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The longitudinal association of the diurnal cortisol rhythm with internalizing and externalizing problems in pre-schoolers. The Generation R Study.

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SUMMARY

Background

Studies investigating the association between diurnal cortisol rhythm and behavioural problems in young children have yielded inconsistent results. We tested the hypothesis that variations in diurnal cortisol rhythm in pre-schoolers are already related to problem behaviour early in life with a cross-sectional and longitudinal design.

Methods

This study was embedded in Generation R, a population-based cohort from foetal life onwards. Parents collected saliva samples from their infant at 5 moments during 1 day. In 322 infants aged 12–20 months, we determined the diurnal cortisol rhythm by calculating the area under the curve (AUC), the cortisol awakening response (CAR), and the diurnal slope. Problem behaviour was assessed at ages 1.5 and 3 years with the Child Behavior Checklist/1.5–5 years.

Results

No cross-sectional associations between the cortisol composite measures and problem behaviour were found at 1.5 years. However, cortisol predicted change in internalizing problems as assessed from 1.5 to 3 years, but not change in externalizing problems. Children with higher AUC levels, flatter slopes and a more positive CAR at baseline were more likely to score higher on the Internalizing Problems scale (β per nmol/L AUC: 0.08, 95% CI: 0.00; 0.17, p = 0.04; β per nmol/L/h slope: 0.57, 95% CI: 0.17; 0.98, p = 0.006; β per nmol/L CAR: 0.04, 95% CI: 0.01; 0.08, p = 0.02) at follow-up.

Conclusions

Variations in diurnal cortisol rhythm are associated with change in internalizing problems in pre-schoolers. The results suggest that variations in diurnal cortisol patterns early in life precede internalizing problems.

INTRODUCTION

The relationship between regulation of the hypothalamic-pituitary-adrenocortical (HPA) axis and behavioural problems in children has been studied repeatedly, as discussed by Granger and Kivlighan (2003) and Jessop and Turner-Cobb (2008). Cortisol, the hormonal end-product of the HPA-axis, is important for a wide variety of adaptive functions and is released in response to stressors. This hormone is also involved in numerous essential bodily functioning involving energy metabolism and immunology (de Kloet, 2003). Cortisol shows a diurnal pattern characterized by post-waking peak cortisol levels (cortisol awakening response) and subsequent declining cortisol levels throughout the day in healthy adults (Edwards et al., 2001).

It is important to know if the developing HPA axis in young children is already related to internalizing and externalizing problems early in life. In adults and adolescents variations in the HPA axis co-occur with many psychiatric disorders such as major depression, bipolar disorder and PTSD (Yehuda and Seckl, 2011 and Spijker and van Rossum, 2012). Thus studying the relationship between HPA axis and problem behaviour in young children can unravel the corresponding temporal sequence. Moreover, many of these severe psychiatric disorders find their origin in childhood (see for example review by Wilkinson and Goodyer, 2011). Also, the concept of the allostatic load model (McEwen and Stellar, 1993) suggests that individuals raised in a more stressful environment accumulate biological consequences, such as alterations in the HPA axis, resulting in higher risk for maladaptive behaviour.

Internalizing and externalizing problems in young children were found moderately stable (Smith et al., 2004 and Bufferd et al., 2012) and also predictive of later maladaptive behavioural outcomes (Keenan et al., 1998 and Bayer et al., 2010). Early childhood or infancy is a developmental period of rapid growth in neurological, physical, and emotional systems. Further according to the allostatic load model, adverse environmental effects are thought to have a lasting impact exceeding the infancy period. These adverse effects affect the infant's frontal brain activity (Dawson et al., 2003), which is involved in emotion regulation and inhibition of inappropriate responses, and also on HPA axis activity (Francis et al., 1996), which is involved in cortisol regulation.

The diurnal cortisol rhythm is not present at birth, but emerges during the first 18 months of life. Watamura et al. (2004) even posited that there is an on-going maturation of the HPA axis up to the third year of life. Although diurnal cortisol levels have been associated with internalizing and externalizing problems or a higher problem score in pre-schoolers, the results of different studies show some inconsistencies. de Haan et al. (1998) found in 2-year-old children that midmorning cortisol levels were positively correlated with internalizing

problem scores and that changes in these cortisol levels after starting preschool were positively associated with externalizing problem scores. The study of Scher et al. (2010) showed that in 12–36 month old children elevated awakening cortisol levels were positively associated with internalizing problem scores but not with externalizing problem scores. The study of Ouellet-Morin et al. (2010) showed with a cross-sectional and longitudinal design that (after 1 year) there was no association between basal cortisol levels and internalizing and externalizing problem scores in 3-year olds.

There are several explanations for these discrepant results. First, the assessments used to characterize the diurnal cortisol rhythm differed in the studies. For example, the study of de Haan et al. (1998) collected one saliva sample midmorning, whereas Essex et al. (2002) collected one saliva sample between 1500 h and 1900 h on at least two of three consecutive days to characterize the diurnal cortisol rhythm. In the study of Dougherty et al. (2009) parents were instructed to collect their child's saliva 30 min after awakening and 30 min before bedtime. The latter instruction was also used in the studies of Ouellet-Morin et al. (2010) and Scher et al. (2010). As timing of sampling and number of samples during the day differed the results of these and other studies are difficult to compare. To obtain a good representation of the diurnal cortisol rhythm it is important to sample at least on three or four different time points during the day (Adam and Kumari, 2009), as was done in the study by Watamura et al. (2004). But not only differences in timing of sampling can be found, differences in the way summary measures are defined also make results hard to compare. Second, most studies in pre-schoolers relating diurnal cortisol levels to internalizing and externalizing problems are cross-sectional in design (e.g. Watamura et al., 2004 and Scher et al., 2010). To assess the directionality of the relationship between diurnal cortisol levels and problem behaviour it is important for studies to have a longitudinal design. Ideally internalizing and externalizing problems at baseline are accounted for in such longitudinal studies to reduce the possibility of reversed causality. Third, some studies only focused on externalizing problems (see meta-analysis of Alink et al., 2008) and did not assess internalizing problems as well. It is important to study diurnal cortisol levels in relation to internalizing and externalizing problems to understand the specificity of any observed relation.

Although there are methodological problems in research on cortisol levels and child development, there is good evidence linking variations in diurnal patterns to problem behaviour. Both the studies of the de Haan et al. (1998) and Scher et al. (2010) showed that higher cortisol levels were positively correlated to internalizing problems. In school-aged children, Cicchetti et al. (2010) found that children with more internalizing symptoms exhibited an attenuated diurnal decrease in cortisol. In adolescents, Shirtcliff and Essex (2008) showed that high cortisol levels predicted internalizing problems 2 years later. Also, Adam et

al. (2010) demonstrated that in adolescents a higher cortisol awakening response predicted major depressive disorder. Thus, diurnal cortisol patterns indicative of higher HPA axis activity have been prospectively related to internalizing symptoms during different developmental periods. The relationship of diurnal cortisol patterns with externalizing problems seems less clear. HPA axis hypoactivity has been related to higher levels of callous-unemotional traits and externalizing problems as antisocial behaviour; however Hawes et al. (2009) pointed out that this association may be characteristic for a particular severe subgroup only. The meta-analysis of Alink et al. (2008) suggests that the relationship between basal cortisol levels and externalizing problems is moderated by age during development. Lower basal cortisol levels (hyporeactivity) were associated with externalizing problems in elementary school-aged children, while this was the case for higher basal cortisol levels (hyperreactivity) in pre-schoolers.

To study the association between diurnal cortisol patterns and behavioural problems early in life we used a population-based sample. This offers the opportunity to study developmental patterns in normally developing children. We focused on the diurnal rhythm and collected several saliva samples for cortisol assessment throughout the day. Several pre-defined summary measures were used to assess different aspects of the diurnal cortisol rhythm. Measuring diurnal cortisol levels in the home environment allows less control than in the laboratory, but defining composite cortisol measures reduces the situational effects and reflects the functioning of the HPA axis in everyday life better (Saxbe, 2008). Internalizing and externalizing problems were measured repeatedly, that is at baseline and follow-up. Our hypothesis is that variations in diurnal patterns signalling hyperactivity of the HPA axis precede higher scores on problem behaviour. More specifically we postulate that diurnal cortisol patterns indicative of higher HPA axis activity during the day precede more internalizing and externalizing problems.

METHODS

Setting

This study was conducted in a subsample of the Generation R Study, a cohort study investigating growth, development and health from foetal life onwards in Rotterdam, the Netherlands. The cohort has been described in detail elsewhere (Jaddoe et al., 2012 and Tiemeier et al., 2012). The Generation R Focus Study, a subcohort within the Generation R Study, is conducted to obtain detailed measurements of the child's development in an ethnically homogeneous subgroup to exclude confounding or effect modification by ethnicity. Only children of Dutch national origin were included in this group, i.e. the children, their

parents and their grandparents were all born in the Netherlands. The participating children were born between February 2003 and August 2005. The children visited the research centre regularly for various somatic and behavioural assessments. Written informed consent was obtained from all participants. The study has been approved by the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam.

Study population

For the current study, children who visited the research centre for the Focus Study around 14 months were eligible for assessment of the diurnal cortisol profile. Parents of 602 children who attended the Focus Cohort examination returned one or more saliva samples. Of these, 236 children had to be excluded, because in these children less than two morning samples or less than three samples during the day were obtained, which is insufficient to compute a cortisol composite measure. The area under the curve was calculated in 277 children, the diurnal cortisol slope in 297 children and the cortisol awakening response in 314 children. For 366 children at least one of these cortisol composite measures could be computed.

Information on problem behaviour at age 18 months was available in 345 children (94% of 366). At age 36 months, information on problem behaviour was available in 332 children (91% of 366). This resulted in a total of 322 children (88% of 366) that were included in one or more analyses of the relation between cortisol and problem behaviour.

Salivary cortisol measurements

An extensive description of the cortisol measurement and analysis was presented previously (Saridjan et al., 2010). Prior to the Focus Study visit at 14 months, parents were instructed to collect five saliva samples at home using Salivette sampling devices (Sarstedt, Rommelsdorf, Germany). Parents received detailed written instructions with pictures concerning the saliva sampling. These saliva samples were collected during one single weekday: immediately after awakening, 30 min later, around noon, between 1500 h and 1600 h, and at bedtime. Supplement Table 4 gives an overview over this schedule. Parents were asked not to let their infant eat or drink 30 min before saliva sampling to avoid disturbances of the cortisol levels. Besides these restrictions, the infants were free to follow their normal daily routines on the sampling day. Parents were asked to record information about sampling times on the Salivette tubes as well as on an enclosed schematic form (see Supplement Table 4 for mean sampling times). On these forms parents were asked to add information about napping time and food intake. Between 97.9% and 99.3% of the parents reported not feeding their child 30 min before sampling. This high adherence to the protocol makes it unlikely that feeding practice influenced the results. The Salivettes were gathered at the laboratory of the Department of Epidemiology at the Erasmus MC, where the samples were centrifuged and frozen at −80 °C.

After completion of the data collection, all frozen samples were sent on dry ice in one batch by courier to the laboratory of the Department of Biological Psychology laboratory at the Technical University of Dresden for analysis. Salivary cortisol concentrations were measured using a commercial immunoassay with chemiluminescence detection (CLIA; IBL Hamburg, Germany). Intra- and interassay coefficients of variation were below 7% and 9%, respectively.

For each time point, cortisol values that were above the 99th percentile (>200 nmol/L) were excluded (n = 18, outliers from 12 children) from the analysis to reduce impact of outliers. Although values >44 nmol/L or 3–4 SD above the mean at each sampling time have been excluded in previous studies (Gunnar and White, 2001 and Gunnar and Talge, 2007), because of positively skewed distributions we used the 99th percentile, equaling 2.5 SD after Z-standardizing the cortisol values, per time point. Our procedure for exclusion of outliers was also used by Dekker et al. (2008). Excluding values >44 nmol/L (n = 12) did not change our results.

We calculated three composite variables of the separate cortisol measurements within a day: the area under the curve (AUC), the diurnal cortisol slope and the cortisol awakening response (CAR). These independent variables characterize different aspects of the HPA axis activity. The AUC was used as a measure of total cortisol secretion during the day (from awakening in the morning until bedtime in the evening). It was determined by the total area under the curve given by the cortisol measurements in nmol/L on the y-axis and the time between the cortisol measurements on the x-axis, in the same way as previously described by Pruessner et al. (2003) using the formula for calculating the area under the curve with respect to the ground. To correct for differences in length of total sampling interval time, the AUC was divided by number of hours between the first cortisol measurement at awakening and the last cortisol measurement before going to bed. The AUC was computed only for those who collected at least three saliva samples. Sleeping hours during the day were not associated with the AUC.

The diurnal cortisol slope was used as a measure of the diurnal cortisol decline. It was calculated by fitting a linear regression line for each child, which predicted the cortisol values from time since awakening. The slope was computed by using the first saliva sample and at least two other cortisol time point measures. To avoid any effect of the CAR (Adam et al., 2006), the second cortisol sample (30 min after awakening) was not included in this measure of the slope. Flatter slopes, as indexed by less negative betas, imply a slower cortisol decline during the day. This can be due to relatively lower morning cortisol levels or relatively higher levels in the afternoon or evening. To determine the influence of the first and last cortisol levels on the slope, the correlation between these cortisol levels and the slope was analysed.

The CAR was also used as an index of the HPA axis activity. It was calculated as the difference between the cortisol value at awakening and the value 30 min after awakening (Kunz-Ebrecht et al., 2004). The CAR was only calculated if the cortisol value 30 min after awakening was taken between 15 min and 60 min after awakening. 95% of the parents reported to have sampled the first saliva sample immediately or within 15 min after awakening. In a previous study, we also found that on average the CAR of children aged 14 months was negative. As older children in this sample were more likely to have a positive CAR, this probably reflects maturation of the HPA-axis (Saridjan et al., 2010).

Child internalizing and externalizing problems

At age 18 and 30 months parents received postal questionnaires to assess their child's problem behaviour. The Child Behavior Checklist (Achenbach and Rescorla, 2000) for toddlers (ages 18 months to 5 years) was used to obtain standardized parental reports of children's internalizing and externalizing problems. This questionnaire contains 99 problem items, which are scored with regard to 7 empirically based syndromes that were derived by factor analyses: Emotionally Reactive, Anxious/Depressed. Somatic Complaints, Withdrawn, Sleep Problems, Attention Problems, and Aggressive Behavior. The summary Internalizing scale is a summary score for items on the first 4 syndrome scales, and the Externalizing scale is a summary score for Attention Problems and Aggressive Behavior. Each item is scored 0, 1, or 2 (0 = not true, 1 = somewhat or sometimes true, 2 = very true or often true) on the basis of the child's behaviour during the preceding 2 months. Higher scores indicated more problems. Good reliability and validity have been reported for the CBCL (Achenbach and Rescorla, 2000). The internal consistency of the Internalizing scale at 18 months in this sample was $\alpha = 0.72$, and $\alpha = 0.88$ for the Externalizing scale. At 36 months this was $\alpha = 0.75$ and 0.89, respectively. Outliers were defined as scores below the 0.1 or higher than the 99.9 percentile and were excluded (n = 9 children) to meet model assumptions. In our study population 3.3% of the children fell in the borderline range and no child was in the clinical range of internalizing problems at age 18 months. For externalizing problems the percentages were respectively 10.7% and 2.6% at 18 months. At 36 months, 3.7% of the children fell in the borderline range and 0.3% in the clinical range of internalizing problems, while 5.3% fell in the borderline range and 1.3% in the clinical range for externalizing problems. The cut-off scores were derived according to Achenbach's manual and based on Dutch norms according to Tick et al. (2007).

Assessment of covariates

The choice of potential confounders was determined a priori and based on earlier literature. Socio-economic status and lifestyle related variables (maternal educational level, maternal smoking during pregnancy), obstetric and neonatal variables (parity, gestational age at birth,

birth weight, Apgar score 5 min after birth) and other known determinants of (mother' reported) infant behavioural problems (maternal age, maternal distress during pregnancy, infant gender, infant age, maternal parenting stress at 18 months) were considered as possible confounders (Gutteling et al., 2005 and Saridjan et al., 2010).

Maternal age, maternal educational level, and parity were determined at enrolment using self-report. Educational level was categorized in three levels: low (no or primary education, and lower vocational training), middle (intermediate and higher vocational training) and high education (university or higher). Information about maternal smoking was obtained by postal questionnaires during pregnancy. Mothers were classified as smokers or non-smokers during pregnancy.

Date of birth, gestational age at birth, birth weight, Apgar score 5 min after birth, and gender of the infant were obtained from community midwife and hospital registries at birth.

Maternal psychiatric symptoms during pregnancy were assessed using the Brief Symptom Inventory (BSI), a validated 53-item (5-point scale) self-report symptom inventory outlined to ascertain the psychological state of individuals (Derogatis and Melisaratos, 1983). Of the 322 mothers in our study population, 305 completed the BSI. The mean total score of the BSI, the Global Severity Index (GSI) and indicator of current psychological distress levels, was obtained by dividing the sum score by the numbers of completed items. The internal consistency of the GSI in this sample was $\alpha = 0.92$.

Maternal parenting stress was measured by the Nijmeegse Ouderlijke Stress Index-Kort (NOSIK; de Brock et al., 1992), the Dutch version of the Parenting Stress Index-Short Form. The NOSIK comprises 25 questions on two domains: parenting stress due to parental factors and parenting stress due to child factors. Only the 11 items of the parental domain were used in the present analyses. In our sample 312 mothers competed these items from the NOSIK. Items were assessed on a four-point Likert scale. Following the manual (de Brock et al., 1992), scores were summed and divided by the number of completed items. Higher scores indicate greater levels of stress. The NOSIK has good reliability (Cronbach's alpha = .95) and validity (de Brock et al., 1992). Internal reliability for the 11 items in the current study, measured by Cronbach's alpha, was 0.69.

Statistical analyses

In the non-response analysis we compared the maternal and child characteristics of our study population with the characteristics of the mothers and infants with no information on the cortisol composite measures and problem behaviour. The following statistical tests were

used in the non-response analyses to compare the children included in the analyses to the children who were excluded because of missing data: independent t-tests for continuous variables approaching a normal distribution, Mann–Whitney U tests for continuous non-normally distributed variables, and chi-square statistics for categorical variables. Analyses of missing data showed that children without information on the cortisol composite measures and without information on their problem behaviour were more often girls (52.9% vs. 42.5%, chi-square = 6.39, df = 1, p = 0.01) and had lower Apgar scores 5 min after birth (Apgar score below 8: 9.0% vs. 4.8%, chi-square = 4.21, df = 1, p = 0.04). The non-responding children were more likely to have less educated mothers as well (% low educational level: 11.6% vs. 6.3%, chi-square = 5.26, df = 1, p = 0.02). However, these children did not differ in any other characteristics from the children in our study population.

The computed variables AUC, slope and CAR, and the CBCL scores showed a slightly skewed distribution. We did not transform these variables since regression residuals were normally distributed and this makes interpretation of the results more straight-forward. The correlation between CBCL scores at 18 months and 36 months was tested for both the Internalizing and Externalizing scale scores using Spearman's correlation coefficient.

We used linear regression models to test the associations between the composite variables of cortisol and the CBCL Internalizing and Externalizing scale scores. First, we tested the associations adjusting for age at cortisol sampling, gender and for age at CBCL measurement (age and gender adjusted analyses). In the final models we additionally adjusted for maternal age, maternal educational level, maternal psychological problems during pregnancy, maternal smoking during pregnancy, parity, gestational age at birth, and maternal parenting stress at 18 months. We did not include birth weight and Apgar score in our models, since these covariates did not change the effect estimates meaningfully (<5%). Percentages of missing values on covariates ranged from 0% to 5.3%. For missing values on continuous variables the median value was imputed and for missing values on categorical variables the median category was used for imputation. In the longitudinal analyses with the Internalizing and Externalizing Problem scores at 36 months as an outcome, CBCL score at 18 months were also added to the models to correct for initial values at 18 months and thereby testing the associations between the composite variables of cortisol and the change in reported problem behaviour. In an alternative approach to test stability of results, we tested the relation of cortisol measures delta-scores (difference scores between 36 and 18 months) of Internalizing and Externalizing scale scores with linear regression models. Delta-scores, however, do not account for the level of problem scores (the same absolute change occurs at low and high levels of problems).

Additionally adjusting for time of waking did not change results; therefore we did not include time of waking in our final models. Also, we performed a sensitivity analysis by calculating the AUC more conservatively by including only children with at least four saliva samples and again by including only children with all five samples. Next we reran the analyses of the CAR by including only those who provided information that the first sample was taken within 10 min after awakening and the second sample was obtained between 30 and 45 min after the first sample, which led to a reduction of the n to 177 because of non-compliance and missing data. This exclusion reduced the power by more than 20% (data not shown).

Also, the interaction between the cortisol composite measures and gender was tested. In post hoc analyses we tested the associations between the composite cortisol variables and the subscales of the CBCL Internalizing scale scores (emotionally-reactive, anxious-depressed, somatic complaints, withdrawn). All statistical analyses were performed with the Statistical Package for the Social Sciences version 17.0 for Windows (SPSS Inc, Chicago, IL, USA).

RESULTS

Table 1 presents the characteristics of the participating mothers and children. 57.5% of this sample was male. Maternal distress during pregnancy did not differ between girls and boys (Mann–Whitney Z = −1.18, df = 1, p = 0.24), but perceived parenting stress at 18 months was lower in girls than in boys (median = 0.10 in girls vs. median = 0.18 in boys, Mann–Whitney Z = −2.11, df = 1, p = 0.035). The following median cortisol values were observed at the different time points during the day: at awakening 15.21 nmol/L (range: 0.08–51.03), 30 min after awakening 13.05 nmol/L (range: 0.07–55.56), at noon 5.45 nmol/L (range: 0.05–47.30), around 1600 h 4.94 nmol/L (range: 0.21–40.48) and at bedtime 2.05 nmol/L (range: 0.09–58.50). These cortisol values and cortisol composite measures did not differ between girls and boys. On average, the children in our study did not show a rise of cortisol after awakening (mean CAR −2.64 nmol/L, range: −19.7; 37.6). There was a correlation between the Internalizing Problem scale scores at 18 and 36 months (Spearman's rho: 0.48, $p < 0.001$), and between the Externalizing Problem scores at 18 and 36 months (Spearman's rho: 0.56, $p < 0.001$). Also, the Internalizing Problem scale scores at 18 months were correlated to the Externalizing Problem scale scores (Spearman's rho: 0.61, $p < 0.0001$); this was also observed at 36 months (Spearman's rho: 0.64, $p < 0.0001$). The scores on the Externalizing Problems scale at 18 months were significantly different between girls and boys (median = 8.17 in girls vs. median = 10.0 in boys, Mann–Whitney Z = −2.13, df = 1, p = 0.03).

Table 1. Subject characteristics.

	Total *N*=322	Mean ± SD (range) or %
Maternal characteristics		
Age (years)		32.0 ± 3.6 (21.0-43.3)
Educational level		
low	20	6.3
middle	170	53.3
high	129	40.4
Smoking during pregnancy (% yes)	34	10.6
Psychiatric symptoms (GSI-score)		0.16 ± 0.18 (0.00-1.67)
Parity (% nulliparous)	193	59.9
Parenting stress at 18 months (Nosik-score)		0.23 ± 0.26 (0.00-1.82)
Child characteristics		
Gender (% boy)	185	57.5
Gestational age at birth (weeks)		40.1 ± 1.56 (34.4-42.9)
Birth weight (grams)		3530 ± 505 (1960-4795)
Apgar-score 5 minutes after birth		9.6 ± 0.6 (5-10)
Age of cortisol sampling (months)		14.4 ± 1.0 (11.7-19.3)
Cortisol values		
AUC (nmol/L)	247	8.27 ± 4.5 (0.21; 27.83)
Slope (nmol/L/h)	265	-1.03 ± 8.3 (-3.82; 2.91)
CAR (nmol/L)	267	-1.73 ± 9.3 (-19.7; 37.6)
Problem behaviour at 18 months		
Internalizing Problems scale	307	3.86 ± 3.3 (0.00-16.0)
Externalizing Problems scale	309	9.95 ± 6.4 (0.00-31.00)
Problem behaviour at 36 months		
Internalizing Problems scale	321	4.02 ± 3.5 (0.00-17.0)
Externalizing Problems scale	320	8.16 ± 5.9 (0.00-30.0)

Values are means ± standard deviations (range) for continuous variables, and percentages for categorical variables.
GSI = Global Severity Index of the Brief Symptom Inventory, measured during pregnancy
Nosik = Nijmegen Parenting Stress Index
AUC = Area under the curve
CAR = Cortisol awakening response

Table 2 shows the cross-sectional associations between the diurnal cortisol rhythm and problem behaviour reported at 18 months. As none of the interactions between gender and the different cortisol composite measures were significant, the results are shown for girls and boys together. The AUC, the slope and the CAR at 14 months were not associated with Internalizing Problem scores at 18 months (see Table 2 for details). Likewise, there were no associations between the cortisol composite measures and the Externalizing Problem scores (see Table 2 for details).

Table 2. Cross-sectional associations between cortisol composite measures and behavioural problems at 18 months.

Cortisol measures	CBCL broadband scales					
	Internalizing Problems [a]			Externalizing Problems [a]		
	N	Beta (95% CI)	*P*	*N*	Beta (95% CI)	*P*
AUC (nmol/L)	260	-0.01 (-0.10; 0.09)	0.91	261	0.02 (-0.15; 0.19)	0.81
Slope (nmol/L/h)	280	-0.15 (-0.65; 0.34)	0.54	282	-0.03 (-0.92; 0.87)	0.96
CAR (nmol/L)	295	-0.03 (-0.07; 0.01)	0.20	296	-0.01 (-0.08; 0.07)	0.84

[a] Models adjusted for age at cortisol sampling, age of CBCL measurement at 18 months, gender, maternal age, maternal educational level, maternal distress during pregnancy, maternal smoking during pregnancy, parity, gestational age at birth, and maternal parenting stress at 18 months

Table 3 presents the associations between the diurnal cortisol rhythm at baseline and problem behaviour reported at 36 months. In the age and gender adjusted analyses, the associations just fell short of reaching the level of significance (β per nmol/L AUC: 0.09, 95% CI: −0.00; 0.19, p = 0.06; β per nmol/L/h slope: 0.42, 95% CI: −0.07; 0.98, p = 0.09; β per nmol/L CAR: 0.04, 95% CI: −0.00; 0.09, p = 0.07; see also Supplement Table 1). Supplement Table 3 shows the association of several important confounders with internalizing and externalizing problems. After additionally adjusting for CBCL scores at 18 months as well as potential confounding factors, associations between the diurnal cortisol rhythm and the Internalizing Problem scores reported at 36 months were found. Children with higher AUC levels, flatter slopes and a more positive CAR at 14 months were more likely to score higher on the Internalizing Problems scale at 36 months (β per nmol/L AUC: 0.08, 95% CI: 0.00; 0.17, p = 0.04; β per nmol/L/h slope: 0.57, 95% CI: 0.17; 0.98, p = 0.006; β per nmol/L CAR: 0.04, 95% CI: 0.01; 0.08, p = 0.02). The associations between the cortisol composite measures and Internalizing Problem scores adjusted for confounders but not for baseline Internalizing Problem scores at 18 months, however, were very similar (see Supplement Table 1).

The sensitivity analyses testing the associations between the cortisol composite measures and delta-scores of the Internalizing and Externalizing Problem scores, showed very similar results (see Supplement Table 2 and Fig. 1). Both the slope and the CAR predict the difference in Internalizing score from baseline to follow-up, in particular the slope showed a very clear dose-response pattern. In the sensitivity analyses calculating the AUC only in children who provided at least four saliva samples and again in those without any missing samples, the results for Internalizing Problem scores remained essentially unchanged. However, they no longer reached the same level of significance in the latter model because of a smaller sample size (4 samples: β per nmol/L AUC: 0.07, 95% CI: −0.02; 0.15, p = 0.12; 5 samples: β per nmol/L AUC: 0.07, 95% CI: −0.03; 0.16, p = 0.19). The association between AUC and Externalizing Problem scores became significant in those who provided all samples (5 samples: β per nmol/L AUC: 0.15, 95% CI: 0.00; 0.31, p = 0.05; 4 samples: β per nmol/L AUC: 0.08, 95% CI: −0.05; 0.21, p = 0.23).

Table 3. Longitudinal associations between cortisol composite measures and behavioural problems at 36 months.

Cortisol measures	Child problem behaviour									
	Internalizing Problems [a]					Externalizing Problems [a]				
	N	Beta (95% CI)	*P*	R^2	F^2	*N*	Beta (95% CI)	*P*	R^2	F^2
AUC (nmol/L)	246	0.08 (0.00; 0.17)	0.04 *	0.351	0.541	245	0.10 (-0.03; 0.23)	0.14	0.400	0.667
Slope (nmol/L/h)	264	0.57 (0.17; 0.98)	0.006 **	0.372	0.592	263	0.42 (-0.26; 1.10)	0.23	0.416	0.712
CAR (nmol/L)	275	0.04 (0.01; 0.08)	0.02 *	0.307	0.443	274	0.03 (-0.03; 0.09)	0.33	0.366	0.577

[a] Models adjusted for age at cortisol sampling, CBCL score at 18 months (continuously), gender, age of CBCL measurement at 36 months, maternal age, maternal educational level, maternal distress during pregnancy, maternal smoking during pregnancy, parity, gestational age at birth, and maternal parenting stress at 18 months.
R^2= adjusted R Square for fully adjusted models.
F^2= Cohen's F-Square effect size.
* Significant at the 0.05 level.
** Significant at the 0.01 level.

By calculating the CAR only in those with documented compliance, the sample size was reduced to 177 and the association with Internalizing Problem Scores was no longer significant (β per nmol/L CAR: 0.02, 95% CI: −0.02; 0.07, p = 0.31). None of the interactions between gender and the different cortisol measures were significant with one exception. Gender moderated the association between the slope and internalizing problems. After stratifying for gender, the slope was positively associated with the Internalizing Problem scores reported at 36 months in girls (β per nmol/L/h slope: 0.90, 95% CI: 0.32; 1.49, p = 0.003) but not in boys (data not shown).
In contrast, we found no association between the cortisol composite measures, i.e. AUC, slopes and CAR with Externalizing Problem scores (see Table 3 for details).

Post hoc analyses showed that the CAR was positively associated with the anxious-depressed subscale of the Internalizing Problems scale (β per nmol/L CAR: 0.02, 95% CI: 0.01; 0.03, p = 0.005) and that the AUC was positively associated with the somatic complaints subscale of the Internalizing Problems scale (β per nmol/L AUC: 0.06, 95% CI: 0.02; 0.09, p = 0.003). However, when corrected for multiple testing, only the latter association remained significant (p = 0.003 < p = 0.05/12).

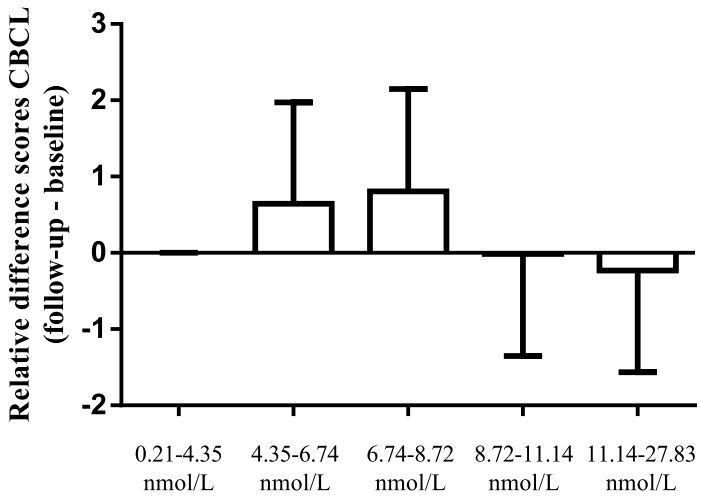
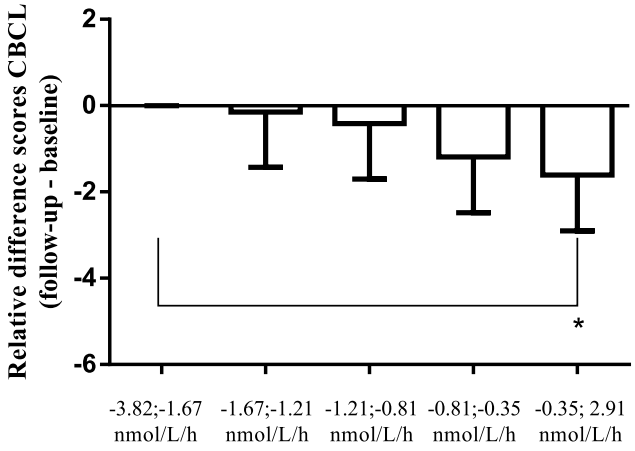


Figure 1. A. Association between AUC at baseline and difference scores in child problem behavior from baseline to follow-up

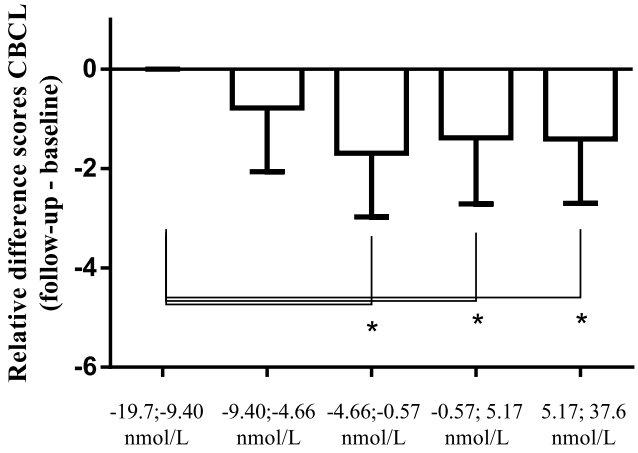
Per quintile of AUC at baseline, the mean change in internalizing problems is calculated and compared with the change of problems in the lowest quintile of AUC (reference) using ANCOVA adjusted for age at cortisol sampling, gender, age of CBCL measurement at 36 months, maternal age, maternal educational level, maternal distress during pregnancy, maternal smoking during pregnancy, parity, gestational age at birth, and maternal parenting stress at 18 months. The mean change in internalizing problems in the reference group over time was 0.25.



B. Association between slope at baseline and difference scores in child problem behavior from baseline to follow-up.

Per quintile of slope at baseline, the mean change in internalizing problems is calculated and compared with the change of problems in the lowest quintile of slope (reference) using ANCOVA adjusted for age at cortisol sampling, gender, age of CBCL measurement at 36 months, maternal age, maternal educational level, maternal distress during pregnancy, maternal smoking during pregnancy, parity, gestational age at birth, and maternal parenting stress at 18 months. The mean change in internalizing problems in the reference group over time was-0.66.

* Relative difference to the reference group was significant ($p < 0.05$).



C. Association between CAR at baseline and difference scores in child problem behavior from baseline to follow-up.

Per quintile of CAR at baseline, the mean change in internalizing problems is calculated and compared with the change of problems in the lowest quintile of CAR (reference) using ANCOVA adjusted for age at cortisol sampling, gender, age of CBCL measurement at 36 months, maternal age, maternal educational level, maternal distress during pregnancy, maternal smoking during pregnancy, parity, gestational age at birth, and maternal parenting stress at 18 months. The mean change in internalizing problems in the reference group over time was-0.88.

* Relative difference to the reference group was significant ($p < 0.05$).

Discussion

This prospective study showed that higher HPA axis activity during the day in infants predicted higher internalizing problem scores in the pre-school age. Children with higher AUC levels, flatter slopes or a more positive CAR were more likely to have higher scores on internalizing problem behaviour at 36 months. Findings were largely similar in boys and girls.

To our knowledge this is one of the few population-based studies to assess diurnal HPA axis activity at an early age in relation to internalizing or externalizing problems. Importantly, this design enabled us to study temporal sequence of the relation between diurnal cortisol patterns and problem behaviour and reduced the possibility of reversed causality. Also, our results contribute to the few studies in young children with a longitudinal design (e.g. Smider et al., 2002), and to those with repeated measurement of internalizing and externalizing problems over a 6-month period (Gunnar et al., 2011).

Although we observed no cross-sectional association between the diurnal cortisol rhythm and internalizing problems at 18 months, we found that higher HPA axis activity during the day at 14 months was related to a significant change of internalizing problems with higher levels at 36 months. Our results are in line with the prospective study of Smider et al. (2002), who found that in boys higher afternoon cortisol levels at age 4.5 years predicted more internalizing symptoms 1.5 years later. Interestingly in 4-year-old children, high basal cortisol levels co-occurring with higher internalizing behaviour scores has been reported in boys also in a cross-sectional study (Pérez-Edgar et al., 2008). First we will discuss possible explanations for the longitudinal associations between infants' diurnal cortisol rhythm and internalizing problems, in the absence of significant cross-sectional associations. At 18 months internalizing problem scores are much less specific and less stable than at 36 months (Rose et al., 1989). Since 18 months is at the very bottom of the standardization range for the CBCL, it is conceivable that persistent behavioural patterns, although present, are not yet clearly recognized by the parent or caregiver. The moderate to high correlation between the internalizing scores at 18 and at 36 months, however, suggests that other factors may account for these results. Alternatively, a bi-directional relation between cortisol and internalizing problems could explain our observations. If the direction of the association between internalizing problems and cortisol levels is in opposite direction (i.e. high internalizing problem scores lead to reduced cortisol levels in some infants) the overall observed association in the cross-sectional models at 18 months would be diluted (but not the baseline adjusted longitudinal association). Given the lack of research in the age group of our participants strong bi-directional effects remain speculative, but this illustrates the importance of adjustment for internalizing problems at baseline. It can never be ruled out that the results of our study are chance findings but

longitudinal analyses with baseline assessment are certainly a powerful design to detect associations because individual variations at baseline, which can also reflect confounding, are adjusted for.

There are also several biological mechanisms, which make our observed longitudinal associations plausible, and constitute possible pathways that may underlie our findings. First, higher cortisol levels have been linked directly to anxiety via activation in the central nucleus of the amygdala (MacMillan et al., 2003), which is involved in both behavioural inhibition and anxiety (Fox et al., 2005). However, higher cortisol levels can also lead to physiological changes in the body, which can be experienced as stress and therefore reinforce anxiety or internalizing problems. Our results showed that the diurnal cortisol slope had a particularly strong relationship with a change in internalizing problem scores. As flatter cortisol slopes have been associated with chronic stress in healthy children (Wolf et al., 2008), this may explain our findings. Second, prenatal and postnatal stress can negatively influence brain development and thereby also HPA activity. Several brain regions, such as the frontal cortex and fusiform gyrus (see review by Nosarti, 2013) have been implicated in internalizing behaviour and neurodevelopmental changes due to stress are a possible explanation for our findings. This concept has been referred to as the allostatic load model (McEwen and Stellar, 1993). Kagan integrated this concept with the well-established psychobiological model of internalizing problems (Kagan et al., 1984) when he demonstrated increased morning cortisol levels in behaviourally inhibited children (Kagan et al., 1987). More recently Buss et al. (2011) showed that increased allostatic load, measured by cumulative indices (in which flatter diurnal cortisol slopes were included), was related to more internalizing problems in young children. Also the study of Ruttle et al. (2011) showed different relationships between morning cortisol levels and internalizing problems analysed concurrently and longitudinally in adolescents. In concurrent analyses internalizing problems were associated with hyperactivity of the HPA axis, whereas in longitudinal analyses internalizing problems predicted hypoactivity. These findings provide a strong case for testing the temporal relation between HPA axis activity and internalizing problems in both directions at different ages. Third, a genetic predisposition could underlie the observed association. It is conceivable that variants of genes involved in brain or endocrine development, such as the serotonin transporter gene with its well documented role in internalizing problems and HPA axis activity in humans (see for example Goodyer et al., 2010), give rise to individual differences in cortisol levels and directly or indirectly also lead to more internalizing problems. This remains speculative, cortisol levels and internalizing problems are moderately heritable but molecular genetic variances influencing cortisol levels or internalizing problems have not been reliably demonstrated. A fourth explanation for our findings is the possible effect of unmeasured confounders in our models. For example socio-economic status or concurrent daily stressors such as family stress can influence internalizing

problems and cortisol levels and affect the relation between HPA activity and internalizing problems. Although we cannot rule out the influence of residual confounding, we tried to minimize the effect of confounding by adjusting our models with several possible indicators of socio-economic background and child development.

In our study we did not find differences between boys and girls in the associations of the cortisol composite measures with internalizing problem scores. There was one exception, girls, but not boys, with a more positive cortisol slope at 14 months (i.e. less decline during the day) had higher internalizing problem scores at 36 months. Our findings are in line with the study of Kryski et al. (2013), who found elevated cortisol reactivity only in girls, and suggested that cortisol reactivity to stress in early childhood has a sex-specific association with girls' internalizing symptoms.

In this study we found no relationship between diurnal cortisol rhythm and externalizing problem behaviour in pre-schoolers. Although the effect of cortisol patterns on externalizing and internalizing problem scores did not differ greatly, our hypothesis that diurnal patterns demonstrating higher HPA axis activity predict more externalizing problems was not supported by our findings. Our results are in line with the findings of the prospective study of Ouellet-Morin et al. (2010), who also found no association between cortisol levels and externalizing problem scores. However, the meta-analysis of Alink et al. (2008) suggests that higher basal cortisol levels are associated with more externalizing problems in pre-schoolers. Possibly our study did not have enough power to find a relationship between diurnal cortisol levels and externalizing problem scores. If not a chance finding, the observed association of HPA axis activity and internalizing problems may reflect that internalizing problems are more reliable at young ages. Internalizing problems in toddlers have been found to be more stable as compared with externalizing problems (Achenbach and Rescorla, 2000). Another explanation could be that externalizing problems are influenced more by environmental risk factors such as low socio-economic status and poverty as compared with internalizing problems in young children (Costello et al., 2003).

The strengths of our study are the large population-based sample and the prospective design. Yet, some limitations of the current study need to be considered. First, the sampling of saliva occurred only on one single day, so day-to-day variability could not be taken into account (Hellhammer et al., 2007). However, to ask parents participating in a large cohort with multiple other assessments to sample on several days increases the risk of drop-out or non-response. Second, the compliance of the saliva sampling was not assessed by an objective measurement such as a timing device, for this we relied on parental report. As can be seen from the sensitivity analyses, our approach to include children from families

who did not adhere well to the protocol increased the power but can also introduce noise to the calculation of the CAR and the AUC. Importantly, the associations largely remained very similar. Furthermore, we relied on parental report for our problem behaviour outcome measures. However, good reliability and validity have been reported for the questionnaire we used to determine problem behaviour (Achenbach and Rescorla, 2000). Another aspect, relevant for clinicians who diagnose child psychiatric disorders, is that we did not use clinical cut-off scores for problem behaviour in our study. This maximizes power and avoids the relying on cut-offs defined arbitrarily or by convention. Our analyses of missing data showed that attrition was not at random. There was a selective dropout of girls, children with lower Apgar scores and children of lower educated mothers. Due to possible selection effects, our results may be less representative of the general population.

In conclusion, our study shows that variations in diurnal cortisol rhythm are longitudinally associated with the change in internalizing problems in pre-schoolers. The study highlights the importance of longitudinal studies that can unravel temporal sequence. Our results suggest that variations in diurnal cortisol patterns are a cause rather than a consequence of internalizing problems in young children.

Supplement table 1. Associations between cortisol composite measures and behavioural problems at 36 months, not adjusted for problem behaviour at 18 months.

A. Basic model, age and gender adjusted without correction for problem behaviour at 18 months

Cortisol measures	Child problem behaviour Internalizing scale scores[a]		Externalizing scale scores[a]	
	Beta (95% CI)	*P*	Beta (95% CI)	*P*
AUC (nmol/L)	0.09 (-0.00; 0.19)	0.06	0.12 (-0.04; 0.28)	0.15
Slope (nmol/L/h)	0.42 (-0.07; 0.92)	0.09	0.28 (-0.59; 1.16)	0.53
CAR (nmol/L)	0.04 (-0.00; 0.09)	0.07	0.04 (-0.03; 0.12)	0.27

[a] Models adjusted for age at cortisol sampling, gender, age of CBCL measurement at 36 months

B. Full models, without correction for problem behaviour at 18 months

Cortisol measures	Child problem behaviour Internalizing scale scores[b]		Externalizing scale scores[b]	
	Beta (95% CI)	*P*	Beta (95% CI)	*P*
AUC (nmol/L)	0.09 (-0.00; 0.18)	0.05	0.12 (-0.04; 0.28)	0.15
Slope (nmol/L/h)	0.49 (0.28; 0.94)	0.04 *	0.33 (-0.52; 1.17)	0.45
CAR (nmol/L)	0.03 (-0.02; 0.07)	0.20	0.02 (-0.05; 0.10)	0.52

[b] Models adjusted for age at cortisol sampling, gender, age of CBCL measurement at 36 months, maternal age, maternal educational level, maternal distress during pregnancy, maternal smoking during pregnancy, parity, gestational age at birth, and maternal parenting stress at 18 months

* Significant at the 0.05 level.

Supplement table 2. Associations between cortisol composite measures and change in behavioural problems between 18 and 36 months.

Cortisol measures	Child problem behaviour Difference scores for Internalizing scale[a]		Difference scores for Externalizing scale[a]	
	Beta (95% CI)	*P*	Beta (95% CI)	*P*
AUC (nmol/L)	0.08 (-0.02; 0.17)	0.11	0.08 (-0.07; 0.23)	0.28
Slope (nmol/L/h)	0.68 (0.19; 1.17)	0.007 **	0.49 (-0.30; 1.29)	0.22
CAR (nmol/L)	0.06 (0.02; 0.11)	0.005 **	0.04 (-0.04; 0.11)	0.32

[a] Models adjusted for age at cortisol sampling, gender, age of CBCL measurement at 36 months, maternal age, maternal educational level, maternal distress during pregnancy, maternal smoking during pregnancy, parity, gestational age at birth, and maternal parenting stress at 18 months. Outcome calculated as CBCL scores at 36 months minus CBCL scores at 18 months.

** Significant at the 0.01 level.

Supplement table 3. Associations between significant covariates in the longitudinal analyses with the slope and behavioural problems at 36 months.

	Child problem behaviour			
Covariates included in	Internalizing scale scores[a]		Externalizing scale scores[a]	
analyses with slope	Beta (95% CI)	*P*	Beta (95% CI)	*P*
Maternal educational level				
low	1.31 (-0.33; 2.95)	0.12	2.83 (0.03; 5.63)	0.0048
middle	0.44 (-0.26; 1.14)	0.43	0.09 (-1.07; 1.23)	0.88
high	ref		ref	
Maternal smoking, yes	-0.17 (-1.37; 1.04)	0.79	0.52 (-1.49; 2.53)	0.61
Maternal psychiatric symptoms	5.24 (3.21; 7.27)	<.001	5.95 (2.22; 9.68)	0.002

[a] Models adjusted for age at cortisol sampling, age of CBCL measurement at 18 months, gender, maternal age, maternal educational level, maternal distress during pregnancy, maternal smoking during pregnancy, parity, gestational age at birth, and maternal parenting stress at 18 months

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Chapter 3.3

Cortisol diurnal rhythm and stress reactivity in constipation and abdominal pain

Kiefte-de Jong JC, Saridjan NS, Escher JC, Jaddoe VW, Hofman A, Tiemeier H, Moll HA.
Cortisol diurnal rhythm and stress reactivity in constipation and abdominal pain:
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ABSTRACT

Objectives

The aim of this study was to assess whether diurnal cortisol rhythm and cortisol stress reactivity were associated with functional constipation and abdominal pain in infancy.

Patients and Methods

This study was embedded in a subset of the Generation R Study, a prospective cohort study from fetal life onward in Rotterdam, the Netherlands. Data of infants between 14 and 24 months of age (N = 483) were used. Salivary cortisol diurnal rhythm and salivary cortisol stress reactivity after a Strange Situation Procedure were assessed at age 14 months. Data on functional constipation were available according to the Rome II criteria, and data on abdominal pain on the basis of the Abdominal Pain Index were available from questionnaire data at 24 months.

Results

In the second year of life, 13% of the infants had functional constipation and 17% had abdominal pain. Only 4% had symptoms of both functional constipation and abdominal pain. Diurnal cortisol rhythm did not differ significantly between children with and children without functional constipation and abdominal pain. Cortisol stress reactivity was slightly higher in infants with abdominal pain than in those without it, but this was not statistically significant (odds ratio 1.41; 95% confidence interval 0.46–4.31). No association was found between the cortisol stress reactivity and functional constipation.

Conclusions

Our results suggest that cortisol as a marker for stress does not play a major role in functional constipation or abdominal pain in infancy.

INTRODUCTION

Functional bowel disorders comprise a large range of gastrointestinal symptoms such as irritable bowel syndrome (IBS), functional constipation, and abdominal pain. These symptoms are frequently seen in Western countries (1). The etiology of these disorders is multifactorial (1) and is a challenge for health care professionals.

Various studies have suggested that functional bowel disorders underlie a complex interaction between psychosocial and physiological factors through the hypothalamic–pituitary–adrenal (HPA) axis (1). The HPA axis regulates the synthesis and secretion of glucocorticoids, which helps to control the metabolism of energy substrates (2). The most important glucocorticoid in humans is cortisol, which is secreted by the adrenal cortex in response to adrenocorticotrophic hormone, which is itself released by the hypothalamus as an effect of the corticotrophic-releasing hormone (CRH) (3). Studies show that psychological stressors activate the HPA axis (3). This can have a direct effect on the motor function of the gastrointestinal tract (4,5). Also, chronic gastrointestinal pain can further enhance activation of the HPA axis, leading to a vicious cycle that may explain the persistence of the symptoms (5,6). Although some studies have indeed shown elevated CRH levels and cortisol response in adults with IBS (7–9), others have claimed the opposite or provided evidence that cortisol responses are blunted in adults with IBS (10,11).

Psychological stressors may affect individuals differently, however, and because the HPA axis is still developing during childhood (6), results on IBS and stress in adults cannot be extrapolated to the pediatric population with functional bowel disorders. Because studies with respect to HPA axis activity and functional bowel disorders in children are extremely scarce, we tested whether infants with functional constipation and abdominal pain have an abnormal profile of the HPA axis after awakening, throughout the day, and in response to a mental stressor.

PATIENTS AND METHODS

Participants and Study Design

This study was embedded in the Generation R Study, a prospective cohort study from early fetal life onward that has been described in detail previously (12,13). An ethnically homogeneous subgroup of Dutch infants was randomly selected from the total cohort to prevent possible confounding or effect modification by ethnicity. Infants were born between February 2003 and August 2005 and 1108 parents gave consent for postnatal follow-up of

their child. The study was approved by the medical ethical review committee at Erasmus University Medical Centre, Rotterdam, the Netherlands.

Collection of Salivary Cortisol Samples

At the age of 14 months, parents visited the Generation R Research Centre. Before this visit parents were asked to collect 5 saliva samples (Salivette Sampling Devices, Sarstedt, Rommelsdorf, Germany) from their infant and to note the sampling times during a normal routine weekday at home: immediately after awakening (mean 07:50 AM; standard deviation [SD] 56 minutes), 30 minutes later (mean 08:25 AM; SD 56 minutes), between 11 AM and 12 PM (mean 11:52 AM; SD 32 minutes), between 3 and 4 PM (mean 15:49 PM; SD 39 minutes), and at bedtime (mean 19:33 PM; SD 57 minutes). Parents received detailed written instructions with pictures concerning the saliva sampling and were asked to keep the samples stored in a freezer until they visited the research center.

To assess how the infant copes with stress, the Strange Situation Procedure (SSP) was used during the visit in the Generation R Research Centre. This is a validated procedure described in detail by Ainsworth and Bell (14). Briefly, the procedure consisted of 7 episodes of 3 minutes each and was designed to evoke mild stress in the infant exposed to the unfamiliar laboratory environment, a female stranger entering the room and engaging with the infant, and the parent leaving the room twice (14). The SSP took place for all of the participants between 8:40 AM and 15:41 PM weekdays (mean 11:31 AM; SD 2 hours). The saliva samples were collected by a research assistant at 3 time points: before, directly after the SSP, and 15 minutes later. The infants were not supposed to eat or drink 30 minutes before sampling.

Missing data after the SSP were the result of technical or procedural problems. Reasons of nonresponse were lack of time and failure to obtain saliva samples because the infant was not familiar with pacifiers.

Samples were centrifuged and stored at -80°C and were sent on dry ice in a single delivery to the laboratory of the Department of Biological Psychology at the Technical University of Dresden. Subsequently, the cortisol levels were assessed by using a commercial immunoassay with chemiluminescence detection (CLIA; IBL, Hamburg, Germany). Intra- and interassay coefficients of variation were <7% and 9%.

To assess the cortisol stress reactivity, a delta was calculated between the last sample (15 minutes post-SSP) and the first sample (pre-SSP). The second assessment, just after the SSP, was not used because it was too close to the onset of stress. We adjusted for baseline cortisol values to take into account the law of the initial values, which indicates that the direction of

physiological response depends to a large degree on the initial levels as a result of variance change or regression to the mean (15).

To assess the total cortisol secretion during the day and to take account of the differences between separate cortisol measurements within each child and the time of the measures from baseline, the area under the curve (AUC) was estimated by calculating the curve of the cortisol measurement in nanomoles per liter on the y-axis and the time between the measurements on the x-axis. To adjust for differences in the duration of the day of measurement, the AUC was divided by the number of hours between the first and the final saliva collections. This method has been described in detail by Pruessner et al (16) and Watamura et al (17), and has been used successfully in previous studies (18,19). The cortisol awakening response (CAR) was estimated as the difference between the cortisol concentrations at awakening and 30 minutes thereafter as described by Kunz-Ebrecht et al (20). As a measure of circadian cortisol decline, the slope was calculated by fitting a linear regression line for each child that predicted the cortisol values from time since awakening by using the first and last saliva samples and at least 1 other cortisol sample.

Functional Constipation

In the second year of life, each child's stool pattern was assessed by questionnaire. Functional constipation in the second year of life was defined according to symptoms of the Rome II criteria (21). To avoid the influence of metabolic disorders, infants were excluded in the analyses because of presence of a congenital heart condition, anemia in the past year, or growth retardation defined as height <-2 SD based on the Netherlands growth curves of infants of 12 to 24 months (22).

Abdominal Pain

A binary definition was defined as the presence or absence of any abdominal pain in the previous 3 months in the second year of life. Additionally, the severity of abdominal pain was classified according to an adapted version of the Abdominal Pain Index as described previously by Walker et al (23). Parents were asked about the frequency of the pain episodes that was rated during the previous 3 months on a 5-point scale (ranging from 0 = not at all to 5-every day). The daily frequencies of the pain episodes were assessed on a 4-point scale (none (1), 1–2 times per day, 3–6 times per day, and throughout the day (4)). The duration of the pain episode was rated on a 4-point scale (a few minutes (1), about half an hour, a few hours, all day (4)). Finally, parents indicated the intensity of the abdominal pain on a 10-point scale (1 = no pain and 10 = the most pain possible). The 5 pain ratings were summed and considered as the index of abdominal pain.

Covariates

Prenatal questionnaires completed by the mother included information on mother's educational level, parity, maternal body mass index (BMI), maternal smoking, and maternal alcohol consumption. Data on sex, birth weight, gestational age, and birth outcomes were available from obstetric records assessed in midwife practices and hospital registries (13). Breast-feeding duration was available from questionnaire data filled in when the child was 6 and 12 months old. The level of parental stress in the child's second year of life was assessed using the Nijmeegse Ouderlijke Stress Index–Kort (NOSIK) (24), the Dutch version of the Parenting Stress Index–Short Form, which has been shown to be reliable and valid (25). The NOSIK comprises 2 domains consisting of 25 items: parenting stress caused by parent factors and parenting stress caused by child factors. Only the items on the parent domain were available in this study (n = 15). Items were assessed on a 4-point scale, and the scores were summed and divided by the number of items that has been filled in. Higher scores indicate greater levels of parental stress.

Analysis Population

From the 882 infants who participated in the Generation R Focus Study and visited our research center between June 2004 and November 2006, information on more than 1 home saliva samples was available in 483 infants. During the SSP procedure, 442 infants had more than 1 saliva samples available and were eligible for analysis. Nonresponse analysis showed that the prevalence of functional constipation and abdominal pain was not different between infants with and without cortisol measurement (8.3% vs 8.1% and 7.6% vs 7.6%, respectively). Mothers of infants with no cortisol measurements were slightly more often smokers during pregnancy (29% vs 18%) and slightly more often lower educated (3% vs 1% low education). Infants with no cortisol measurement were slightly more often girls (52% vs 44%) and were slightly more often breast-fed for longer than 6 months (46% vs 36%). No difference between infants with and without cortisol measurement was found on birth weight (3517 vs 3509 g), gestational age (40.1 vs 40.1 weeks), parity (61% vs 68% nulliparous), and parental stress score (0.26 vs 0.25). To prevent bias associated with attrition, missing data of the infants who had at least more than 1 saliva sample available (either from home sampling or during the SSP, n = 483) were multiple imputed (n = 5 imputations) on the basis of the correlation between each variable with missing values and the other patient characteristics as described previously by Sterne et al (26). To obtain the desired effect sizes and standard errors, data were analyzed in each dataset separately. Subsequently, the results of the 5 imputed analyses were pooled and are reported in this article.

Data Analysis

Differences in characteristics between infants with and without functional constipation and abdominal pain were tested with the [chi]2 test for categorical variables and the Mann-Whitney U test for continuous variables.

To assess how diurnal cortisol rhythm and cortisol reactivity were associated with functional constipation and abdominal pain, logistic regression analyses were performed with functional constipation and abdominal pain as dependent variables. Linear regression analyses were performed with the Abdominal Pain Index as a dependent variable (normally distributed). Tests for linear trend were carried out fitting the indicators of cortisol diurnal rhythm and stress response as a continuous variable. To test for nonlinear trends, a quadratic term was added to the model that included the linear term. Because both the linear term and the quadratic term were not statistically significant (see the supplementary table), analyses were performed after stratification of AUC, CAR, cortisol slope, and delta stress into tertiles.

Additional adjustment for potential confounders such as sex, maternal educational background, parity, maternal smoking, maternal alcohol consumption, maternal BMI, birth weight, gestational age, breast-feeding duration, and parental stress were on the basis of literature followed by the change in effect size (ie, >=10% change in regression coefficient). Effect modification by sex was evaluated by adding the product-term of cortisol variables and sex (eg, AUC*sex) as an independent variable to the model.

Results were reported as odds ratios (ORs) and 95% confidence interval (95% CI) for the analyses on abdominal pain and functional constipation and as regression coefficients ([beta]) and 95% CI for the analyses on the Abdominal Pain Index. Complete case analyses and analyses after the multiple imputation procedure were performed. Similar results were found after the complete case analyses, but 95% CIs for the effect estimates were narrower after the multiple imputation procedure. Therefore, only the data after the multiple imputation procedure are presented. $P < 0.05$ was considered as statistically significant. Statistical analyses were carried out by using SPSS 17.0 for Windows (SPSS Inc, Chicago, IL).

RESULTS

Patient Characteristics

Patient characteristics are presented in Table 1. Of 483 infants with cortisol data, 13% and 17% had functional constipation and abdominal pain, respectively. Four percent of the infants had symptoms of both functional constipation and abdominal pain. The mean (SD) index for abdominal pain was 6.61 (1.61). The mean (SD) age of cortisol sampling during the SSP and at home throughout the day was 14.5 (0.87) and 14.4 (1.07) months, respectively.

Table 1. Maternal and child characteristics (*N* = 483).

	Abdominal pain			Functional constipation		
	Yes, *N* = 80	No, *N*= 403	*P*	Yes, *N* = 62	No, *N* = 421	*P*
Mother						
Parity N (% nulliparous)	46 (58)	227 (56)	0.48	31 (50)	242 (57)	0.16
Educational level of mother, N (%)						
Low	7 (9)	31 (8)	0.09	6 (10)	33 (8)	0.41
Mid	65 (81)	274 (68)		44 (71)	295 (70)	
High	7 (9)	97 (24)		13 (21)	93 (22)	
Maternal Smoking, N (%)	21 (26)	105 (26)	0.63	14 (23)	112 (27)	0.36
Maternal alcohol consumption, N (%)	41 (51)	216 (54)	0.40	28 (45)	230 (55)	0.26
NOSIK score median (rage)	0.18 (0.0-1.8)	0.18 (0.0-1.0)	0.46	0.18 (0.0-1.8)	0.18 (0.0-1.0)	0.57
BMI mean (SD)	24 (3.4)	25 (4.3)	0.04	24 (3.4)	24 (4.3)	0.47
Child						
Male, N (%)	44 (55)	200 (50)	0.40	33 (53)	211 (50)	0.58
Birth weight, g, mean (SD)	3400 (580)	3459 (534)	0.28	3456 (488)	3453 (547)	0.61
Gestational age, wk, mean (SD)	40.0 (1.7)	40.0 (1.7)	0.64	39.8 (1.5)	40.0 (1.7)	0.41
BMI mean (SD)	17.3 (1.7)	17.3 (1.4)	0.65	17.3 (1.5)	17.3 (1.4)	0.42

BMI = body mass index; NOSIK = Nijmeegse Ouderlijke Stress Index-Kort; SD = standard deviation.

Diurnal Rhythm

Mean (SD) cortisol levels at time of awakening was 15.80 (9.70) nmol/L in the total study group. Between 11 and 12 PM this decreased to 7.26 (6.45) nmol/L, declining further to 3.42 (5.80) nmol/L at bedtime.

Similar diurnal patterns of cortisol were found in infants with functional constipation and abdominal pain (Fig. 1). Compared to infants with no functional constipation or abdominal pain, no significant difference was found in AUC, CAR, and circadian cortisol decline (Table 2). There was no significant association found between indices of diurnal cortisol rhythm and the abdominal pain index (Table 3). Additional adjustment for age of cortisol sampling, sex, maternal educational background, parity, maternal smoking, maternal alcohol consumption, maternal BMI, birth weight, gestational age, breast-feeding duration, and parental stress did not change these results (data not shown).

Stress Response

Before the SSP, mean (SD) levels in the study group were 6.26 (5.21) nmol/L. Directly after the SSP, mean (SD) cortisol levels remained relatively stable at 6.19 (5.21) nmol/L but increased to 7.18 (6.29) nmol/L 15 minutes after the SSP. The increase in cortisol levels after the SSP was higher in infants with functional constipation and abdominal pain (Fig. 2), but this was not statistically significant after adjustment for baseline cortisol levels (Table 2). No statistically significant association was found between stress reactivity and the abdominal pain index (Table 3). Additional adjustment for age of cortisol sampling, sex, maternal educational background, parity, maternal smoking, maternal alcohol consumption, maternal BMI, birth weight, gestational age, breast-feeding duration, and parental stress did not alter the results (data not shown).

No statistical interaction with sex was found in the analyses between cortisol stress response, diurnal rhythm, and functional constipation and abdominal pain (data not shown).

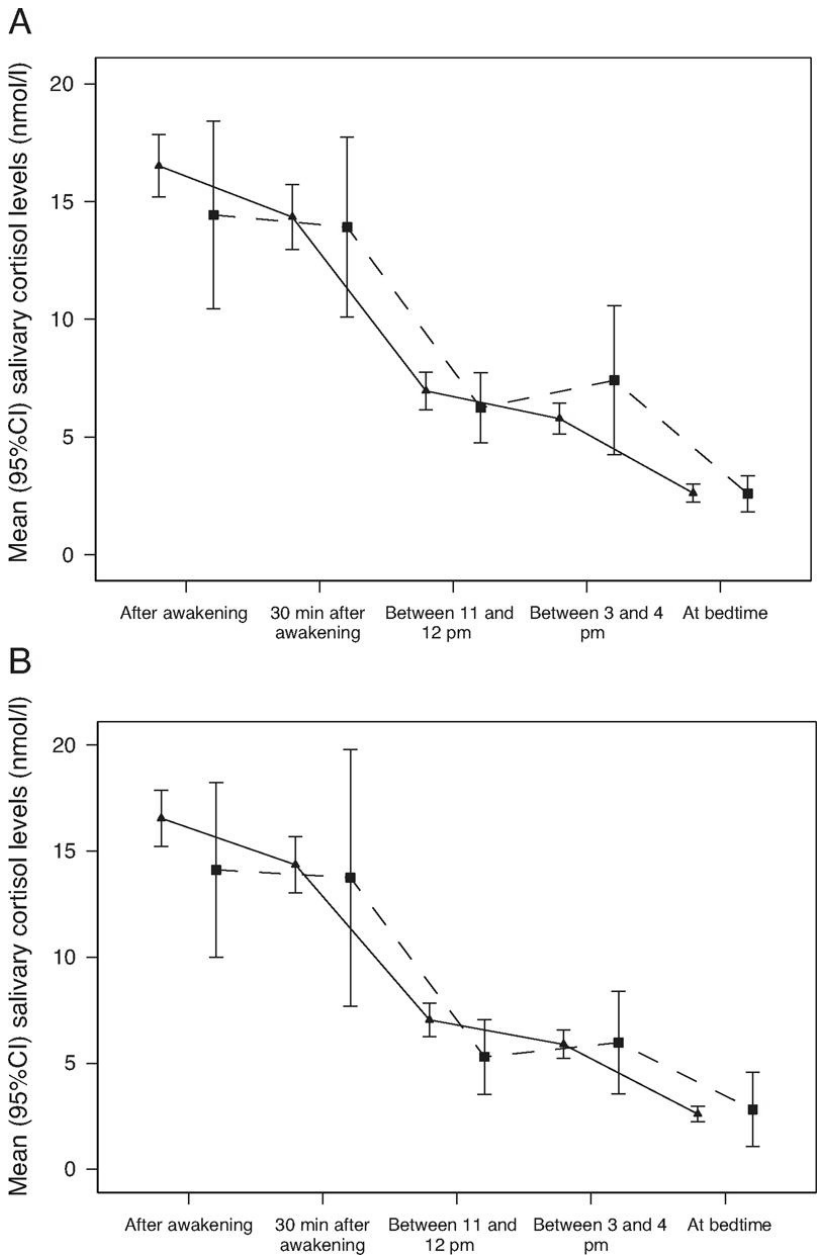

Figure 1. A, Circadian cortisol rhythm according to infants with and without functional constipation (▲,no constipation; ■, functional constipation). B, Circadian cortisol rhythm according to infants with and without abdominal pain (▲, no abdominal pain; ■, abdominal pain).

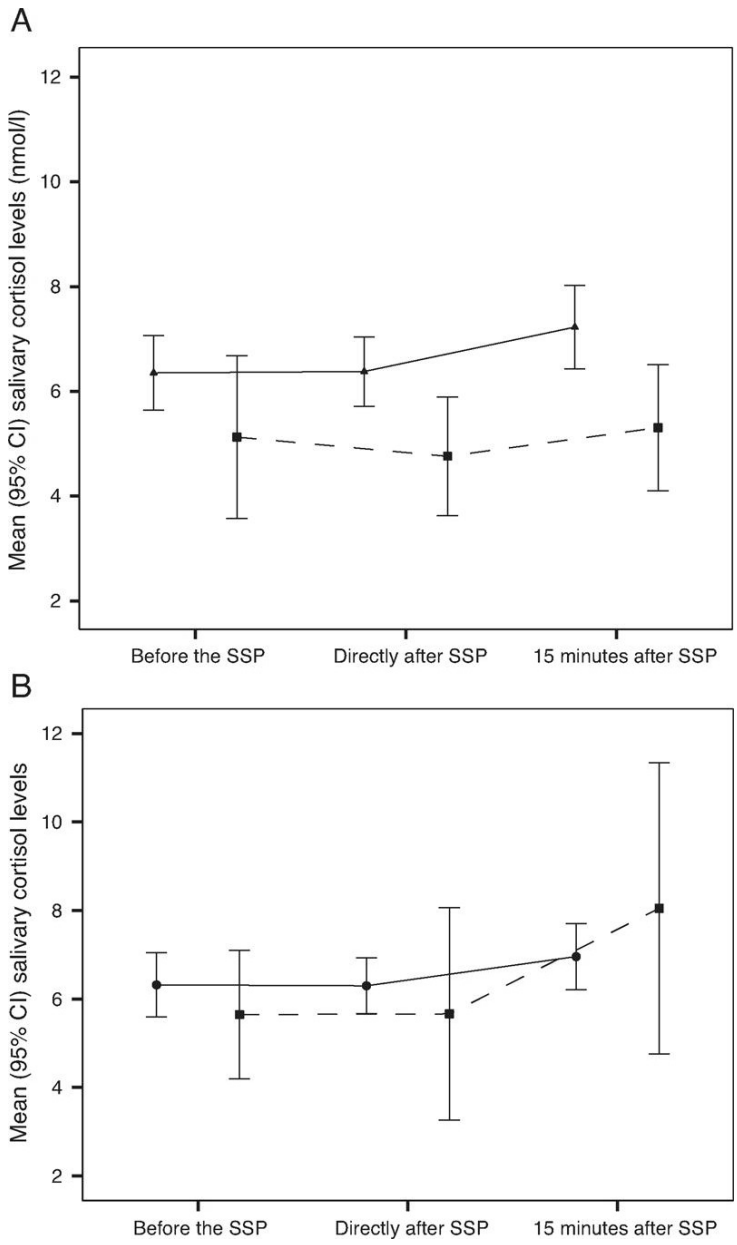

Figure 2. A, Salivary cortisol response after a Strange Situation Procedure (SSP) in infants with and without functional constipation (▲, no constipation; ■, functional constipation). B, Salivary cortisol response after an SSP in infants with and without abdominal pain (▲, no abdominal pain; ■, abdominal pain).

Table 2. Logistic regression analyses on categories of diurnal cortisol rhythm and stress reactivity in children with and without functional constipation and abdominal pain (N=483).

	Abdominal pain OR (95% CI)	Functional constipation OR (95 CI)
Delta stress *		
≤-1.40 nmol/L	Reference	Reference
-1.39 to 2.53 nmol/L	1.08 (0.39-2.99)	0.92 (0.40-2.09)
≥ 2.53 nmol/L	1.41 (0.46-4.31)	0.88 (0.38-2.04)
CAR		
≤-2.94 nmol/L	Reference	Reference
-2.93 to 0.28 nmol/L	1.13 (0.43-2.93)	1.35 (0.55-3.31)
≥ 0.29 nmol/L	0.72 (0.27-1.97)	0.93 (0.40-2.17)
Slope		
≤-1.28 nmol/L	Reference	Reference
-1.27 to-0.68 nmol/L	1.21 (0.28-2.50)	1.39 (0.54-3.62)
≥-0.67 nmol/L	1.66 (0.74-3.72)	1.19 (0.39-3.59)
AUC		
≤ 6.01 nmol/L	Reference	Reference
6.02- 9.45 nmol/L	0.98 (0.40-2.44)	0.97 (0.25-3.70)
≥ 9.46 nmol/L	1.04 (0.40-2.71)	1.37 (0.44-4.23)

AUC=area under the curve; CAR=cortisol awakening response;OR = odds ratio; 95% CI = 95% confidence interval.
* Adjusted for baseline cortisol levels.

Table 3. Linear regression analyses on categories of diurnal cortisol rhythm, stress reactivity, and the abdominal pain index (N=483).

	Abdominal Pain Index β (95% CI)
Delta stress *	
≤-1.40 nmol/L	Reference
-1.39 to 2.53 nmol/L	0.16 (-0.58 to 0.89)
≥ 2.53 nmol/L	0.01-(1.02 to 1.03)
CAR	
≤-2.94 nmol/L	Reference
-2.93 to 0.28 nmol/L	0.12 (-0.58 to 0.82)
≥ 0.29 nmol/L	0.04 (-0.66 to 0.74)
Slope	
≤-1.28 nmol/L	Reference
-1.27 to-0.68 nmol/L	0.29 (0.09-0.48)
≥-0.67 nmol/L	0.10 (-0.44 to 0.63)
AUC	
≤ 6.01 nmol/L	Reference
6.02- 9.45 nmol/L	0.03 (-0.58 to 0.63)
≥ 9.46 nmol/L	0.02 (-0.70 to 0.713)

AUC = area under the curve; CAR = cortisol awakening response; 95% CI = 95% confidence interval. β: Regression coefficient indicating the mean difference in abdominal pain score compared with the reference group.
* Adjusted for baseline cortisol levels.

DISCUSSION

In this study, we demonstrated that the diurnal rhythm of cortisol and stress reactivity is not significantly associated with either functional constipation or abdominal pain. The effect of the HPA axis in young infants with functional bowel disorders has been studied relatively little. Even the results reported in adults are conflicting. For example, although FitzGerald et al (27) recently showed that controls had a higher cortisol response after acute stress than women with IBS did, Chang et al (28) showed that patients with IBS had higher cortisol levels than controls but that there was no association with baseline cortisol levels. Similarly, an earlier study in children with recurrent abdominal pain found that cortisol levels were blunted relative to the levels in controls (29).

A recent study of young children with IBS demonstrated that cortisol stress reactivity is related more to adverse life events than to the presence of IBS in children (30), suggesting that the association between cortisol and functional bowel disorders in previous studies may be confounded by psychological status. Several studies have suggested that the prevalence of depression and anxiety disorders is higher in adults with functional bowel and chronic life stress can contribute to functional bowel disorders (31–34). Although the amount of stress is difficult to quantify in young children, Dorn et al (35) showed that scores on social stress found in children with anxiety scores were similar to those found in children with recurrent abdominal pain.

Because blunted and increased cortisol levels have both been shown in patients with functional bowel disorders, we expected to find differences in cortisol levels in infants with functional constipation and abdominal pain compared to those without these complaints; however, HPA response in people with functional bowel disorders may vary according to their psychological condition. For instance, patients with IBS without psychiatric comorbidity are more sensitive to stress than those with severe depression (36). Similarly, because the HPA axis is still developing early in life and has a high intraindividual instability (37), the influence of the HPA axis in early childhood constipation and abdominal can be difficult to explore. Intervention studies also suggest that colon motility increases, not decreases, after administration of CRH to subjects with IBS (38). If cortisol levels were inversely associated with functional constipation in a subset of our study group but elevated cortisol levels could also occur in infants who had these symptoms, then the association may be canceled out.

Other neurological pathways such as the autonomic nervous system also have been suggested to play a role in functional bowel disorders. Not only has increased activity of the autonomic nervous system been found in adults with IBS (39,40) but also differences in autonomic

activity in response to stress have been found in children with and without chronic abdominal pain (41). Because the autonomic nervous system responds to stress much faster than the HPA axis (42), this may have a more prominent role in functional bowel disorders, but this needs further elucidation.

The strength of this study is that the study population was not selected on the basis of the medical care they had received. As a result of reverse causality, a selected population may increase bias because children with abdominal pain seeking medical care may already have elevated cortisol levels because of the constant pain or symptoms.

Despite this strength of the study, different methodological considerations must be taken into account when interpreting our results. First, we used criteria from Rome II (21) to define functional constipation, and we were not able to specify this outcome according to the most recent evidence-based Rome III criteria (43). As a result, our results preclude conclusions on the role of the HPA axis in more severe functional constipation according to the Rome III criteria.

Second, studies also have shown that cortisol reactivity to stress collapses with increasing age and it has been proposed that the difference in cortisol levels in response to stress becomes smaller as a child ages (42). Because we had only 17% cases with abdominal pain, the small difference in cortisol stress response that we were not able to detect as statistically significant may be influenced by the small sample size. On the contrary, differences in cortisol stress reactivity are in accordance with results from other studies in the same age group, but these studies were much smaller than our study group (44,45). Third, the cortisol stress response and its physical change are thought to be time limited (6). The time lag between cortisol sampling and the assessment of gastrointestinal symptoms in our study may, therefore, explain our results because the association between cortisol secretion and functional bowel disorders may be applicable only when it is measured in short succession. At last, differences between our study and results from other studies may be caused by different types of cortisol measurement (salivary, urinary, or total serum cortisol). Nevertheless, it is thought that only the free (unbound) forms of cortisol are biologically active, and we used salivary cortisol measurements, which correlate with free (unbound) serum cortisol levels in healthy subjects (46).

In conclusion, these data do not support the hypothesis that cortisol plays a significant role in functional constipation and abdominal pain in infants age 24 months. Further studies should clarify whether other branches of the brain–gut axis may be involved in these conditions and whether there is any influence of adverse life events.

Supplementary table. Analyses on diurnal cortisol rhythm and stress reactivity in children with and without functional constipation and abdominal pain by using a linear term and a quadratic term (N=483).

	Abdominal pain OR (95% CI)	Functional constipation OR (95% CI)	Abdominal pain index β (95% CI)
Delta stress *			
Linear term	1.04 (0.98-1.10)	0.99 (0.93-1.05)	-0.005 (0.63-1.56)
Quadratic term	1.00 (0.99-1.00)	0.99 (0.99-1.00)	0.00 (-0.002-0.002)
CAR			
Linear term	0.98 (0.91-1.05)	0.99 (0.93-1.06)	0.001 (0.93-1.08)
Quadratic term	1.00 (0.99-1.01)	1.00 (0.98-1.01)	0.00 (-0.07-0.07)
Slope			
Linear term	1.40 (0.82-2.40)	1.15 (0.60-2.00)	0.077 (0.65-1.79)
Quadratic term	0.98 (0.63-1.52)	0.61 (0.22-1.66)	-0.013 (-0.17-0.14)
AUC			
Linear term	1.02 (0.63-1.65)	1.18 (0.64-2.17)	0.008 (0.71-1.44)
Quadratic term	1.04 (0.54-1.99)	1.22 (0.37-4.05)	-0.19 (0.60-1.63)

OR: odds ratio per unit of the cortisol measure (i.e. deltastress, CAR, slope or AUC); 95% CI: 95% Confidence interval; * Adjusted for baseline cortisol levels. β: Regression coefficient indicating the mean difference in abdominal pain score per unit of the cortisol measure.

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CHAPTER 4

The diurnal cortisol rhythm and sleep

Chapter 4.1

Does disturbed sleeping precede symptoms of anxiety or depression in toddlers?

Jansen PW, Saridjan NS, Hofman A, Jaddoe VW, Verhulst FC, Tiemeier H.
Does disturbed sleeping precede symptoms of anxiety or depression in toddlers?
The Generation R study.

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ABSTRACT

Objective

To examine whether sleep problems in infancy and early toddlerhood precede symptoms of anxiety or depression at 3 years.

Methods

Data on specific sleep problems at 2 months and 24 months were available for 4,782 children participating in a population-based cohort in The Netherlands. The Child Behavior Checklist for toddlers containing the Anxious/Depressed syndrome scale was assessed at 36 months. We adjusted the logistic regression analyses for several confounding factors; the analyses with sleep problems at 24 months were additionally adjusted for preexisting anxiety or depressive symptoms (at 18 months).

Results

Dyssomnia and parental presence during sleep onset at 2 months and 24 months were associated with anxiety or depressive symptoms at 3 years (e.g., parental presence: odds ratio2 months, 1.22; 95% confidence interval, 1.04–1.44; odds ratio24 months, 1.58; 95% confidence interval, 1.30–1.92). Parasomnia, short sleep duration, and absence of set bedtime at 24 months, but not at 2 months, also preceded anxiety or depressive symptoms. These significant associations were not due to children's anxiety or depressive symptoms at 18 months. Rhythmicity and co-sleeping were not associated with later anxiety or depressive symptoms. Additional analyses provided little evidence for a bidirectional association with anxiety or depressive symptoms preceding later sleep problems.

Conclusions

Our findings highlight the importance of sleep problems early in life, because different sleep problems are associated with the frequency of anxiety or depressive symptoms. Therefore, healthcare practitioners must be particularly attentive to these problems in young children. Future research should address possible mechanisms underlying the association between disturbed sleeping and anxiety or depressive symptoms.

INTRODUCTION

Anxiety disorders and depression are among the most common psychiatric diagnoses in children and adolescents with prevalence rates estimated between 2% and 10% (1–3). It is well established that children with these disorders often have comorbid sleep problems. Several studies in clinical samples indicated that anxious and depressed children are troubled sleepers who are characterized by difficulties in falling asleep, reluctance to sleep alone, frequent awakenings, nightmares, and overtiredness (4–7). Alfano and colleagues (5) indicated that 88% of children with an anxiety disorder experienced at least one sleep-related problem. Sleep problems are a common and important symptom of anxiety disorders and depression.

Yet, there is emerging evidence from longitudinal research in the general population that disturbed sleep might also predispose children to anxiety and depression: It was repeatedly indicated that sleep problems during childhood preceded later anxiety disorders and depression with follow-up periods varying from 1 year to 15 years (8–14). Several studies (10,12,13), however, did not account for preexisting anxiety or depressive symptoms in children. Thus, in these studies, it cannot be ruled out that symptoms of anxiety or depression caused sleep problems before the baseline measure was obtained. In addition, previous research was mainly conducted in school-aged children. This further complicates our ability to understand the interrelationship between sleep and anxiety or depression, as their development often begins early in life (2). Another feature of most longitudinal research on sleep problems and anxiety or depressive symptoms is the use of composite scores of disturbed sleeping or insomnia (8–12). These composite scores give a good overall notion of the association, but it is not possible to determine from these composites which specific sleep problem precedes anxiety and depression. This knowledge might facilitate the identification of children at risk for these disorders.

In a population-based prospective study, we examined whether sleep problems in infancy and early toddlerhood precede anxiety or depressive symptoms at the age of 3 years. The specific sleep problems and habits under study were dyssomnia, parasomnia, sleep duration, and rhythmicity. We also examine sleep-related parenting behavior (set bedtime, parental presence during sleep onset, co-sleeping), as these behaviors may interfere with the development of autonomous sleep habits (15–17).

METHODS

Design and Study Population

This study was embedded in Generation R, a population-based cohort from fetal life onward (18). All pregnant women (expected delivery date April 2002–January 2006) living in Rotterdam, The Netherlands, were invited to participate by their midwife or obstetrician during routine visits. The participation rate was estimated at 61%.

We obtained written informed consent from all participants. The Medical Ethical Committee of the Erasmus Medical Center, Rotterdam approved the study.

Data on sleep problems at 2 months or 24 months were available for 6,367 children. Those without information on child behavior at 36 months (n = 1585) were excluded, yielding a sample size of 4,782 children for the present study (response rate 75%; n = 4782 of 6367). In the analyses, the study population varies due to missing data on the individual items.

Child Behavior Checklist for Toddlers

Child behavior was assessed, using the Dutch version of the Child Behavior Checklist for toddlers (CBCL/1½-5) (19) when the children were 1½ years old and again when they were 3 years old. At 1½ years, the CBCL/1½-5 was mostly filled out by the mothers (82.5%). When the children were 3 years, we asked mothers and fathers to each report the behavior of their child by completing separate questionnaires. The CBCL/1½-5 is a 99-item questionnaire in which parents rate the occurrence of their child's behavior within the past 2 months on a 3-point scale. Among others, the CBCL/1½-5 includes the Anxious/Depressed syndrome scale (eight items: too dependent; feels hurt quickly; upset when separated from parents; unhappy; tense; doesn't feel at ease; anxious; sad). The CBCL/1½-5 Anxious/Depressed syndrome scale was highly skewed and could not be normalized. Hence, the sum score of this scale was dichotomized (20% highest scores defined as having anxiety or depressive symptoms).

Good reliability and validity have been reported for the CBCL/1½-5 (19). High construct validity is supported by concurrent and predictive associations with a variety of other measures, e.g., referral to mental health facilities, later behavioral problems, and psychiatric classifications using the Diagnostic and Statistical Manual of Mental Disorders (19,20). In this study, the internal consistency of the Anxious/Depressed syndrome scale at 3 years was [alpha] = 0.69.

Sleep Problems/Habits and Sleep-Related Parenting Behavior

Sleep problems of the children were assessed by parental questionnaire at two different time points, namely, at the ages of 2 months and 24 months. We asked the parents to report about

specific sleep problems or habits at that moment. The assessment of sleep problems was performed with the same questionnaire protocol at the different time points.

The presence of dyssomnia was indicated by the average number of awakenings during the night: Not once; 1 to 2 times; 3 to 4 times; and >=5 times. Due to low occurrence rates, the latter two categories were combined as ">=3 times." Average sleep duration of children per 24 hours was based on the number of sleeping hours during both night and day. For ease of interpretation and comparability across ages, sleep duration was divided into quartiles. Parasomnia was assessed by the question whether a child had nightmares. Again, we combined the categories and scored the presence of nightmares as No (Never) or Yes (Sometimes and Often). Parasomnia was only assessed at 24 months, as the presence of nightmares cannot easily be determined in 2-month-old baby. Rhythmicity was determined by the question whether the child had a stable sleep pattern (Yes; No).

We also assessed parenting behavior related to bedtime rituals and sleep habits of the children. "Set bedtime" was defined as whether or not the child went to bed around the same time every night (Yes; No). The presence of a set bedtime was only assessed at 24 months, as this item was considered not age appropriate for 2-month-old babies. Parents reported whether they stayed present during sleep onset of their child (Yes; No). Finally, "co-sleeping" was defined present (Yes) if a child slept with his/her parents in one bed.

Covariates

The following variables (indicated in *italics*) were considered as possible confounders in the association between sleep habits and child behavior, because these variables are known determinants of anxiety and depression (21) and might also be associated with disturbed sleeping (17,22). Information on *gender* of the children was obtained from the medical records completed by community midwives and obstetricians. We obtained *age of the children* from the date the questionnaires were filled out. In accordance with Statistics Netherlands, child ethnicity was based on country of birth of the children's parents, which was assessed by questionnaire (23).

Information on maternal characteristics was obtained by questionnaire mainly during pregnancy. *Maternal age* was expressed in years. *Maternal education* was defined by the highest attained educational level and classified into four categories in line with the definition of Statistics Netherlands (24). Furthermore, *civil status* was categorized as Married/cohabiting and Single. Finally, *maternal psychopathology*, such as anxiety, depressive symptoms, hostility, and psychoticism, was assessed using the Brief Symptom Inventory at 2 months after birth (25). The 20% highest total sum scores were labeled as high levels of psychopathology, which

corresponds closely with the cutoffs used to describe "above average" psychopathological symptoms in the Dutch norm population (26).

Besides the Anxious/Depressed syndrome scale, the CBCL/1½-5 also included the Sleep Problems syndome scale (details about CBCL/1½-5 described above) (19). We calculated the sum score of this seven-item scale, using the CBCL assessment at 18 months.

Statistical Analyses

The main outcome measure was the Anxious/Depressed syndrome scale CBCL/1½-5) as filled out by mothers when their children were 3. Multivariate logistic regression analyses were performed to calculate odds ratios (ORs) for the association of sleep problems or sleep-related parenting behavior with symptoms of anxiety or depression among 3-year-old toddlers. Analyses adjusted for confounding factors are presented as model 1. Next, we additionally adjusted the analyses for the CBCL/1½-5 Anxious/Depressed syndrome score of children at 18 months (presented as model 2), to demonstrate whether sleep problems precede anxiety or depression independent of symptoms of these disorders that were already present before the baseline assessment of sleep problems. Thus, for each sleep problem, model 2 gives the risk of incident anxiety or depressive symptoms as it emerged between the ages of 18 months and 3 years. The analyses with disturbed sleeping at 2 months could not be controlled for anxiety or depressive symptoms at 2 months, as this behavior cannot be scored with the CBCL at such a young age. Hence, model 2 is not applicable for sleep problems at 2 months. Next, the possibility of a bidirectional association of sleep problems with anxiety or depression was examined: We calculated ORs for the risk of sleep problems at 24 months associated with anxiety or depressive symptoms at 18 months. The analyses were adjusted for confounding factors (model 1) and additionally for sleep problems that were already present (respective sleep problem at 2 months and CBCL/1½-5 Sleep Problems syndrome scale at 18 months, model 2).

Missing values on the covariates (variables adjusted for in models 1 and 2) were estimated, using multiple imputation techniques that were based on linear and logistic regression analyses (27). The reported ORs are the pooled results of five imputed data sets. All statistical analyses were performed, using the Statistical Package of Social Sciences version 17.0 for Windows (SPSS Inc., Chicago, Illinois).

Nonresponse Analyses

Children with missing data on the Anxious/Depressed syndrome scale (CBCL/1½-5) at the age of 3 years (n = 1585) were compared with children for whom this information was available (n = 4782). Data were more often missing in children with a non-Dutch background,

[chi]2(2,6120) = 255, p < .001. Furthermore, mothers of children with missing data were younger, F(1,6367) = 348, p < .001, more often low educated, [chi]2(3,5980) = 369, p < .001, more often single parent, [chi]2(1,5971) = 119, p < .001, and had more psychopathological problems, [chi]2(2,6367) = 49, p < .001. Gender of the children did not differ between those with and without information on the Anxious/Depressed syndrome scale, [chi]2(1,6367) = 0.21, p = .647.

RESULTS

Characteristics of the children and their mothers are presented in Table 1. Children with anxiety or depressive symptoms at 3 years were more often of non-Dutch origin than children without these symptoms, [chi]2(2,4687) = 218, p < .001. Mothers of children with anxiety or depressive symptoms were more often low educated, [chi]2(3,4603) = 115, p < .001, and reported more psychopathological symptoms, [chi]2(2,4782) = 138, p < .001, than mothers of children without these symptoms.

Table 1. Characteristics of Children With and Without Anxiety or Depressive Symptoms at 3 Years.

Child Characteristics	*n*[a]	Anxiety or Depressive Symptoms (Maternal Report, at 3 Years)		*p*[b]
		Absent (*n* = 3622)	Present (*n* = 1160)	
Sex (% boy)	4782	49.6	51.5	.272
Ethnicity: Dutch %	3163	72.8	50.4	<.001
Other Western	439	8.9	10.9	
Non-Western	1085	18.3	38.6	
CBCL/1½-5 scale scores, median [95% range]				
Anxious/depressed (18 months)	4231	1.00 [0-4.0]	2.00 [0-6.0]	<.001
Sleep problems (18 months)	4237	1.00 [0-7.0]	2.00 [0-9.0]	<.001
Father anxious depressed (36 months)	3930	0.00 [0-4.0]	2.00 [0-7.0]	<.001
Maternal Characteristics				
Age (years), mean (SD)	4782	31.9 (4.4)	30.7	<.001
Educational level: High (%)	1538	36.2	24.5	<.001
Mid-high	1162	25.9	23.0	
Mid-low	1244	26.3	29.5	
Low	659	11.6	23.0	
Civil status (% single)	4583	6.8	11.7	<.001
Psychopathology symptoms (% present)	779	16.6	34.7	<.001

CBCL = Child Behavior Checklist for toddlers; SD = standard deviation.

[a] Data were missing on ethnicity (*n* = 95), CBCL Anxious/depressed 18 months (*n* = 551), CBCL sleep problems 18 months (*n* = 545), CBCL father report anxious/depressed (*n* = 852), educational level (*n* = 179), civil status (*n* = 199), and psychopathology (*n* = 1055).

b The *p* value indicates statistically significant differences between children with and without anxiety or depressive symptoms. The *p* values are derived from χ^2 tests (categorical variables), Kruskal-Wallis tests (CBCL scores), or analysis of variance (age).

The prospective relationship between sleep problems and anxiety or depressive symptoms at the age of 3 years is presented in Table 2. We show the analysis of a specific sleep problem at age 2 months, followed by the respective analysis of the same sleep problem at 24 months. Dyssomnia at 2 months preceded later anxiety or depressive symptoms (e.g., OR>=3 awakenings, 1.66; 95% confidence interval [CI], 1.23–2.24). Dyssomnia at 24 months had a very similar effect; this association was independent of symptoms of anxiety or depression that were already present at 18 months. At 2 months of age, sleep duration was not associated with later anxiety or depressive symptoms. At 24 months, however, a relative short sleep duration (lowest quartile) was a risk factor for anxiety or depressive symptoms at the age of 3 years (OR, 1.32; 95% CI, 1.07–1.62). The association between parasomnia and anxiety or depression could not be examined at the age of 2 months, but parasomnia at 24 months preceded later anxiety or depressive symptoms (OR, 1.34; 95% CI, 1.13–1.61). Finally, rhythmicity at 2 months was not related with anxiety or depressive symptoms at 3 years (OR, 1.20; 95% CI, 0.99–1.41). In contrast, lack of rhyhmicity at 24 months increased the risk of later symptoms of anxiety or depression (OR, 1.38; 95% CI, 1.08–1.76), although this association became nonsignificant after adjusting for the Anxious/Depressed syndrome scale at 18 months (OR, Table 3 shows the association between sleep-related parenting behavior and CBCL Anxious/Depressed score at 3 years. The effect of a set bedtime on symptoms of anxiety or depression was not examined at the age of 2 months. The absence of a set bedtime at 24 months was a risk factor for later symptoms of anxiety or depression, which was independent of anxiety or depressive symptoms at 18 months (OR, 1.34; 95% CI, 1.09–1.65). At both 2 months and 24 months, we observed that children whose parents stayed with them during sleep onset had an increased risk of later developing symptoms of anxiety or depression as compared with children who fell asleep without their parents nearby (OR2 months, 1.22; 95% CI, 1.04–1.44; OR24 months, 1.58; 95% CI, 1.30–1.92). This association remained significant after adjusting for anxiety or depressive symptoms at 18 months (OR24 months, 1.36; 95% CI, 1.10–1.69). Co-sleeping at 2 months and 24 months was not related with the Anxious/Depressed score at 3 years (Table 3).1.23; 95% CI, 0.95–1.60).

Table 2. Sleep Problems Early in Life and Anxiety or Depressive Symptoms at 3 Years.

Sleep Problems	*n*	OR for Anxiety or Depressive Symptoms	
		Model 1[a]	Model 2[b]
Dyssomnia			
Average number of awakenings at night			
2 months: Never	1262	Reference	—
1-2 times	2162	**1.21** (1.01-1.45)	
≥3 times	281	**1.66** (1.23-2.24)	
24 months: Never	1855	Reference	Reference
1-2 times	2349	**1.32** (1.14-1.54)	**1.25** (1.06-1.46)
≥3 times	286	**1.90** (1.43-2.51)	**1.61** (1.19-2.17)
Sleep duration			
Average number of sleeping hours per day[c]			
2 months: >16.5 hrs	953	Reference	—
14.5-16.5 hrs	998	1.11 (0.88-1.38)	
12.5-14.5 hrs	912	1.00 (0.79-1.26)	
<12.5 hrs	875	1.09 (0.86-1.37)	
24 months: >13.5 hrs	1040	Reference	Reference
13.0-13.5 hrs	725	1.01 (0.79-1.29)	0.93 (0.72-1.20)
12.5-13.0 hrs	1105	1.17 (0.94-1.45)	1.12 (0.90-1.40)
<12.5 hrs	1424	**1.47** (1.20-1.79)	**1.32** (1.07-1.62)
Parasomnia			
Presence of nightmares			
2 months: —	—	—	—
24 months: No	2621	Reference	Reference
Yes	1063	**1.48** (1.24-1.75)	**1.34** (1.13-1.61)
Don't know	791	**1.44** (1.19-1.74)	**1.40** (1.15-1.70)
Rhythmicity			
Stability of sleep pattern			
2 months: stable	2317	Reference	—
Unstable	1445	1.20 (0.99-1.41)	
24 months: stable	4125	Reference	Reference
Unstable	369	**1.38** (1.08-1.76)	1.23 (0.95-1.60)

Values are odds ratios (ORs) (95% confidence intervals). Significant ORs ($p < .05$) are indicated in bold font.

[a] Model 1: analyses adjusted for child age (36 months and 2 months or 24 months), ethnicity and gender, and maternal age, maternal educational level, civil status, and maternal psychopathological symptoms.

[b] Model 2: model 1 additionally adjusted for Child Behavior Checklist for toddlers (CBCL) Anxious/Depressed syndrome scale at 18 months.

[c] Variables were split into quartiles.

The prospective relation of anxiety or depressive symptoms preceding sleep problems is presented in Table 4. Anxiety or depressive symptoms at 18 months were associated with several sleep problems at 24 months (e.g., lack of rhythmicity: ORmodel 1, 1.54; 95% CI, 1.14–2.09). However, none of these associations remained statistically significant once we adjusted for sleep problems at 2 months and 18 months (e.g., lack of rhythmicity: ORmodel 2, 1.09; 95% CI, 0.79–1.51). The exception to this was parasomnia: Independent of sleep problems at 18 months, parents were more often unable to provide information about parasomnia, if their child had symptoms of anxiety or depressive at 18 months (ORmodel 2, 1.28; 95% CI, 1.00–1.65).

Table 3. Association Between Sleep Related Parenting Behavior Early in Life and Anxiety or Depressive Symptoms at 3 Years.

Sleep-Related Parenting Behavior	*n*	OR for Anxiety or Depressive Symptoms	
		Model 1[a]	Model 2[b]
Does child have set bedtime?			
2 months: —	—	—	—
24 months: Yes	3389	Reference	Reference
No	635	**1.40** (1.15-1.70)	**1.34** (1.09-1.65)
Parents present during child sleep onset			
2 months: No	2424	Reference	—
Yes	1398	**1.22** (1.04-1.44)	
24 months: No	3852	Reference	Reference
Yes	676	**1.58** (1.30-1.92)	**1.36** (1.10-1.69)
Co-sleeping			
2 months: No	3049	Reference	—
Yes	807	1.13 (0.94-1.37)	
24 months: No	4052	Reference	Reference
Yes	475	**1.31** (1.05-1.63)	1.16 (0.92-1.47)

Values are odds ratios (95% confidence intervals). Significant ORs ($p < .05$) are indicated in bold font.
[a] Model 1: analyses adjusted for child age (2 months, 24 months, and 36 months), ethnicity and gender, and maternal age, maternal educational level, civil status, and maternal psychopathological symptoms.
[b] Model 2: model 1 additionally adjusted for Child Behavior Checklist for toddlers (CBCL) Anxious/Depressed syndrome scale at 18 months.

Supplementary analyses confirmed the robustness of the longitudinal association between specific sleep problems and symptoms of anxiety or depression. First, the association between child sleep problems with the Anxious/Depressed score (presented in Table 2) was additionally adjusted for sleep-related parenting behaviors (i.e., set bedtime, parental presence during child sleep onset, and co-sleeping) to ascertain the individual effects of the specific sleep problems. To achieve the same goal, we adjusted the relation between sleep-related parenting behaviors and Anxious/Depressed score (presented in Table 3) for child sleep problems (i.e., dyssomnia, sleep duration, parasomnia and rhythmicity). The results of these additional analyses were very similar to the results presented in Tables 2 and 3, indicating that the specific sleep problems have an effect on anxiety or depressive symptoms independent of parenting behavior. Likewise, sleep-related parenting behavior was associated with symptoms of anxiety or depression independently of child sleep problems. Second, we repeated the analyses presented in Tables 2 and 3 with the Anxious/Depressed score at 3 years dichotomized by the borderline (93rd percentile) and clinical (98th percentile) cutoff points of this scale (maternal report). These analyses showed similar tendencies as the analyses based on the 20% cutoff, although several of the associations did not reach statistical significance due to a smaller number of children being defined as having anxiety

or depressive symptoms. Third, we repeated the logistic regression analyses between sleep problems and anxiety or depressive symptoms, using the paternal report of child behavior at 3 years instead of the maternal report (n = 3930). The results were approximately the same for the paternal and maternal report, with one exception. The absence of a set bedtime at 24 months was significantly associated with mother reported, but not with father reported Anxious/Depressed score at 3 years (ORmother, 1.30; 95% CI, 1.03–1.65; ORfather, 1.13; 95% CI, 0.91–1.41).

Table 4. Anxiety or Depressive Symptoms at 18 Months as Determinant of Sleep Problems at 24 Months.

	n	OR for Specific Sleep Problem	
		Model 1[a]	Model 2[b]
Dyssomnia			
Average number of awakenings at night			
Never	1855	Reference	Reference
1-2 times	2349	**1.29** (1.05-1.58)	0.89 (0.67-1.18)
≥3 times	286	**1.94** (1.36-2.78)	1.05 (0.63-1.74)
Sleep duration			
Average number of sleeping hours per day[c]			
>13.5 hrs	1040	Reference	Reference
13.0-13.5 hrs	725	1.36 (0.98-1.90)	1.25 (0.89-1.76)
12.5-13.0 hrs	1105	1.05 (0.77-1.43)	0.85 (0.62-1.17)
<12.5 hrs	1424	**1.61** (1.22-2.11)	0.98 (0.73-1.32)
Parasomnia			
Presence of nightmares			
No	2621	Reference	Reference[d]
Yes	1063	**1.38** (1.10-1.72)	1.00 (0.79-1.27)
Don't know	791	**1.41** (1.10-1.82)	**1.28** (1.00-1.65)
Rhythmicity			
Unstable sleep pattern (versus stable)	4494	**1.54** (1.14-2.09)	1.09 (0.79-1.51)
Sleep-related parenting behavior			
Presence set bed time (versus absent)	4024	1.22 (0.94-1.57)	1.04 (0.80-1.36)[d]
Parents present at sleep onset (versus absent)	4528	**1.80** (1.41-2.31)	1.13 (0.87-1.48)
Co-sleeping (versus no co-sleeping)	4527	**1.39** (1.05-1.83)	0.87 (0.64-1.18)

Values are odds ratios (ORs) (95% confidence intervals). Significant ORs ($p < .05$) are indicated in bold font.

[a] Model 1: analyses adjusted for child age (18 months and 24 months), ethnicity and gender, and maternal age, maternal educational level, civil status, and maternal psychopathological symptoms.

[b] Model 2: model 1 additionally adjusted for the respective sleep problem at 2 months and for sleep problems in children at 18 months (Child Behavior Checklist for toddlers [CBCL] sleep problems scale). For example, analyses with dyssomnia at 24 months as outcome were adjusted for all variables in model 1, for dyssomnia at 2 months, and for CBCL Sleep problems scale at 18 months.

[c] Variable was split into quartiles.

[d] Analyses were only adjusted for all variables in model 1 and for CBCL sleep problems scale at 18 months, as parasomnia and presence of a set bedtime were not assessed at 2 months.

DISCUSSION

This large population-based study showed that dyssomnia, parasomnia, and short sleep duration in infancy and early toddlerhood are associated with an increased risk of anxiety or depressive symptoms at 3 years. In addition, certain sleep-related parenting behaviors, like absence of a set bedtime and parental presence during sleep onset of the child, precede later symptoms of anxiety or depression. These associations could not be explained by the measured confounding factors nor by children's anxiety or depressive symptoms that were already present at the time the sleep problems were assessed. Furthermore, we found little evidence for a bidirectional association with anxiety or depressive symptoms preceding later sleep problems. Only a single relation was observed: Symptoms of anxiety and depression increased the chance of later lack of parental information on parasomnia. This may reflect parental involvement or could be due to chance.

Strengths and Limitations

Before we discuss these findings, some methodological considerations should be addressed. Strengths of the present study are the large number of participating children, its population-based design, and the use of the age-appropriate and validated CBCL/1½-5 to obtain information on child behavior (19). Other strengths are the adjustment for anxiety or depressive symptoms that were already present when the sleep problems at 24 months were measured and examination of a possible bidirectional relation, which enables us to infer about the direction of the association between sleep problems and anxiety or depression.

Our research also has limitations. First, our nonresponse analyses indicated an under-representation of children in the most disadvantaged groups. Moreover, nonparticipation at the start of the study might also have been selective. This selective inclusion can limit generalizability of our study results. Generation R was, however, not designed to be a nationally representative sample and, with any generalization, the sample characteristics should be considered carefully. A second limitation is that we had to rely on parent reports of sleep problems and symptoms of anxiety or depression, while the objectivity of these reports is discussed (28). Parental perceptions are, however, predictive of poor outcome later in life (29). Moreover, the use of both maternal and paternal reports of child behavior reduces the shared method variance problem (reporter bias). Third, sleep patterns can change rapidly early in life and might also be dependent of temporary circumstances, such as sickness or teething (15,30). Unfortunately, we lacked information on intensity of sleep problems throughout the first 2 years of life and on temporary factors possibly influencing the presence of sleep problems. Finally, we assessed sleep problems by single item measures. The

psychometric properties of these items have not been established. The constructs measured seem, however, fairly unambiguous. In general, sleep research in children is hampered by the lack of criteria for defining "sleep problems." Although we based our assessment of sleep problems on previous studies (5,31–33), distinguishing between "poor" and "normal" sleep remains arbitrary. Although these methodological issues should not affect the nature of the observed associations, care must be taken when the effect sizes are interpreted as they are linked to the definitions of the items used.

Comparison With Previous Studies

Our finding that disturbed sleeping was associated with symptoms of anxiety or depression compares well with previous research in clinical samples and the general population (4–14). However, only few studies (8,9,11,14) showed a longitudinal relationship between sleep problems and anxiety or depressive symptoms at the same time reducing the possibility of anxiety or depression affecting sleep. Recall that these studies mostly focused on a composite score of disturbed sleeping (8–9, 11) and specific sleep problems were examined only once (14). Gregory and colleagues (14) showed that parasomnia (defined by presence of nightmares) and a relatively short sleep duration in school-aged children increase the risk of self-reported symptoms of anxiety or depression 14 years later. The effect of parasomnia was, however, explained by other factors like socioeconomic status and symptoms of anxiety and depression that were already present before the age of 24 months (14). This contrast with our results is possibly due to differences in follow-up period: Parasomnia may precede anxiety or depression at short term, but not in the long run. Research with a longer follow-up period than our study is necessary to confirm this hypothesis. Another possible explanation for the contrast in findings regarding parasomnia is that Gregory et al. assessed anxiety and depressive symptoms by means of a self-report, whereas we used a maternal report. Mothers who notice parasomnia in their offspring are perhaps oversensitive toward their children's symptoms of anxiety and depression. Yet, this potential explanation is hardly supported by our supplementary analyses pointing out that parasomnia was associated with both mothers' and fathers' reported anxiety or depressive symptoms of their children.

Explanation of the Findings

Different mechanisms may underlie our findings of dyssomnia, parasomnia, short sleep duration, and sleep-related parenting behavior preceding toddlers' symptoms of anxiety or depression.

A causal sequence from disturbed sleeping to symptoms of anxiety and depression could be biologically determined. Previous research (34) suggested that short sleep duration and frequent awakenings during the night affect the hypothalamic-pituitary-adrenal (HPA) axis.

Dysregulation of the HPA axis, expressed by distinct stress-related hormonal levels and an unusual pattern of cortisol secretion, has been linked with anxiety and depression in children (35). Alternatively, it is also plausible that short sleep duration and frequent awakenings interfere with the development of regulatory neuronal systems that control daytime vigilance, e.g., regions of the prefrontal cortex in the brain. Decreased vigilance results in agitation and affective disturbances in children (36). Finally, a causal, nonbiological explanation for the association between a short sleep duration and frequent awakenings stems from findings that sleep restrictions of only half an hour already affect performance during daytime (37). This poor functioning might lead to feelings of failure that result eventually in anxiety or depressive symptoms.

On the other hand, the reported prospective association can also be explained by an underlying factor causing both sleep disturbances and symptoms of anxiety or depression, although such an explanation is difficult to match with our results. If a factor caused both disturbed sleeping and anxiety or depressive symptoms, then adjustment for preexisting symptoms of anxiety or depression would have resulted in attenuation of the association between sleep and later anxiety or depressive symptoms. Only if the underlying cause leads to sleep problems at a young age and to anxiety or depression only later in life, this becomes plausible. Genetic variation might be such an underlying cause, as genes associated with anxiety, depression, and general sleep problems overlap partially (38) and because genetic phenotypes emerge at different ages. Alternatively, underlying child characteristics might also be involved. Frequent awakenings and parental presence during sleep onset are known consequences of poor sleep consolidation and little self-soothing abilities in children. These characteristics have all been associated with a difficult temperament (17). At later ages, children with temperamental difficulties often have problems with their emotion regulation, which is an important feature of anxiety and depression (39). In contrast with parental presence during sleep onset, co-sleeping was not associated with anxiety or depressive symptoms. Perhaps, co-sleeping is also an indication of other phenomena, such as the number of rooms or cultural tradition, rather than only parenting behavior associated with children's self-soothing abilities. Finally, the sleep-related parenting behaviors we studied could be indicators of a general parenting style. Presence during sleep onset may reflect parental overprotectiveness, and absence of a set bedtime may be due to family disorganization or parental inability to set rules. Possibly, these parental behaviors cause aspecific symptoms early in life, such as disturbed sleeping (17,38), and lead to more specific psychiatric profiles, like anxiety and depression, only later during childhood.

Implications

Our findings highlight the importance of sleep problems early in life, because dyssomnia, parasomnia, and sleep-related parenting behaviors are associated with the frequency of anxiety and depressive symptoms. Therefore, general practitioners and staff at the well-baby clinics should be particularly attentive for young children's sleep problems and parenting behaviors indicating unhealthy sleep hygiene. An intriguing hypothesis that needs testing, however, is whether intervention aimed at reducing sleep problems in infancy reduces the risk of later anxiety disorders or depression in children with sleep problems. Furthermore, future research should address the possible mechanisms underlying the association between disturbed sleeping and anxiety or depressive symptoms, such as dysregulation of the HPA axis and altered neuronal development.

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Chapter 4.2

The longitudinal association of the diurnal cortisol rhythm with sleep duration and perceived sleeping problems in pre-schoolers

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ABSTRACT

Objective

Cortisol, the end-product of the hypothalamic-pituitary-adrenal (HPA) axis, plays an important role in modulating sleep, which in turn is important for mental health. Yet, studies investigating the association between diurnal cortisol rhythm and sleep patterns in young children are scarce. We tested the hypothesis that the diurnal cortisol rhythm is associated with shorter sleep duration and more sleep problems across early childhood.

Methods

This study was embedded in Generation R, a population-based cohort from fetal life onwards. Parents collected saliva samples from their infant at 5 moments during one day. In 322 infants aged 12-20 months, we determined the diurnal cortisol rhythm by calculating the area under the curve (AUC), the cortisol awakening response (CAR), and the diurnal slope. Sleep duration and sleep behaviour were repeatedly assessed across ages 14 months - 5 years. Generalized estimating equation models were used to assess related cortisol measures to sleep duration and sleep behaviour.

Results

The diurnal cortisol slope and the CAR, but not the AUC, were associated with sleep duration across childhood. Children with flatter slopes and children with a more positive CAR were more likely to have shorter night-time sleep duration (β per nmol/L/h slope: -0.12, 95% CI:-0.19;-0.05, p=0.001; β per nmol/L CAR:-0.01, 95% CI:-0.02;-0.00, p=0.04). Cortisol measures did not predict sleep problems.

Conclusions

The present study suggests that a flatter diurnal cortisol slope and a more marked morning rise, which can indicate stress (or HPA dysregulation), have long-term effects on sleep regulation.

INTRODUCTION

A good night's sleep is considered to be beneficial for the physical and mental health of children. Cross-sectional studies have shown that shorter sleep duration is associated with overweight and obesity in children (1, 2). Also, problems with sleeping are related to both externalizing and internalizing problems in pre-schoolers and older children (e.g. 3, 4). Such an association has also been found in prospective studies of older children and adolescents (see review of 5). Longitudinal studies in young children, however, are scarce. Recently, Sivertsen et al. (6) showed that early sleep problems at 18 months predicted externalizing and internalizing problems at 5 years.

Assessment of determinants of specific sleep patterns in young children to understand mechanisms underlying poor sleep are particularly scarce. Epidemiological research demonstrated that sleep patterns are influenced by environmental factors such as child-rearing situation, socio-economic status and stressful events (7, 8). However, the biological determinants of child sleep, such as cortisol, melatonine and other hormones are mostly inferred from clinical studies (e.g. 9, 10). Also, where cross-sectional studies only demonstrated that disturbed sleep is associated with hormonal variations, less is known whether cortisol predicts changes in sleep duration or sleep problems.

Cortisol is the hormonal end-product of the HPA-axis and is important for a wide variety of adaptive functions and is released in response to stressors. This hormone is also involved in numerous essential bodily functioning involving energy metabolism and immunology which are intrinsically related to sleep (11). Cortisol shows a diurnal pattern characterised by post-waking peak cortisol levels (cortisol awakening response) and subsequent declining cortisol levels throughout the day in healthy adults (12). Cortisol levels reach their lowest point during the first half of the sleep period (13). During sleep, cortisol levels remain low and then rise again until morning awakening.(12, 13).

Several studies show the close association between poor sleep and higher cortisol levels (14), for example in infants (15, 16), preschoolers (17, 18), older children (19), and children suffering from obstructive apnea syndrome (20). Also, less sleep has been associated with a blunted or flatter diurnal cortisol rhythm in infants with colic (21). However, all these studies focus on the effect of sleep patterns on cortisol (changes) but do not address cortisol secretion as a biological risk factor for poor sleep. Only recently, Kiel et al. (22) showed that high morning cortisol levels predict increasing sleep problems from age 2 to age 3, and to our knowledge, no studies explored the association between cortisol levels and child sleep duration. In summary, few studies assessed the association between the developing diurnal cortisol rhythm in infancy and sleeping patterns later in childhood.

In this population-based prospective study, we examined whether the diurnal cortisol rhythm in infancy, i.e. at age 14 months, is associated with night time sleep duration or sleep problems as measured repeatedly between 2 to 5 years. We tested the hypothesis that flatter slopes as part of the diurnal cortisol rhythm are associated with shorter sleep duration and more sleep problems in early childhood.

METHODS

Setting

This study was conducted in the Generation R Focus Cohort, a subcohort of the Generation R Study. This study investigates growth, development and health from fetal life onwards in Rotterdam, the Netherlands. The cohort has been described in detail elsewhere (23, 24). The Generation R Focus Study is conducted to obtain detailed measurements of the child's development in an ethnically homogeneous subgroup to exclude confounding or effect modification by ethnicity. Only children of Dutch national origin were included, i.e. the children, their parents and their grandparents were all born in the Netherlands. The participating children were born between February 2003 and August 2005. The children visited the research center regularly for various somatic and behavioural assessments. Written informed consent was obtained from all participants. The study has been approved by the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam.

Study population

For the current study, children who visited the research center for the Focus Study around 14 months were eligible for assessment of the diurnal cortisol profile. Parents of 602 children who attended the Focus Cohort examination returned one or more saliva samples. Of these, 236 children had to be excluded, because in these children less than two morning samples or less than three samples during the day were obtained, which is insufficient to compute a cortisol composite measure. The area under the curve was calculated in 277 children, the diurnal cortisol slope in 297 children and the cortisol awakening response in 314 children. For 366 children at least one of these cortisol composite measures could be computed.

Information on night time sleep duration at age 14 months was available in 352 children (96% of 366), at age 24 months in 349 children (95%), and at age 36 months in 328 children (90%). At the ages of 1.5, 3 and 5 years, information on sleep behaviour was available in 345 (94%), 325 (89%) and 342 children (91% of 366) respectively. This resulted in a total of 364 children (99% of 366) that were included in one or more analyses of the relation between cortisol and sleep duration or sleep behaviour.

Salivary cortisol measurements

An extensive description of the cortisol measurement and analysis was presented previously (25). Prior to the Focus Study visit at 14 months, parents were instructed to collect five saliva samples at home using Salivette sampling devices (Sarstedt, Rommelsdorf, Germany). Parents received detailed written instructions with pictures concerning the saliva sampling. These saliva samples were collected during one single weekday: immediately after awakening, 30 minutes later, around noon, between 1500h and 1600h, and at bedtime. For the noon saliva sample collection, parents reported a mean deviation time of 0.42h (equalling 26 minutes). Parents were asked not to let their infant eat or drink 30 min before saliva sampling to avoid disturbances of the cortisol levels. Besides these restrictions, the infants were free to follow their normal daily routines on the sampling day. Parents were asked to record information about sampling times on the Salivette tubes as well as on an enclosed schematic form. Questions assessing napping time, food intake and sleep duration were added to this form. The Salivettes were gathered at the laboratory of the Department of Epidemiology at the Erasmus MC, where the samples were centrifuged and frozen at -80^{o}C. After completion of the data collection, all frozen samples were sent on dry ice in one batch by courier to the laboratory of the Department of Biological Psychology laboratory at the Technical University of Dresden for analysis. Salivary cortisol concentrations were measured using a commercial immunoassay with chemiluminescence detection (CLIA; IBL Hamburg, Germany). Intra- and interassay coefficients of variation were below 7% and 9%, respectively.

For each time point, cortisol values that were above the 99th percentile (>200 nmol/L) were excluded (n=18, outliers from 12 children) from the analysis to reduce the impact of outliers.

We calculated three composite variables of the separate cortisol measurements within a day: the area under the curve (AUC), the diurnal cortisol slope and the cortisol awakening response (CAR). These independent variables characterize different aspects of the HPA axis activity. The AUC was used as a measure of total cortisol secretion during the day (from awakening in the morning until bedtime in the evening). It was determined by the total area under the curve given by the cortisol measurements in nmol/L on the y-axis and the time between the cortisol measurements on the x-axis, in the same way as previously described by Pruessner, Kirschbaum, Meinlschmid, & Hellhammer (26) using the formula for calculating the area under the curve with respect to the ground. To correct for differences in length of total sampling interval time, the AUC was divided by number of hours between the first cortisol measurement at awakening and the last cortisol measurement before going to bed. The AUC was computed only for those who collected at least three saliva samples. Sleeping hours during the day were not associated with the AUC.

The diurnal cortisol slope was used as a measure of the diurnal cortisol decline. It was calculated by fitting a linear regression line for each child, which predicted the cortisol values from time since awakening. The slope was computed by using the first saliva sample and at least two other cortisol time point measures. To avoid any effect of the CAR (27, 28), the second cortisol sample (30 minutes after awakening) was not included in this measure of the slope. Flatter slopes, as indexed by less negative betas, imply a slower cortisol decline during the day. This can be due to relatively lower morning cortisol levels or relatively higher levels in the afternoon or evening. To determine the influence of the first and last cortisol levels on the slope, the correlation between these cortisol levels and the slope was analysed.

The CAR was also used as the third summary index of the HPA axis activity. It was calculated as the difference between the cortisol value at awakening and the value 30 minutes after awakening (29). The CAR was only calculated if the cortisol value 30 min after awakening was taken between 15 min and 60 min after awakening. 95% of the parents reported to have sampled the first saliva sample immediately or within 15 minutes after awakening.

Sleep duration and sleep behaviour

At age 14 months information about sleep duration was derived from the schematic form enclosed with the saliva sampling. Parents were asked to report average sleep duration per night during the past week. At age 24 and 36 months parents received postal questionnaires from where information about sleep duration was assessed. At 24 months, the number of hours a child slept during the night was derived from an open question of the average hours of sleep. At 36 months, mothers were asked to report the usual bedtime and wake-up time of their child, on weekdays and on weekends. From these questions weighted average sleep duration at 36 months was calculated.

Information about sleep behaviour was assessed using postal questionnaires at ages 1.5, 3 and 5 years, containing The Child Behavior Checklist (30) for toddlers (ages 18 months to 5 years). This questionnaire contains problem items on problem behaviour rated on a 3-point scale: 0 (not true), 1 (somewhat or sometimes true) or 2 (very true or often true). Sleep behaviour was directly derived from the Sleep Problems scale, which contains 7 items (Doesn't want to sleep alone; Has trouble getting to sleep; Nightmares; Resists going to bed at night; Sleeps less than most kids during day and/or night; Talks or cries out in sleep; Wakes up often at night). These items were summed to weighted scores according to the manual to obtain the Sleep Problems scale (30). Internal reliability of the Sleep Problems scale in the current sample, measured by Cronbach's alpha, was between 0.69 and 0.74.

Assessment of covariates

The choice of potential confounders was determined a priori and based on earlier literature (7, 8, 31). Socio-economic status and lifestyle related variables such as maternal educational level, maternal smoking during pregnancy, and other known determinants of child sleep duration and behaviour such as maternal age, maternal psychiatric symptoms during pregnancy, infant gender, infant BMI, and maternal parenting stress at 18 months were considered as possible confounders (25).

Maternal age and maternal educational level were determined at enrolment using self-report. Educational level was categorized in three levels: low (no or primary education, and lower vocational training), middle (intermediate and higher vocational training) and high education (university or higher). Information about maternal smoking was obtained by postal questionnaires during pregnancy. Mothers were classified as smokers or non-smokers during pregnancy.

Date of birth, birth weight, and gender of the infant were obtained from community midwife and hospital registries at birth. Child BMI around 2 and 3 years were assessed at child health centers and age- and sex-adjusted Z-scores were calculated using national growth curves (32).

Maternal psychiatric symptoms during pregnancy were assessed using the Brief Symptom Inventory (BSI), a validated 53-item (5-point scale) self-report symptom inventory outlined to ascertain the psychological state of individuals (33). The mean total score of the BSI, the Global Severity Index (GSI) and indicator of current psychological distress levels, was obtained by dividing the sum score by the numbers of completed items. The internal reliability of the GSI in this sample was α=0.92.

Maternal parenting stress was measured by the Nijmeegse Ouderlijke Stress Index – Kort (NOSIK; 34), the Dutch version of the Parenting Stress Index – Short Form. The NOSIK comprises 25 questions on two domains: parenting stress due to parental factors and parenting stress due to child factors. Only the 11 items of the parental domain were used in the present analyses. Items were assessed on a four-point Likert scale. Following the manual (34), scores were summed and divided by the number of completed items. Higher scores indicate greater levels of stress. The NOSIK has good reliability (Cronbach's alpha = 0.95) and validity (34). Internal reliability for the 11 items in the current study, measured by Cronbach's alpha, was 0.69.

Statistical Analyses

In the non-response analysis we compared the maternal and child characteristics of our study population with the characteristics of the eligible mothers and children with no information on the cortisol composite measures (366 vs. 236). As there were only 2 children with information on the cortisol composite measures and no information on sleep measurements, it was not possible to statistically compare these children (lost to follow-up) to our study population. For continuous variables approaching a normal distribution we used independent t-tests, for continuous non-normally distributed variables Mann-Whitney U tests and for categorical variables chi-square statistics. Analyses of missing data showed that children without information on the cortisol composite measures and without information on sleep measurements were more often girls (52.9% vs. 42.5%, chi-square=6.39, df=1, p=0.01) and had lower Apgar scores 5 minutes after birth (Apgar score below 8: 9.0% vs. 4.8%, chi-square=4.21, df=1, p=0.04). The non-responding children were more likely to have lower educated mothers as well (% low educational level: 11.6% vs. 6.3%, chi-square=5.26, df=1, p=0.02). However, these children did not differ in any other characteristics from the children in our study population.

The computed variables AUC, slope and CAR, and the CBCL scores showed a slightly skewed distribution. We did not transform these variables since regression residuals were normally distributed and this makes interpretation of the results more straight-forward.

We tested the associations between the composite variables of cortisol with night time sleep duration and sleep behaviour measured at the different ages using linear regression models. First, we tested the associations adjusting for age at cortisol sampling and gender. In the final models we additionally adjusted for waking time at 14 months, maternal age, maternal educational level, maternal psychiatric symptoms during pregnancy, and maternal parenting stress at 18 months. We did not include maternal smoking during pregnancy, child's napping time and BMI in our models, since these covariates did not change the effect estimates meaningfully (<5%). Percentages of missing values on covariates ranged from 0% to 12% (average 7.4%). For missing values on continuous variables the median value was imputed and for missing values on categorical variables the median category was used for imputation. Also, the interaction between the cortisol composite measures and gender was tested in the linear regression models with sleep duration and sleep behaviour as outcome measures.

To analyze the repeated measures of sleep duration and sleep behaviour during the follow-up period, we used generalized estimating equation models (GEEs) which yields an overall estimate of the associations between the diurnal cortisol rhythm and sleep duration in the first 3 years, and with sleep behaviour in the first 5 years. With GEE analyses, repeated

measures over time can be analyzed, taking into account that these repeated measurements within the same subject are correlated. For the analysis examining associations of diurnal cortisol rhythm at 14 months and sleep duration, parent reports of night time sleep duration between the ages of 14 and 36 months were used as continuous outcome measures. For the analysis examining associations of diurnal cortisol rhythm at 14 months and sleep behaviour, CBCL Sleep Problems weighted sum scores between 1,5 and 5 years of age were used as outcomes.

All statistical analyses were performed with the Statistical Package for the Social Sciences version 21.0 for Windows (SPSS Inc, Chicago, IL, USA).

RESULTS

Table 1 presents the characteristics of the participating mothers and children; 56.9% of this sample was male. The following median cortisol values were observed at the different time points during the day: at awakening 15.33 nmol/L (range: 0.08-51.03), 30 minutes after awakening 13.05 nmol/L (range: 0.07-55.56), at noon 5.41 nmol/L (range: 0.05-47.30), around 1600h 4.88 nmol/L (range: 0.21-40.48) and at bedtime 2.03 nmol/L (range: 0.09-58.50). These cortisol values and cortisol composite measures did not differ between girls and boys. On average, the children in our study did not show a rise of cortisol after awakening (mean CAR-1.87 nmol/L, range:-22.1; 37.6).

Sleep duration did not differ between girls and boys at 24 and 36 months, at 14 months sleep duration was longer in girls than in boys (median=12:00h in girls vs. median=11:00h in boys, Mann-Whitney Z=-2.75, df=1, p=0.006). The scores on the Sleep Problem scale did not differ between girls and boys measured at 1.5, 3 and 5 years. As none of the interactions between gender and the different cortisol composite measures were significant in the linear regression models with the sleep measures as outcomes, the results are shown for girls and boys together.

Table 1. Subject characteristics.

	Total N=364	Mean ± SD (range) or %
Maternal characteristics		
Age (years)		31.9 ± 3.7 (16.22-43.3)
Educational level		
low	25	6.9
middle	193	53.0
high	141	38.7
Smoking during pregnancy (% yes)	39	10.7
Psychiatric symptoms (GSI-score)		0.16 ±0.18 (0.00-1.67)
Parenting stress at 18 months (Nosik-score)		0.23± 0.26 (0.00-1.82)
Child characteristics		
Gender (% boy)	207	56.9
Birth weight (grams)		3520 ±515 (1670-4795)
BMI SDS		0.19± 0.96 (-2.1;6.1)
Age of cortisol sampling (months)		14.4± 1.1 (11.7-19.3)
Cortisol values		
AUC (nmol/L)	277	8.30 ± 4.5 (0.21; 27.83)
Slope (nmol/L/h)	297	-1.04± 8.1(-3.82; 2.91)
CAR (nmol/L)	312	-1.87± 9.3 (-22.1; 37.6)
Time at awakening (hh:mm)		7:37 ± 0:45(5:35-10:00)
Napping time during the day (hh:mm)		2:54 ± 0:59(0:50-6:50)
Sleep duration (hh:mm)		
At 14 months	350	11:24 ± 0:50 (9:00-14:00)
At 24 months	349	11:13 ± 0:48 (8:30-13:30)
At 36 months	328	11:30 ± 0:37(9:00-14:36)
Sleep problems		
At 1.5 years	345	1.28± 1.8(0.00-11.0)
At 3 years	325	1.60± 2.1(0.00-12.0)
At 5 years	342	1.10± 1.73(0.00-9.00)

Values are means ± standard deviations(range) for continuous variables, and percentages for categorical variables.
GSI = Global Severity Index of the Brief Symptom Inventory, measured during pregnancy
Nosik = Nijmegen Parenting Stress Index
AUC = Area under the curve
CAR = Cortisol awakening response

Table 2 presents the associations between the diurnal cortisol rhythm and sleep duration reported repeatedly between 14 and 36 months.

Children with flatter slopes and a more positive CAR at 14 months were more likely to have shorter sleep duration (β per nmol/L/h slope: -0.12, 95% CI: -0.19; -0.05, p=0.001; β per nmol/L CAR: -0.01, 95% CI: -0.02; -0.00, p=0.04). The AUC at 14 months was not related to

sleep duration measured between 14 and 36 months (see Table 2). Adjusting for prenatal maternal psychiatric symptoms and maternal parenting stress at child's age 18 months, both potential antecedents of the diurnal cortisol rhythm and also potential confounders, did not change any of the observed associations substantially (fully adjusted data, including maternal psychiatric symptoms and parenting stress, shown only). The appendix table shows the separate associations between the cortisol composite measures and sleep duration at the different measurement time points.

Table 2. Associations between cortisol composite measures and repeatedly measured sleep duration.

Cortisol measures	Sleep duration measured at 14, 24 and 36 months			
	Model 1		Model 2	
	Beta (95% CI)	*p*	Beta (95% CI)	*p*
AUC (nmol/L)	0.005 (-0.011; 0.020)	0.56	0.007 (-0.009; 0.023)	0.397
Slope (nmol/L/h)	-0.120 (-0.189;-0.051)	**0.001**	-0.105 (-0.173;-0.037)	**0.002**
CAR (nmol/L)	-0.008 (-0.015;-0.000)	**0.041**	-0.007 (-0.014;-0.000)	**0.045**

Model 1: adjusted for age at cortisol sampling and gender
Model 2: as model 1, additionally adjusted for waking time at 14 months, maternal age, maternal educational level, maternal psychiatric symptoms during pregnancy, and maternal parenting stress at 18 months
Betas are derived from GEE (generalized estimating equation) linear regression models.

Table 3 shows the associations between the diurnal cortisol rhythm and sleep behaviour reported repeatedly between 1.5 and 5 years. The AUC, the slope and the CAR at 14 months were all not associated with Sleep Problem scores from 1.5 to 5 years (see Table 3 for details). Likewise, there were no associations between the cortisol composite measures and the Sleep Problem scores at the different measurement time points (results not shown).

Table 3. Associations between cortisol composite measures and repeatedly measured sleeping problems.

Cortisol measures	CBCL sleeping problems measured at 18 months, 36 months and 5 years			
	Model 1		Model 2	
	Beta (95% CI)	*p*	Beta (95% CI)	*p*
AUC (nmol/L)	-0.002 (-0.036; 0.031)	0.89	-0.004 (-0.037; 0.029)	0.83
Slope (nmol/L/h)	0.062 (-0.111; 0.236)	0.48	0.093 (-0.066; 0.253)	0.25
CAR (nmol/L)	0.012 (-0.003; 0.028)	0.12	0.013 (-0.001; 0.027)	0.063

Model 1: adjusted for age at cortisol sampling and gender
Model 2: as model 1, additionally adjusted for waking time at 14 months, maternal age, maternal educational level, maternal psychiatric symptoms during pregnancy, and maternal parenting stress at 18 months
Betas are derived from GEE (generalized estimating equation) linear regression models.

DISCUSSION

This prospective population-based study showed that the infants with a flatter cortisol slope and those with a more marked morning cortisol rise have shorter night time sleep duration - as measured repeatedly across early childhood. However, none of these cortisol summary measures predicted pre-school sleep problems.

Although cortisol levels have been cross-sectionally associated with sleep problems, few studies have assessed longitudinally whether cortisol levels in infants or toddlers predict sleep problems at later age. Recently, Kiel et al. (22), however, reported that variation in cortisol secretion patterns at ages 18-20 months predicted an increase of sleep problems from age 2 to 3 years. These authors studied a composite of diurnal, nocturnal and morning levels of cortisol. However, there was no main effect of cortisol, rather the direction of the association between cortisol and sleep depended on the parenting style; i.e. both a positive and a negative association between blunted cortisol secretion and sleep problems in this sample was observed. Thus, our results are in line with those of Kiel et al. (22), who also observed no main effect and only detected an association when stratifying the sample on parental control.

There are different explanations possible for the negative findings on sleep problems in our study and those from Kiel et al. (22). First, it could be that we must reject our hypothesis as there is no association. Second, the association could depend to such an extent on parenting style that not stratifying (or otherwise accounting for interaction) in the present study obscured the association. In the present study, however, we did not observe any confounding or effect modification by parenting stress. Third, our study and that of Kiel et al. (22) may not have enough power to show a main effect association. Indeed, Kiel et al. (22) included 51 toddles in their study, which were additionally grouped, whereas our study with more than 300 children had a larger sample size, but this would still be insufficient to show very small effects. Fourth, the Child Behavior Checklist (CBCL) is not a very precise measure of sleep problems, although it is a validated instrument and the Sleep Problems scale is based on 7 items that has repeatedly been used as a stand-alone sleep problem measure (30, 35) and had a fairly good internal reliability in our study population (Cronbach's alpha between 0.69 and 0.74).

In contrast, we clearly confirmed our hypothesis that the diurnal cortisol rhythm was longitudinally associated with sleep duration; infants with a flatter cortisol slope and those with a more marked morning cortisol rise slept shorter at night from age 14 months to age 3 years. In our study we used night time sleep duration as a separate outcome measure instead

as part of a construct of poor sleep. Our findings are in line with the results of cross-sectional studies, e.g. those from Lemola et al. (36) who reported that morning cortisol secretion was negatively associated with sleep duration in children. El-Sheikh et al. (37), as well, found that higher afternoon cortisol levels were associated with shorter sleep duration in children. Also, Räikkönen et al. (38) found, in line with our results, that children with short sleep duration displayed higher cortisol awakening response and less decrease in cortisol across the day. These cross-sectional studies must be interpreted cautiously as consistency with the temporal direction of association cannot be evaluated. To our knowledge, only Hatzinger et al. (39) conducted a longitudinal study in pre-schoolers. However they investigated whether poor sleep, objectively measured with sleep-actigraphy recordings, was prospectively associated with cortisol secretion 12 months later. Although they, like the above mentioned studies, showed a cross-sectional association between poor sleep and high morning cortisol levels, no longitudinal association between sleep as a predictor of cortisol secretion was observed. This strongly suggests that the direction of association may be the reverse.

Several potential mechanisms could explain our findings. First, stress (acute and chronic, as well as physical and psychological) is known to alter cortisol secretion in children (40) and it has been shown that sleep, stress and cortisol secretion are highly intertwined (14). Infants who experience more stress, such as an adverse intra-uterine environment or early life adversity due to low socio-economic status or maternal depression, are more likely to have altered cortisol secretion patterns (41, 42, 43) and sleeping patterns (44). In this study we assessed several potential environmental stressors for the child, such as prenatal maternal psychiatric symptoms and maternal parenting stress at child's age 18 months, as potential antecedents and/or confounders. However, including these specific factors in our models did not change the observed associations and this cannot explain why cortisol is prospectively related to shorter sleep duration in early childhood. Second, and certainly not an exclusive mechanism, the diurnal cortisol secretion has a close association with the sleep-wake cycle. Mostly the impact of disturbed sleep on the diurnal cortisol rhythm has been the focus of research (review 45). However, it is important to realize that a bidirectional relation between sleep and HPA system has been discussed repeatedly (review 46). Yet, these studies in adults cannot easily be translated to children as sleep duration is a highly developmentally determined phenomenon (47). Third, genetic differences could explain both different cortisol secretion patterns and later sleep problems in children (review 48). Genetics, gene-environment interaction and other common causes can, even in a longitudinal study, not be ruled out as explanations for our results. Likewise residual confounding is always possible, although we tried to minimize the effect of external variables by adjusting our models with several possible indicators of socio-economic background, including maternal age and maternal educational level, as well as adverse circumstances, such as maternal psychiatric symptoms during pregnancy and parenting stress at 18 months.

The results of the current study should be carefully considered in light of several methodological strengths and limitations. The strengths of our study are the large population-based sample, its prospective design, and the repeated measures over time of both sleep duration and sleep behaviour. Yet, some limitations also need to be considered. First, the sampling of saliva occurred only on one single day, so day-to-day variability could not be taken into account (49). However, to ask parents participating in a large cohort with multiple other assessments to sample on several days increases the risk of drop-out or non-response. Second, the compliance of the saliva sampling was not assessed by an objective measurement such as a timing device, for this we relied on parental report. Furthermore, we relied on parental report for sleep duration and sleep behaviour which is the best available measure in large population based studies but cannot be seen as a gold standard such as polysomnography , or as objective as actigraphy. Also, at the ages of assessment we had no information on parenting style, thus stratification as performed by Kiel et al. (22) was not possible. Our analyses of missing data showed that attrition was not at random. There was a selective dropout of girls, children with lower Apgar scores and children of lower educated mothers. Due to possible selection effects, our results may be less representative of the general population.

These results have both scientific and clinical implications. Firstly, they suggest that variations in cortisol precede a shorter sleep duration and may thus help understand the biological mechanisms from stressors, such as social disadvantage and early (family) adversity, to sleep deficits. Also, it is conceivable that, if replicated, a flatter cortisol slope (implying blunted HPA axis activity) may be used as a predictor of sleep deficits in certain subgroups of stressed infants.

In conclusion, a flatter cortisol slope in infants and a more marked morning cortisol rise in infants was associated with shorter sleep at pre-school age(s). The underlying mechanism cannot easily be inferred from this study, however, if infant cortisol levels remain high across the day this may indicate stress or HPA dysregulation with long-term effects on sleep.

Appendix Table 1A. Associations between cortisol composite measures and sleep duration at 14 months.

Cortisol measures	Sleep duration measured at 14 months			
	Model 1		Model 2	
	Beta (95% CI)	*p*	Beta (95% CI)	*p*
AUC (nmol/L)	-0.003 (-0.03; 0.02)	0.82	0.002 (-0.02; 0.02)	0.83
Slope (nmol/L/h)	-0.12 (-0.24;-0.00)	**0.047**	-0.11 (-0.22; 0.01)	0.07
CAR (nmol/L)	-0.01 (-0.02; 0.00)	**0.04**	-0.01 (-0.02; 0.001)	0.08

Model 1: adjusted for age at cortisol sampling and gender
Model 2: as model 1, additionally adjusted for waking time at 14 months, maternal age, maternal educational level, maternal psychiatric symptoms during pregnancy, and maternal parenting stress at 18 months

Appendix Table 1B. Associations between cortisol composite measures and sleep duration at 24 months.

Cortisol measures	Sleep duration measured at 24 months			
	Model 1		Model 2	
	Beta (95% CI)	*p*	Beta (95% CI)	*p*
AUC (nmol/L)	0.012 (-0.011; 0.034)	0.32	0.013 (-0.011; 0.036)	0.29
Slope (nmol/L/h)	-0.161 (-0.27;-0.037)	**0.006**	-0.152 (-0.27;-0.037)	**0.010**
CAR (nmol/L)	-0.011 (-0.02;-0.001)	**0.027**	-0.010 (-0.02;-0.001)	**0.033**

Model 1: adjusted for age at cortisol sampling and gender
Model 2: as model 1, additionally adjusted for waking time at 14 months, maternal age, maternal educational level, maternal psychiatric symptoms during pregnancy, and maternal parenting stress at 18 months

Appendix Table 1C. Associations between cortisol composite measures and sleep duration at 36 months.

Cortisol measures	Sleep duration measured at 36 months			
	Model 1		Model 2	
	Beta (95% CI)	*p*	Beta (95% CI)	*p*
AUC (nmol/L)	0.009 (-0.01; 0.03)	0.30	0.01 (-0.007; 0.026)	0.254
Slope (nmol/L/h)	-0.10 (-0.18;-0.01)	**0.02**	-0.085 (-0.165;-0.006)	**0.035**
CAR (nmol/L)	-0.003 (-0.01; 0.005)	0.41	-0.004 (-0.011; 0.004)	0.33

Model 1: adjusted for age at cortisol sampling and gender
Model 2: as model 1, additionally adjusted for waking time at 14 months, maternal age, maternal educational level, maternal psychiatric symptoms during pregnancy, and maternal parenting stress at 18 months

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CHAPTER 5

General discussion

GENERAL DISCUSSION

RATIONALE

Dysregulation of hypothalamic-pituitary-adrenal (HPA) axis activity can be determined by studying patterns of the diurnal cortisol rhythm. Certain diurnal cortisol patterns have been associated with different types of stress in adults, adolescents and children (Lucas-Thompson & Hostinar 2013, Räikkönen et al., 2010, Sieh et al., 2012). Several stressors have also been associated with negative (mental) health outcomes, whereby dysregulation of the HPA axis activity is considered a plausible mechanism to explain this relationship (e.g. reviews of Herbert 2013, and Shah & Malla 2015, incl. erratum). The two objectives of this thesis was 1) to extend the knowledge on determinants of the developing diurnal cortisol rhythm in infants and 2) to study the relationship between this diurnal cortisol rhythm and child's psychosocial functioning. The first part of this thesis described the relations between early risk factors and the diurnal cortisol rhythm in infants. The second and third part focused on how the diurnal cortisol rhythm affects cognitive functioning, internalizing and externalizing problems, as well as sleep in pre-schoolers. The current chapter reviews our main findings and discusses major methodological issues with regards to HPA axis activity research in children and epidemiological studies. The chapter concludes with implications for clinical practice and future research.

MAIN FINDINGS

Influence of early risk factors on the infant diurnal cortisol rhythm

Dysregulation of diurnal cortisol secretion patterns may explain the link between adversities early in life and later mental health problems. However, few studies have investigated the influence of social disadvantage and family adversity on the HPA axis early in life. In chapter 2 we showed that effects of social disadvantage and early adversity on the diurnal cortisol rhythm are already observable in infants aged 12-20 months from a population-based sample. The area under the curve (AUC), the cortisol awakening response (CAR) and the diurnal cortisol slope were calculated as different composite measures of the diurnal cortisol rhythm. We found that older infants showed lower AUC levels; moreover, infants with a positive CAR were significantly older. Both the AUC and the CAR were related to indicators of social disadvantage and early adversity. Infants of low-income families, in comparison to high-income families, showed higher AUC levels and a positive CAR. Infants of mothers who smoked during pregnancy were also significantly more likely to show a positive CAR.

Furthermore, infants of mothers experiencing parenting stress showed higher AUC levels. The results of our study showed that effects of social disadvantage and early adversity on the diurnal cortisol rhythm are already observable in infants. This may reflect the influence of early negative life events on early maturation of the HPA axis. The results of our study have been used in a recent meta-analysis (Pearson et al., 2015) in which they concluded that there is strong evidence that adverse prenatal factors influence HPA axis activity in the offspring. This suggests that our findings contribute to extending the knowledge on this topic. The importance of understanding the influence of early life stress on the development of emotional and behavioural problems have been elaborately described in the review of Loman and Gunnar (2010). In this review they describe how translation of evidence from basic animal early experience research can be applied to child research. Research on this topic is continued, as recently Simons et al. (2015) reported that prenatal and postnatal maternal stress influences child diurnal cortisol secretion.

Infant diurnal cortisol rhythm and psychosocial functioning

Infant diurnal cortisol rhythm and cognitive functioning

Little is known about the relationship between diurnal cortisol secretion patterns and cognitive functioning early in life. In chapter 3.1 we showed that a more positive CAR was associated with a lower risk of delay in language comprehension and a lower risk of nonoptimal fine motor development at age 18 months, and with a lower risk of delay in nonverbal cognitive development at age 30 months. Also, children with flatter diurnal cortisol slopes had a lower risk of delay in nonverbal cognitive development. Higher AUC levels were associated with a higher risk of delay in language production. These results show that variations in diurnal cortisol rhythms are already associated with variations in cognitive functioning at a young age. Infants with a diurnal cortisol pattern indicative of less stress and more cortisol reactivity, that is, lower AUC levels and a more positive CAR, show a lower risk of delay in cognitive functioning as toddlers. Although the relationship between cortisol and cognition have been studied extensively in adults and elderly people (Lupien et al., 2005), only few studies have linked cortisol production early in life to cognitive development (Annett et al, 2005, Blair et al., 2005, van Bakel & Riksen Walraven, 2004). However, in these studies they assessed cortisol reactivity, that is the response of cortisol after a stressor, and not the diurnal cortisol rhythm. Thus, the findings of our cross-sectional study contribute to the knowledge of another aspect of HPA axis activity early in life and its relation with cognitive development. Needless to say, research on this topic is currently still ongoing, as is demonstrated by Forns et al. (2014), who also reported on child cortisol levels in saliva and neuropsychological development during the second year of life. This group described that higher cortisol levels were associated with better cognitive scores, whereas no association was found between cortisol levels and psychomotor development.

Association of infant diurnal cortisol rhythm with internalizing and externalizing problems
Studies investigating the association between diurnal cortisol rhythm and behavioural problems in young children have yielded inconsistent results (de Haan et al., 1998; Scher et al., 2010; Ouellet-Morin et al., 2010). In chapter 3.2 we showed that no cross-sectional associations between the cortisol composite measures (AUC, CAR and diurnal slope) and problem behaviour were found at 1.5 years. However, cortisol predicted change in internalizing problems as assessed from 1.5 to 3 years, but not change in externalizing problems. Children with higher AUC levels, flatter slopes and a more positive CAR at baseline were more likely to score higher on the Internalizing Problems scale (from the Child Behavior Checlist; Achenbach and Rescorla, 2000) at follow-up. Thus, variations in diurnal cortisol rhythm were associated with change in internalizing problems in pre-schoolers. The results suggest that variations in diurnal cortisol patterns early in life precede internalizing problems. This seems in contrast with our findings mentioned in chapter 3.1, where we found that a more positive CAR show lower risk of delay in cognitive functioning as toddlers. However, it is very well possible that children with higher intelligence are more prone to internalizing problems, because they tend to ”overthink” matters. Although assessed in a clinical sample of children with ASD, Estes et al. (2007) found that higher intelligence at age 6 was associated with increased internalizing symptoms by age 9. Also, our study in chapter 3.1 was, although not exactly cross-sectional, but temporal and not longitudinal in its design, so results are not easily comparable to the longitudinal study in chapter 3.2.

Infant diurnal cortisol rhythm and stress reactivity in constipation and abdominal pain
Functional bowel disorders comprise a large range of gastrointestinal symptoms such as irritable bowel syndrome (IBS), functional constipation, and abdominal pain (Ammoury et al., 2009). Psychological stress is widely believed to play a major role in functional bowel disorders, especially irritable bowel syndrome (IBS). Various studies have suggested that functional bowel disorders underlie a complex interaction between psychosocial and physiological factors through the hypothalamic–pituitary–adrenal (HPA) axis (Ammoury et al., 2009). If variations in the developing infant HPA axis already exists, it would be interesting to see if cortisol can be used as a marker for stress early in life. In chapter 3.3 we showed that in the second year of life, 13% of the infants had functional constipation and 17% had abdominal pain. Only 4% had symptoms of both functional constipation and abdominal pain. Diurnal cortisol rhythm did not differ significantly between children with and children without functional constipation and abdominal pain. Cortisol stress reactivity was slightly higher in infants with abdominal pain than in those without it, but this was not statistically significant. No association was found between the cortisol stress reactivity and functional constipation.

Our results suggest that cortisol as a marker for stress does not play a major role in functional constipation or abdominal pain in infancy. Although this study provides no clues for using cortisol as a marker for stress in normal developing children, completely discarding cortisol as a possible useful tool would be prematurely.

Infant diurnal cortisol rhythm and sleep

Disturbed sleep preceding anxiety or depression

Sleep problems are highly intertwined with mental health problems. In chapter 4.1 we studied whether sleep problems in infancy and early toddlerhood precede symptoms of anxiety or depression at 3 years.

Dyssomnia and parental presence during sleep onset at 2 months and 24 months were associated with anxiety or depressive symptoms at 3 years. Parasomnia, short sleep duration, and absence of set bedtime at 24 months, but not at 2 months, also preceded anxiety or depressive symptoms. These significant associations were not due to children's anxiety or depressive symptoms at 18 months. Rhythmicity and co-sleeping were not associated with later anxiety or depressive symptoms. Additional analyses provided little evidence for a bidirectional association with anxiety or depressive symptoms preceding later sleep problems. Our findings highlight the importance of sleep problems early in life, because different sleep problems are associated with the frequency of anxiety or depressive symptoms. Therefore, healthcare practitioners must be particularly attentive to these problems in young children. Future research should address possible mechanisms underlying the association between disturbed sleeping and anxiety or depressive symptoms. Although we discuss possible biological and nonbiological explanations for our findings in this chapter, the possibility that HPA axis dysregulation could be the underlying mechanism of this association is further examined in chapter 4.2.

Infant diurnal cortisol rhythm and sleep

Cortisol, the end-product of the hypothalamic-pituitary-adrenal (HPA) axis, plays an important role in modulating sleep, which in turn is important for mental health. Yet, studies investigating the association between diurnal cortisol rhythm and sleep patterns in young children are scarce. In chapter 4.2 we showed that the diurnal cortisol slope and the CAR, but not the AUC, were associated with sleep duration across childhood. Children with flatter slopes and children with a more positive CAR were more likely to have shorter sleep duration. Cortisol measures did not predict sleep problems.

The present study suggests that a flatter cortisol slope and a more marked morning rise, which can indicate stress (or HPA axis dysregulation), have long-term effects on sleep

regulation. Although we found associations between cortisol and sleep duration, we did not find a relationship between cortisol and sleep problems. However, it is too early to draw the conclusion that HPA axis activity is not an explanation for the underlying mechanism in the relationship between disturbed sleep and internalizing problems. It is important to realize that the study sample's size in chapter 4.1 was tenfold higher than the size of the study sample in this chapter. Therefore we cannot exclude that the study in this chapter did not have enough power to find small effects.

METHODOLOGICAL CONSIDERATIONS

Measuring cortisol

Investigating HPA axis activity in humans, and children in particular, has been challenging since cortisol research commenced in large clinical and epidemiological studies around the 1950s (Doom & Gunnar, 2013). Activation of the HPA axis starts with neurons in the hypothalamus releasing a hormone called corticotropin-releasing hormone (CRH). The release of CRH in turn subsequently triggers the release of another hormone namely adrenocorticotropin (ACTH) from the pituitary gland, also located in the brain. This ACTH travels in the blood to the adrenal glands, located above the kidneys, and triggers the secretion of cortisol. The latter is the hormonal end-product of the HPA axis, which is important for a wide variety of adaptive functioning and is released in response to stressors. Markers of HPA activation are thus ACTH, CRH and cortisol levels. However, ACTH can only be measured in blood and CRH can only be measured in cerebrospinal fluid. Only cortisol is relatively easily measurable in blood and urine.

Repeated cortisol sampling from blood is a stressful and invasive procedure and is only justified to use in clinical studies. In large population-based studies in children sampling cortisol repeatedly and multiple times from blood is not feasible.

Although sampling cortisol from urine may seem a convenient non-invasive method in comparison to sampling from blood, urinary cortisol provides levels that are pooled across multiple hours. This allows only total "average" exposure to cortisol across 12 or 24 h available as a measure (Seeman et al., 1997), which is not very precise. Also, in young children who are not yet toilet trained, sampling of urine is not feasible without a urinary catheter which makes this sampling method rather invasive and therefore not suitable for large observational studies. Even in somewhat older children who are already toilet trained, collecting all urinary outflow in 24 hour period is hardly achievable.

Since the late 1980s cortisol is also measurable in saliva. This not only provides for a non-invasive sampling method, it also does not require the collaboration of skilled personnel for the installation of an intravenous canule or a catheter. It allows for a more uncomplicated and less troublesome sample collection, which can be used in larger observational studies. Over the past 25 years, salivary cortisol has frequently been used as a biomarker of psychological stress in stress studies (Kirschbaum & Hellhammer, 1994). Although salivary cortisol measures are an indirect assessment of HPA axis activity, it has proven to be a useful and validated tool not only in research settings but also seems a promising tool for clinical use (Inder et al., 2012).

The diurnal rhythm of cortisol can be easily measured in both adults and children using saliva sampling multiple times throughout the day. To capture the diurnal rhythm it is necessary to sample saliva during several different time points during the day and preferably on multiple consecutive days. From this the diurnal decline of cortisol during the day (slope) can be calculated as well as the cortisol awakening response (CAR) (see below). This makes saliva sampling a unique tool to assess the diurnal rhythm and provide information about activity of the HPA axis in regular day-to-day life. Ultimately, cortisol saliva sampling is best suited to give information about the central regulation of the HPA axis activity. This method of sampling can also be used in studying effects of short-term intervention studies which target HPA axis regulation of participants in community settings. Furthermore, saliva cortisol sampling is also useful to study the reactivity of the HPA axis after a stressor in a laboratory settting (such as the Trier Social Stress Test).

More recently, another non-invasive sampling method has been developed through measuring cortisol levels in hair in adults (Manenschijn et al., 2011) and in healthy children (Noppe et al., 2014). As hair cortisol levels provide an integrated index of cumulative stress exposure across an extended period of time, it certainly better reflects overall cumulative HPA axis activity in contrast to measurements of cortisol in blood or saliva that represent cortisol at one point in time. Moreover, the convenience and non-invasiveness of this sampling method makes this a promising tool for future epidemiological research.

Salivary cortisol measures

As Doom and Gunnar nicely described in their review (2013) research on cortisol in children has been a major focus of scientists since cortisol saliva sampling became available more than 25 years ago. Although saliva sampling provides a non-invasive way to examine cortisol levels in children (and adults), there are numerous challenges in the field of cortisol research resulting from the many different cortisol measures. In the review of Adam and Kumari (2009) they describe the frequently used diurnal cortisol measures in field-based research

in adults (CAR, diurnal cortisol slope, AUC, waking cortisol level, cortisol level at specific time points across the day, bedtime cortisol level, and cortisol reactivity to momentary or daily stressors). Not coincidentally, the three main cortisol (summary) measures used in this thesis (CAR, diurnal cortisol slope, and AUC) are the most commonly employed and the most robustly related to psychosocial processes and to health outcomes. It is very likely that these three cortisol measures capture different aspects of HPA axis activity, since the observed associations differ in most published studies per measure. For example both heightened and blunted CARs have been related to psychosocial stress and poor health outcomes (reviews of Chida & Steptoe 2009, and Incollingo Rodriguez et al., 2015). Flatter diurnal cortisol slopes, indicative of HPA dysregulation, are typically associated with poorer psychosocial and physical health (see also review of Incollingo Rodriguez et al., 2015). However, in the present thesis we found that children with flatter diurnal cortisol slopes had a lower risk of delay in nonverbal cognitive development (chapter 3.1). The AUC gives an estimate of average cortisol exposure but provides no indication of diurnal change. Higher and lower total cortisol levels have been associated with depression and obesity (reviews of Jessop & Turner-Cobb 2008, and Incollingo Rodriguez et al., 2015). Besides these diurnal cortisol summary measures, individual cortisol levels at certain time points have also been studied by many investigators. However, these individual time points are closely tied to summary measures. Yet, cortisol levels at awakening may give an indication of the steepness of the diurnal cortisol slope and are related to the CAR, these levels are a poor proxy. The same can be said for bedtime cortisol levels, which reflect total cortisol secretion and the steepness of the diurnal cortisol slope. Finally, cortisol levels at even more random time points across the waking the day have been used (e.g. 10 AM or 10 PM, or in the afternoon at 4 PM) as single exposure or outcome measure in prior research. The results of these studies are difficult to interpret, if there is no information about waking and bedtime levels. In particular elevated afternoon and evening cortisol levels are thought to be associated with negative outcomes. However, results are not only inconsistent across measures but results of individual studies are not very consistent for each individual measure either. This suggests some degree of chance finding, publication bias or measurement problems.

In general, it is important to realize that the secretion of cortisol is very adaptive and characterized by intra- and inter-individual variability. Also, the compliance of sampling saliva at home, especially in the morning, is low (Kudielka et al., 2003). Therefore, sampling saliva on one certain time point to obtain a specific cortisol level is of limited use in cortisol research because this gives rise to substantial error or unexplained variance (noise) in the statistical models. This may well explain why cortisol levels have low heritability (unpublished Generation R and Rotterdam Study data).

In summary, sampling saliva on several time points during the day, preferably on more consecutive days, seems to be desirable to obtain a more precise measure of cortisol.

Moreover, if several cortisol summary measures or individual cortisol levels are used (although reference or "normal" levels are not well-defined), this gives another problem, the issue of multiple testing. Therefore, as already mentioned before, a few pre-specified cortisol summary measures (CAR, diurnal cortisol slope, and AUC) should be used to reduce the issue of multiple testing. As the compliance of saliva cortisol sampling at home is low, this is not as easy as it seems. It is hard to calculate the cortisol summary measures if samples are missing. Also, to make the cortisol summary measures comparable one must take into account the timing of the samples at home, which is a challenge as compliance of time recording also is low.

Furthermore, although the CAR, diurnal cortisol slope, and AUC are the most commonly employed cortisol summary measures in cortisol research, it is still unknown what the exact biology is behind these summary and advocated measures. Despite extensive research on the CAR, the function of this steep increase of cortisol after awakening remains unknown (Fries et al., 2008). Also, the calculation of these summary measures is not always straightforward. For example, it is important to realize not to use the saliva samples within one hour after awakening to calculate the AUC, otherwise the correlation between the CAR and AUC would be too large. Taking all this into account, it is not surprising that the summary measures of saliva cortisol samples have yielded very inconsistent results.

Besides sampling salivary cortisol multiples times on a typical day, cortisol reactivity to momentary stressors have also been used in cortisol research (also in this thesis). Adam and Kumari (2009) made a distinction between cortisol reactivity to momentary stressors and cortisol reactivity to daily stressors. The first are elevations in cortisol levels above typical diurnal level for that time of day for that person, which have been associated with cross-sectional negative mood states. Cortisol reactivity to daily stressors have been described as changes in cortisol levels from 1 day to the next, which has been associated with changes of HPA axis activity in daily demands and events. Cleary, measuring cortisol reactivity to stressors gives valuable information of HPA axis activation, but this cannot be easily compared to cortisol summary measures depicting the diurnal cortisol rhythm. However, it is evident that information of the diurnal cortisol level is needed for a correct interpretation of changes in cortisol levels to a stressor, so both diurnal cortisol measures and cortisol reactivity must be investigated in cortisol research. It must also be noted, that adequate (psychological) stressors in cortisol research remain challenging as certain paradigms for social stress not always evoke the same cortisol responses when slightly altered (Wiemers et al., 2013). Also,

for research in children it is a challenge to expose them to adequate stressors which will influence cortisol levels, but are still ethically acceptable.

Despite the challenges described in sampling saliva cortisol in children, repeated salivary sampling to obtain summary cortisol measures remains a useful tool to study both the diurnal cortisol rhythm and HPA axis reactivity to a stressor. Especially when enhancing compliance by making sampling at home easier, for example by using eye sponges instead of cotton rolls (Donzella et al., 2008), using objective measures of time such as an electronic monitor that date- and time-stamps bottle opening or even using an accelerometer to capture the precise measurement for time of awakening (Rotenberg & McGrath, 2014).

Confounding in cortisol research

Another challenge in cortisol research is controlling for possible confounding variables. In the earlier days of cortisol research in children studies examining diurnal cortisol secretion frequently had participant numbers in the tens, thus lacking sufficient degrees of freedom to adequately control for possible variables that may account for or obscure the found relationship. For this discussion I provide an overview of frequently examined covariates in child cortisol research that have been related to diurnal cortisol secretion is given (see table 1). A distinction can be made between covariates that were typically collected on day of sampling regarding the schedule (**D**ay of sampling covariates) and lifestyle factors (**L**ifestyle covariates) and covariates not specific to day of sampling regarding demographic (**B**ackground covariates) and medical/health factors (**M**edical and health covariates).

Other covariates are typically controlled for by exclusion. Exclusion criteria in adult cortisol research are use of steroid medications, 3rd trimester pregnancy, illness on day of testing and presence of endocrine disorder. Also, many confounders are selected based on adult literature (for an overview of the most frequently examined covariates in adult research see review of Adam & Kumari, 2009). Although research from adults provides useful information for possible confounding factors in cortisol research in children, not all factors are relevant early in life whereas other factors may be of specific influence on diurnal cortisol secretion during certain developmental periods. In the table below an overview is given of confounders used from a selection of cited references throughout this thesis. Although this is a starting point for identifying confounding factors, when investigating HPA axis activity as a potential biomarker for later negative (mental) health outcomes, it is important to also realize that with different outcome measures other potential confounding factors could be influencing the relationship of interest. Obviously, there can be no consensus about the confounding factors for a certain relationship between cortisol and any of the (mental) health outcomes.

Table 1. Potential confounders in cortisol research [a]

Reference	Child age at baseline (range)	Cortisol measures	Outcome	Confounders
Early life adversity				
Bruce et al. (2009)	3-6 years	Morning cortisol levels on 2 consecutive days	Maltreatment	Gender (**B**) Ethnicity (**B**) Household income (**B**)
Dozier et al. (2006)	20 to 60 months	Cortisol levels at 3 timepoints during the day for 2 days	Cortisol levels	Gender (**B**) Age (**B**) Family income (**B**) Ethnicity (**B**)
Halligan et al. (2004)	13 years	Cortisol levels at 8:00 AM and 8:00 PM over 10 days	Cortisol levels	Gender (**B**) Tanner stage (**B**) Breast feeding status (**B**)
Kertes et al. (2008)	7-11 years	Cortisol levels 30 min after wakeup and before bedtime	Morning cortisol levels Diurnal cortisol decrease	Gender (**B**) Parent education (**B**) Family income (**B**)
Oskis et al. (2009)	9-18 years (only females)	Cortisol awakening response (CAR) Diurnal cortisol decline	CAR Diurnal cortisol decline	Age (**B**) BMI (**B**) Anxiety: trait (**B**), state (**L**) Season of sampling (**D**) Self-reported waking time and waking method (**D**)
Cognitive development				
Rosmalen et al. (2002)	10-12 years	Cortisol levels at 07:00 AM, at 07:30 AM and 20:00 PM AUC	Individual cortisol levels AUC	Season of sampling (**D**) Gender (**B**) (potential confounders not in models: age, pubertal development, perinatal variables, BMI)
Watamura et al. (2003)	10 to 30 months	Cortisol levels at 10:00 AM and 4:00 PM	Individual cortisol levels Change in cortisol levels	Age (**B**) Gender (**B**)

Behavioural problems				
Dougherty et al. (2009)	3-4 years	Cortisol levels in the morning and evening	Morning cortisol levels	Age (**B**) Gender (**B**) Child current negative emotionality (**L**) Child current depressive symptoms (**L**) Parental hostility (**B**) Child life stress (**B**)
Essex et al. (2002)	4.5- year olds	Cortisol levels in the afternoon	Cortisol levels	Collection time (**D**) Medications Gender (**B**)
Kryski et al. (2013)	3-year-olds	Cortisol reactivity to stress task	Internalizing symptoms	Family SES/income (**B**) Gender * (**B**)
Perez-Edgar et al. (2008)	4-year-olds	Morning cortisol levels (within 30 min of rising on 3 consecutive days)	Behavioural maladjustment	Gender * (**B**)
Shirtcliff et al (2008)	11 years	Cortisol levels at waking, afternoon and at bedtime on 3 consecutive days	Mental health symptoms	Gender (**B**) Puberty (**B**) Medications (**M**)
Smider (2002)	4.5 years	Afternoon cortisol levels on 3 consecutive days	Internalizing and externalizing behaviour	Gender (**B**) Medication (**M**)
Wolf (2008)	8-18 years	Cortisol levels 1, 4, 9 and 11h after awakening	Cortisol slope (and alpha-amylase)	Age (**B**) Gender (**B**) Asthma medication (**M**)
Sleep				
Brand et al (2011)	8 weeks	Morning cortisol levels	Crying and sleeping	Age (**B**) Birth weight (**B**) Apgar score (**B**) Gender (**B**) Birth (**B**) Birth order (**B**) Mothers' age or delivery experience (**B**)

Table 1. *(Continued)*

Fernandez-Mendoza et al. (2014)	5-12 years	Evening and morning cortisol levels	Individual cortisol levels	Gender (**B**) Age (**B**) Ethnicity (**B**) Waist circumference (**M**) Apnea/hypopnea index (**M**) Anxiety and depression (**L**)
Lemola et al. (2015)	6-10 years	CAR (0-10-20-30 min after awakening)	Sleep pattern	Age (**B**) Gender (**B**) Prematurity status (**B**) Awakening time (**D**)
El-Sheik et al. (2008)	8 years	Afternoon cortisol levels	Subjective sleep problems, objective measures of shorter sleep duration and poorer sleep quality	Age (**B**) Gender (**B**) Ethnicity (**B**) Puberty status (**B**) SES (**B**) BMI (**B**) Time of saliva sampling (**D**)
Räikkönen et al. (2010)	8-year-olds	Diurnal cortisol (CAR, slope) Cortisol reactivity (TSST)	Diurnal cortisol levels Cortisol levels after TSST	Time at awakening (**D**) Gender (**B**) Age (**B**) BMI (**M**) Mother's occupational status (**B**) Mother's licorice use during pregnancy (**B**)

[a] This list is not exhaustive, but is based on references cited in this thesis.

* treated as intermediate/effect modifier

Abbreviations: CAR = cortisol awakening response, AUC = area under the curve, TSST = Trier Social Stress Test, BMI = body mass index, SES = socioeconomic status

(**B**): Background or demographic covariates

(**D**): Day of sampling covariates

(**L**): Lifestyle covariates

(**M**): Medical and health covariates

Cited references: see reference list general discussion.

Theoretically, there are numerous factors that could act as a confounder in the relationship between cortisol and any psychosocial outcome measure. Trying to find small effects and simultaneously taking into account several (potential) confounding factors, could lead to type II errors (failure to reject a false null hypothesis) due to insufficient power. Another problem rises if a factor acts as an intermediate and mediates the relationship between cortisol and the outcome measure, this leads to overcorrection in the statistical models and also can lead to type II errors.

Also important, with adjustment for confounding, even in large, well-defined studies, which rigorously control for socio-economic and other factors, it is virtually impossible to rule out residual confounding. This can occur when the variable used to adjust is an imperfect match for the underlying confounding variable, when a confounder remains unaccounted for in study design and analysis, or when there are unknown confounders.

It is therefore important in cortisol research to measure possible confounding factors as precisely as possible in a large enough study population in order to properly adjust for confounders.

SCIENTIFIC AND CLINICAL IMPLICATIONS

Even limited success in the prevention of negative (mental) health outcomes would not only decrease the costs of (mental) health care enormously, but would also increase quality of life for many people. Especially in children, a very vulnerable group and the future of our society, the gain would be tremendous. However, in order to prevent certain outcomes, understanding of the development of as well as the road to adverse outcomes with possible clues for intervention is key. Unfortunately, the real world is utterly complex and knowledge of normal development is also necessary in order to understand abnormal development. So to understand the mechanisms through which dysregulation of HPA activity leads from different types of stress to adverse (mental) health outcomes, it is also important to understand the development of HPA axis activity early in life in normal developing children. The results of the studies presented in this thesis contribute to our knowledge of the development of the diurnal cortisol rhythm in the second year of life of normal developing Dutch children. We showed that it is possible to examine salivary cortisol at a young age in a relatively large population based sample (of more than 300 children). We also showed that several determinants influence the cortisol diurnal rhythm at this young age. Moreover, we showed that variations in cortisol are not only a correlate of cognitive functioning, internalizing problems and shorter night-time sleep duration, but we also showed the temporal relationship of cortisol variations

and these outcomes. From a scientific point of view this thesis contributes to fundamental knowledge of the normal developing HPA axis in young children and to our knowledge of the role of cortisol in developmental psychopathology.

Certainly, replication of our findings in other epidemiological studies will render more proof that the found associations are sustainable. Furthermore, additional studies, not only from observational but also from experimental designs (with each their own strengths and limitations), are needed to fully understand the complex mechanisms through which different types of stress influence dysregulation of HPA activity and ultimately can lead to adverse (mental) health outcomes.

Evidently, it will take time before these fundamental findings on the normal development of HPA axis activity, will be of direct use for the clinical practice. Therefore, direct implications of the presented studies for the clinical practice are limited. A possible implication in the future could be that with more knowledge of the normal development of the diurnal cortisol rhythm reference values for abnormal developing diurnal patterns can be developed for (young) children, which in turn can identify vulnerable risk groups in which early intervention can take place to prevent adverse (mental) health outcomes. It is not unthinkable that in combination with certain genetic profiles identification of certain risk groups will eventually be possible.

FUTURE DIRECTIONS

The field of cortisol research is an exciting area. As is often the case in research, trying to answer specific research questions usually generates more questions that are still left unanswered. As our understanding of the development of the cortisol diurnal rhythm is growing, the need for more longitudinal studies becomes clearer. Furthermore, different kinds of cortisol measures depict specific aspects of HPA activity and more longitudinal studies addressing these specific measures will contribute to the overall knowledge of HPA axis functioning. In children, repeated salivary sampling to obtain summary cortisol measures remains a useful tool to study the diurnal cortisol rhythm and HPA axis reactivity to a stressor. These longitudinal studies should be large enough to ensure that the power of the studies are sufficient enough to also assess the effects of several possible confounders and mediators. Another promising method to measure cortisol levels is through hair samples. This is a convenient, non-invasive method and thus feasible in large observational studies in children. By providing an integrated index of cumulative stress exposure across an extended period of time, hair cortisol levels certainly add another dimension to the cortisol research, which

needs to be explored thoroughly, especially in developing children. Also, the interaction of the HPA system with other bodily systems, environmental (stress) factors as well as (epi) genetic factors, and combined gene-environment interaction, needs to be studied to gain more understanding of how these interactions work.

In conclusion, this thesis focused on the development of mainly the diurnal cortisol rhythm in the second year of life as a measure of HPA axis activity and its associations with child development in pre-schoolers in the general population. Our studies provide a small but important contribution to overall knowledge of the development of HPA axis activity and it's possible influence on child development. Future research should not only focus on adequate study designs, such as prospective designs with repeated measurements and multiple informants while taking into account possible confounding factors, but also how different measures of cortisol must be interpreted in the context of HPA axis development. In addition, studies focusing on the underlying possible mechanisms of how dysregulation of HPA axis activity leads to adverse mental health outcomes are needed in order to gain more clues that can be used for prevention and intervention.

Of cortisol and children; the story is still ongoing.

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CHAPTER 6

Summary / samenvatting

SUMMARY

Child psychiatric disorders are among the most significant public health problems affecting a large part of the population. In pre-school children both externalizing problems, such as aggressive behaviour and hyperactivity, and internalizing problems, such as anxiety, are the most common problems. Often, these childhood disorders affect the individual lastingly, most adult mental health problems have their onset or a precursor in childhood. The development of internalizing and externalizing problems is assumed to be the result of multiple causes, influenced by genetic and environmental factors and their interaction(s). One of the factors associated with both internalizing and externalizing problems is stress, in the broadest sense of the term. Although it has been shown that more stressful events such as early social adversity or neglect leads to a higher risk of both internalizing and externalizing problems, the psychobiological mechanisms underlying these associations are poorly understood. One of the most important stress systems in the human body, and the primary focus of this thesis, is the hypothalamic-pituitary-adrenal (HPA) axis. This HPA axis is the main neuroendocrine system that is activated in response to physical or psychosocial stress. Cortisol is the hormonal end product of the HPA axis and cortisol levels have since the 1950s been used as a proxy for HPA axis activity. An important characteristic of the HPA axis is its diurnal rhythm. The diurnal secretion of cortisol is characterized by high levels in the morning due to a steep rise in cortisol shortly after awakening, followed by a decline in cortisol levels toward the evening. The HPA axis is a very delicate system and it is involved in regulating a range of bodily functions such as energy metabolism and immune system functioning. Also important, the HPA axis develops and matures in early childhood. Epidemiological studies of the HPA-axis in early life are lacking. Few studies have addressed the question whether early alterations in the HPA-axis precede child developmental problems such as internalizing, externalizing or cognitive problems. Most of these studies focussed on cortisol reactivity but have not addressed the diurnal rhythm. The present thesis is an attempt to close this gap.

This thesis aims to extend the knowledge on determinants of the developing diurnal cortisol rhythm in infants and the associations between the infant's diurnal cortisol rhythm and child development in a general population study. To this end, we measured HPA axis activity at the age of 14 months through saliva sampling in a population-based sample. The area under the curve (AUC), the cortisol awakening response (CAR) and the diurnal cortisol slope were calculated as different composite measures of the diurnal cortisol rhythm. The studies in this thesis were embedded in The Generation R Study, a large prospective population-based cohort from foetal life onwards in the city of Rotterdam, the Netherlands.

In chapter 2, we study the effects of social disadvantage and early adversity on the diurnal cortisol rhythm in infants and found that these are already observable in infants from a population-based sample. We found that older infants showed lower AUC levels; moreover, infants with a positive CAR were significantly older. Both the AUC and the CAR were related to indicators of social disadvantage and early adversity. Infants of low-income families, in comparison to high-income families, showed higher AUC levels and a positive CAR. Infants of mothers who smoked during pregnancy were also significantly more likely to show a positive CAR. Furthermore, infants of mothers experiencing parenting stress showed higher AUC levels. The results of our study showed that effects of social disadvantage and early adversity on the diurnal cortisol rhythm are already observable in infants. This may reflect the influence of early negative life events on early maturation of the HPA axis.

In chapter 3, we examine the associations between the diurnal cortisol rhythm in infants and their psychosocial functioning. A more positive CAR was associated with a lower risk of delay in language comprehension, a lower risk of nonoptimal fine motor development, and a lower risk of delay in nonverbal cognitive development. Also, children with flatter diurnal cortisol slopes had a lower risk of delay in nonverbal cognitive development. Higher AUC levels were associated with a higher risk of delay in language production (chapter 3.1). These results show that variations in diurnal cortisol rhythms are already associated with variations in cognitive functioning at a young age. Infants with a diurnal cortisol pattern indicative of less stress and more cortisol reactivity, that is, lower AUC levels and a more positive CAR, show a lower risk of delay in cognitive functioning as toddlers. Furthermore, we showed that no cross-sectional associations between the cortisol composite measures (AUC, CAR and diurnal slope) and problem behaviour were found at 1.5 years (chapter 3.2). However, cortisol predicted change in internalizing problems as assessed from 1.5 to 3 years, but not change in externalizing problems. Children with higher AUC levels, flatter slopes and a more positive CAR at baseline were more likely to score higher on the Internalizing Problems scale at follow-up (chapter 3.2). Thus, variations in diurnal cortisol rhythm were associated with change in internalizing problems in pre-schoolers. The results suggest that variations in diurnal cortisol patterns early in life precede internalizing problems.

In the second year of life, 13% of the infants had functional constipation and 17% had abdominal pain (chapter 3.3). Only 4% had symptoms of both functional constipation and abdominal pain. Diurnal cortisol rhythm did not differ significantly between children with and children without functional constipation and abdominal pain (chapter 3.3). Cortisol stress reactivity was slightly higher in infants with abdominal pain than in those without it, but this was not statistically significant. No association was found between the cortisol stress reactivity and functional constipation (chapter 3.3). Our results suggest that cortisol as a

marker for stress does not play a major role in functional constipation or abdominal pain in infancy.

In chapter 4, we study the associations between sleep problems and internalizing problems early in life, as well as the relationship between the diurnal cortisol rhythm with sleep duration and sleep problems in pre-schoolers. Dyssomnia and parental presence during sleep onset at 2 months and 24 months were associated with anxiety or depressive symptoms at 3 years (chapter 4.1). Parasomnia, short sleep duration, and absence of set bedtime at 24 months, but not at 2 months, also preceded anxiety or depressive symptoms (chapter 4.1). These significant associations were not due to children's anxiety or depressive symptoms at 18 months. Rhythmicity and co-sleeping were not associated with later anxiety or depressive symptoms. Additional analyses provided little evidence for a bidirectional association with anxiety or depressive symptoms preceding later sleep problems (chapter 4.1). Our findings highlight the importance of sleep problems early in life, because different sleep problems are associated with the frequency of anxiety or depressive symptoms. Furthermore, we showed that the diurnal cortisol slope and the CAR, but not the AUC, were associated with sleep duration across childhood (chapter 4.2). Children with flatter slopes and children with a more positive CAR were more likely to have shorter sleep duration. Cortisol measures did not predict sleep problems (chapter 4.2). These results suggest that a flatter cortisol slope and a more marked morning rise, which can indicate stress (or HPA axis dysregulation), have long-term effects on sleep regulation.

In chapter 5, the main findings of the studies in this thesis are summarized and methodological issues with regard to epidemiological cortisol research in children are discussed. The chapter concludes with possible clinical implications and suggestions for future studies.

SAMENVATTING

Kinder- en jeugdpsychiatrische stoornissen behoren tot de belangrijkste volksgezondheidsproblemen die een groot deel van de bevolking treft. In jonge kinderen zijn zowel externaliserende problemen, zoals agressief gedrag en hyperactiviteit, en internaliserende problemen, zoals angst, de meest voorkomende problemen. Vaak hebben deze stoornissen bij kinderen een blijvende invloed op het individu, aangezien de meeste volwassenen psychiatrische problemen hun begin of een voorloper daarvan in de kindertijd hebben. Er wordt aangenomen dat de ontwikkeling van internaliserende en externaliserende problemen het resultaat is van meerdere oorzaken, die worden beïnvloed door genetische en omgevingsfactoren en hun interactie(s). Een van de factoren die samenhangen met zowel internaliserende als externaliserende problemen is stress, in de breedste zin van het woord. Hoewel is aangetoond dat meer stressvolle gebeurtenissen, zoals sociale tegenspoed vroeg in het leven of verwaarlozing, leidt tot een hoger risico op zowel internaliserende en externaliserende problemen, worden de psychobiologische mechanismen die ten grondslag liggen aan deze associaties onvoldoende begrepen. Een van de belangrijkste stresssystemen in het menselijk lichaam en de primaire focus van dit proefschrift, is de hypothalamus-hypofyse-bijnier (HPA) as. De HPA-as is het belangrijkste neuro-endocriene systeem dat wordt geactiveerd als reactie op fysieke of psychosociale stress. Cortisol is het hormonale eindproduct van de HPA-as en cortisol waarden worden sinds de jaren 50 gebruikt als een proxy voor de HPA-as activiteit. Een belangrijk kenmerk van de HPA-as is het dagritme. De dagelijkse secretie van cortisol wordt gekenmerkt door hoge waarden in de ochtend als gevolg van een sterke stijging van cortisol kort na het ontwaken, gevolgd door een daling van de cortisol waarden gedurende de dag. De HPA-as is een zeer delicate systeem en is betrokken bij het reguleren verschillende lichaamsfuncties zoals energiemetabolisme en werking van het immuunsysteem. Ook belangrijk is dat de HPA-as zich ontwikkelt en uitrijpt in de vroege kindertijd. Epidemiologische studies van de HPA-as op jonge leeftijd ontbreken. Er zijn maar weinig studies die zich hebben gericht op de vraag of vroege veranderingen in de HPA-as voorafgaan aan ontwikkelingsproblemen in kinderen zoals internaliserende, externaliserende of cognitieve problemen. De meeste van deze studies hebben zich gefocust op cortisol reactiviteit, maar hebben niet specifiek het cortisol dagritme onderzocht. Dit proefschrift is een poging om deze kloof te dichten.

Dit proefschrift heeft als doel om de kennis uit te breiden ten aanzien van de determinanten van de ontwikkeling van het cortisol dagritme bij jonge kinderen en de associaties tussen dit cortisol dagritme en de ontwikkeling van het kind in een algemene bevolkingsstudie. Hiertoe hebben we de activiteit van de HPA-as op de leeftijd van 14 maanden gemeten via speekselmonsters in een steekproef uit de algemene bevolking. De oppervlakte onder

de curve (AUC), de cortisol awakening response (CAR) en de cortisol "slope" (afname van cortisol gedurende de dag) werden berekend als verschillende samengestelde maten van het cortisol dagritme. De studies in dit proefschrift werden verricht binnen de Generation R Study, een groot bevolkingsonderzoek in Rotterdam waarin kinderen en hun ouders worden gevolgd vanaf de zwangerschap.

In hoofdstuk 2 bestuderen we de effecten van sociale achterstand en tegenslagen vroeg in het leven op het cortisol dagritme bij jonge kinderen en vonden dat deze reeds zichtbaar zijn bij jonge kinderen in een steekproef van de algemene bevolking. We toonden dat oudere kinderen lagere AUC waarden hadden; bovendien waren kinderen met een positieve CAR significant ouder. Zowel de AUC en de CAR waren gerelateerd aan indicatoren van sociale achterstand en vroege tegenslag. Kinderen uit gezinnen met lage inkomens, in vergelijking met kinderen uit gezinnen met een hoog inkomen, vertoonden hogere AUC waarden en een positieve CAR. Kinderen van moeders die tijdens de zwangerschap rookten hadden ook significant meer kans op een positieve CAR. Bovendien vertoonden kinderen van moeders die meer stress ervaren van het ouderschap hogere AUC waarden. De resultaten van onze studie toonden aan dat de effecten van sociale achterstand en tegenslagen vroeg in het leven op het cortisol dagritme reeds merkbaar zijn in de eerste helft van het tweede levensjaar. Dit kan de invloed van vroege negatieve gebeurtenissen in het leven op de ontwikkeling van de HPA-as weergeven.

In hoofdstuk 3 onderzoeken we de associaties tussen het cortisol dagritme bij kinderen en hun psychosociaal functioneren. Een meer positieve CAR werd geassocieerd met een lager risico op achterstand in taalbegrip, een lager risico op niet optimale fijne motorische ontwikkeling en een lager risico op achterstand in de non-verbale cognitieve ontwikkeling. Ook kinderen met een minder steile cortisol slope hadden een lager risico op achterstand in de non-verbale cognitieve ontwikkeling. Hogere AUC waarden waren geassocieerd met een hoger risico op achterstand in de taalproductie (hoofdstuk 3.1). Deze resultaten tonen aan dat variaties in het cortisol dagritme reeds geassocieerd zijn met variaties in cognitief functioneren op jonge leeftijd. Kinderen met een cortisol dagritme dat indicatief is voor minder stress en meer cortisol reactiviteit, dat wil zeggen, lagere AUC waarden en een meer positieve CAR, tonen een lager risico op achterstand in cognitief functioneren als peuters. Verder hebben we laten zien dat er geen cross-sectionele relaties werden gevonden tussen de samengestelde cortisol maten (AUC, CAR en cortisol slope) en probleemgedrag op de leeftijd van 1,5 jaar (hoofdstuk 3.2). Echter, cortisol voorspelde verandering in internaliserende problemen zoals gemeten van 1,5 tot 3 jaar, maar geen verandering in externaliserende problemen. Kinderen met hogere AUC waarden, minder steile cortisol slopes en een positievere CAR bij de uitgangssituatie hadden meer kans om hoger op de internaliserende problemen schaal te scoren bij de follow-up (hoofdstuk 3.2). Op deze manier werden variaties in het cortisol

dagritme geassocieerd met veranderingen in internaliserende problemen bij peuters. De resultaten suggereren dat variaties in het cortisoldagritme op jonge leeftijd voorafgaan aan internaliserende problemen.

In het tweede jaar van het leven, had 13% van de kinderen functionele obstipatie en 17% had buikpijn (hoofdstuk 3.3). Slechts 4% had symptomen van zowel functionele obstipatie als buikpijn. Het cortisol dagritme verschilde niet significant tussen kinderen met en kinderen zonder functionele obstipatie en buikpijn (hoofdstuk 3.3). Cortisol stress-reactiviteit was iets hoger bij kinderen met buikpijn dan die zonder, maar dit was statistisch niet significant. Er werd geen verband gevonden tussen de cortisol stress-reactiviteit en functionele obstipatie (hoofdstuk 3.3). Onze resultaten suggereren dat cortisol als een marker voor stress geen belangrijke rol speelt bij functionele obstipatie of buikpijn op jonge leeftijd.

In hoofdstuk 4 bestuderen we de associaties tussen slaapproblemen en internaliserende problemen op jonge leeftijd, alsook de relatie van het cortisol dagritme met slaapduur en slaapproblemen bij peuters en kleuters. Dyssomnie en ouderlijke aanwezigheid tijdens het inslapen op de leeftijd van 2 maanden en 24 maanden werden in verband gebracht met angst of depressieve symptomen na 3 jaar (hoofdstuk 4.1). Parasomnia, korte slaapduur, en de afwezigheid van een vaste bedtijd voor het slapen bij 24 maanden, maar niet bij 2 maanden, werden ook voorafgegaan aan angst of depressieve klachten (hoofdstuk 4.1). Deze significante associaties waren niet te wijten aan angst van of depressieve symptomen van kinderen op de leeftijd van 18 maanden. Slaapritme en samen slapen waren niet geassocieerd met latere angst of depressieve symptomen. Aanvullende analyses gaven weinig bewijs voor een bi-directionele associatie van angst of depressieve symptomen voorafgaande aan latere slaapproblemen (hoofdstuk 4.1). Onze bevindingen benadrukken het belang van slaapproblemen vroeg in het leven, omdat verschillende slaapproblemen gerelateerd zijn aan de frequentie van angst en depressieve symptomen. Verder hebben we aangetoond dat de cortisol slope en de CAR, maar niet de AUC, waren geassocieerd met de slaapduur gedurende de kindertijd (hoofdstuk 4.2). Kinderen met een minder steile cortisol slopes en kinderen met een meer positieve CAR hadden meer kans op een kortere slaapduur. De verschillende samengestelde cortisol maten voorspelden geen slaapproblemen (hoofdstuk 4.2). Deze resultaten suggereren dat een minder steile cortisol slope en een meer uitgesproken stijging van cortisol in de ochtend, wat kan duiden op stress (of HPA-as ontregeling), lange-termijn effecten op de slaap regulatie kan hebben.

In hoofdstuk 5 worden de belangrijkste bevindingen van de studies in dit proefschrift samengevat en worden de methodologische aspecten met betrekking tot epidemiologisch cortisol onderzoek bij kinderen besproken. Het hoofdstuk wordt afgesloten met mogelijke klinische implicaties en suggesties voor toekomstige studies.

CHAPTER 7

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Dankwoord

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PhD Portfolio

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ABOUT THE AUTHOR

Nathalie Siti Saridjan was born on May 25th 1978 in Paramaribo, Surinam. She grew up in Hengelo (the Netherlands) and in 1996 obtained her Gymnasium degree from Lyceum de Grundel in the same city. Unfortunately she was unable to start medical school the same year due to the then applicable lottery system ("numerus fixus"). Instead, she started the study Biomedical Sciences at the Radboud University in Nijmegen (then known as the Catholic University Nijmegen) in 1996. In 1998 she was also allowed to start medical school at the same university. In 2000 she obtained her Master's degree in Biomedical Sciences, minor Pharmacology and major Toxicology, after research internships at the Netherlands Cancer Institute in Amsterdam and at the University of California San Francisco (UCSF) affiliated Ernest Gallo Clinic and Research Center in the Bay Area. In 2005 she obtained her medical degree. As part of her internships, she worked as a trainee at the paediatrician department at the Diakonessenhuis in Paramaribo, Surinam. Her first job was as MD at the Stichting Kinder- en Jeugdpsychiatrie Oost Nederland (SKJPON, now part of Karakter Child and Adolescent Psychiatry) in Nijmegen and Apeldoorn, were she worked under supervision of prof.dr. RJ. van der Gaag and drs. J. Visser. She then worked as an MD at the paediatrician department of the Canisius-Wilhelmina Ziekenhuis in Nijmegen for almost a year and a half. In January 2007, she started the work described in this thesis under supervision of Prof.dr. H. Tiemeier and Prof. dr. F.C. Verhulst. In September 2009, she started her residency in psychiatry at UMC Utrecht under supervision of Prof.dr. R. Kahn, and later dr. J. Wijkstra. In 2014 she received her degree as child and adolescent psychiatrist and started working at the outpatient clinic at Karakter Child and Adolescent Psychiatry in Almelo.
Nathalie is married to Bas Braamhaar since July 7th 2007 and they are happy that since September 2014 they live closer to their relatives in Twente.

PHD PORTFOLIO SUMMARY

Summary of PhD training and teaching activities

Name PhD student: Nathalie S. Saridjan Erasmus MC Department: Child & Adolescent Psychiatry/Psychology	PhD period: Jan 2007- Dec 2015 Promotors: Prof.dr. F.C. Verhulst Prof.dr. H. Tiemeier

1. PhD training		
	Year	**ECTS**
General academic skills		
- Biomedical English Writing and Communication	2008	4
Research skills		
- Statistics	2007/2008	14.2
- Methodology	2007	0.7
(Inter)national conferences		
- Dutch Psychiatry Conference (NVvP voorjaarscongres), Amsterdam, the Netherlands *Oral presentation*	2008	0.3
- International Society of Psychoneuroendocrinology (ISPNE), Dresden, Germany *Oral presentation*	2008	1
- International Federation of Psychiatric Epidemiology (IFPE), Vienna, Austria, 2009 *Oral presentation*	2009	1
- International Society of Psychoneuroendocrinology (ISPNE), Leiden, the Netherlands *Oral presentation*	2013	0.8
Seminars and workshops		
- 2nd DGPA Spring School, Dresden, Germany *Poster presentation*	2009	1
2. Teaching activities		
	Year	**ECTS**
Teaching		
- Vaardigheidsonderwijs 'VO.3: Ontwikkeling van 0-18 jaar' medical students, Erasmus MC, Rotterdam	2008/2009	1
Supervising Master's thesis		
- "The association between the circadian rhythm of salivary cortisol and sleeping and crying behaviour at 14 months", Fatima Khan, psychology student, Erasmus University, Rotterdam	2008	2
Other		
- Teaching medical students and interns during residency psychiatry at UMC Utrecht	2009-2014	10

Note: 1 ECTS (European Credit Transfer System) is equal to a workload of 28 hours.