THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS IN PRETERM BIRTH: SHORT- AND LONG-TERM CORRELATES

Bibian van der Voorn

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It is easier to build strong children than to repair broken men.
Frederick Douglass

Dedicated to
Prof. Dr. Thomas Addison (1793 – 1860) and
Prof. Dr. David J.P. Barker (1938 – 2013)

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ABSTRACT

Very preterm (i.e., <32 wks of gestation) infants admitted to the neonatal intensive care unit are compromised in their abilities to respond adequately to common threats like hemodynamic changes and reduced energy supplies, which is partly attributable to adrenocortical insufficiency. Conversely, later in life, these infants show features of increased glucocorticoid bioactivity, such as abdominal fat distribution, raised blood pressure, insulin resistance and diabetes mellitus type 2. It has been suggested that the very preterm newborn responds to the adverse postnatal environment with a sustained elevation in HPA axis activity that persists beyond infancy. This has implications for subsequent growth, body composition, metabolism, neurodevelopment and, ultimately, long-term disease risk. The mechanisms underpinning these associations are not fully elucidated yet.

This review gives a brief summary of studies that investigated adrenocortical function in very preterm newborns and how the axis changes with age, as a possible explanation for the association between prematurity and long-term outcome.

INTRODUCTION

The 'fetal cortisol hypothesis' was postulated in 1993 as an explanation for the association between low birth weight and certain chronic diseases, such as cardiovascular diseases and diabetes mellitus type 2 (DM2) [1]. In brief, it was hypothesized that abnormally low activity of the placental barrier enzyme 11βHSD type 2 allows a larger proportion of maternal cortisol to reach the fetus, resulting in IUGR, increased HPA axis activity and predisposition to hypertension and DM2. In support of this hypothesis, in a variety of animal models, birth weight was reduced by prenatal exposure to dexamethasone, which escapes inactivation by 11βHSD type 2, or carbenoxolone, which inhibits 11βHSD type 2 [2]. The offspring exhibited persistent increases in HPA axis activity, blood pressure, glucose intolerance and anxiety-like behavior [2]. In line with animal experiments, studies in humans showed that prenatal exposure to maternal anxiety or depression, by exposure of the fetus to excess glucocorticoids, was associated with a similar phenotype [2]. Furthermore, children born to mothers who regularly consumed liquorice – a potent inhibitor of 11βHSD type 2 – during their pregnancies had greater HPA axis activity, reduced performance at several cognitive tasks and more externalizing behavior [3,4].

Reduced 11βHSD type 2 activity has also been implicated to play a role in the length of gestation. Two independent studies found that heavy use of liquorice was associated with shorter gestation [5,6]. Others found that the expression and activity of 11βHSD type 2 were reduced in the placentas of mothers with known risk factors for preterm delivery, such as preeclampsia and IUGR [7,8].

In healthy pregnancies, the fetal HPA axis is still immature in the third trimester, and in pathological pregnancies this may even be more evident because of the suppressive effects of excess maternal cortisol [9]. Therefore, it is of no surprise that the HPA axis is not fully functional among preterm infants in their first weeks of life; a proportion of them manifest clinical signs of adrenocortical insufficiency [10]. Conversely, later in life, survivors show features of increased glucocorticoid bioactivity, such as abdominal fat distribution, raised blood pressure, insulin resistance and DM2 [11-15]. This poses the question as to whether the HPA axis could also be programmed postnatally by adversities associated with preterm birth.

GLUCOCORTICOIDS AND RESILIENCE TO NEONATAL THREATS

Worldwide, of all live-born children, 11.1% (range: 5–18%) are born prematurely (i.e., <37 wks of gestation) [16]. Approximately 1–2% of all babies are born very preterm (i.e., <32 wks) [17,18], necessitating admission to a NICU. In this review, we will focus specifically on the latter group. Although pregnancy dating can be reliably assessed with ultrasound these days, birth weight is still being used as a surrogate for gestational age by many studies. Therefore, we also included studies in infants with very low birth weight (VLBW; i.e., <1,500 g) or extremely low birth weight (ELBW; i.e., <1,000 g).

Owing to improvements in neonatal care, the chances for survival of infants born between 26 and 32 wks of gestation have improved from 70% in the early 1980s to more than 90% nowadays [19]. The limit of viability has been set at 22–24 wks. Overall, infants born at the threshold of viability experience a more complicated neonatal course and, consequently, carry much higher risks of neonatal mortality and long-term morbidities [20].

Cortisol is necessary for the maintenance of both blood pressure and glucose homeostasis. It influences the sensitivity of the peripheral tissues to the actions of insulin, glucagon and catecholamines. Other mechanisms of action include inhibition of nitric oxide-induced vasodilatation, inhibition of vasodilator prostanoids, upregulation of angiotensin II receptors and, at higher molar concentrations, activation of renal mineralocorticoid receptors [21].

Hypotension is a common threat during the early postnatal course of very preterm infants. Among the causes of hypotension are abnormalities in the regulation of the vascular tone (e.g., by sepsis or adrenocortical insufficiency), left-to-right shunting through a persistent ductus arteriosus, volume depletion and myocardial dysfunction [22,23]. Although systemic hypotension has been associated with adverse neurological outcome, it is unclear whether low blood pressure alone, in the absence of other signs of hemodynamic instability, is harmful for the very preterm infant's brain [22,23].

Hypoglycemic episodes are frequently observed in the early postnatal course of very preterm infants. Acute illnesses, such as the respiratory distress syndrome, infections and necrotizing enterocolitis, and asphyxia increase the glucose demands in tissues. The endogenous glucose production can only partly compensate for sudden declines in the circulating glucose level [24], attributable to low hepatic glycogen content [25], poor availability of the gluconeogenic substrates alanine and glycerol [26], impaired glucose-6-phosphatase activity (the final step in both glycogenolysis and gluconeogenesis) [27] and a reduced capacity to secrete counter-regulatory hormones like cortisol [28,29]. Also lipolysis and ketogenesis are severely impaired, even at low blood glucose levels [26,30].

Relative adrenal insufficiency is common among very preterm infants in their first weeks of life and occurs when the HPA axis is unable to produce sufficient cortisol for the degree of illness. However, there is lack of consensus about the definition of a normal cortisol value. A cortisol level ≥15 μg/dL (i.e., ≥414 nmol/L) has been considered adequate for ill very preterm infants [29]. According to this definition, a considerable proportion fail to mount an adequate adrenocortical response to stress (**table 1**). Still, their cortisol levels are higher than in healthy fetuses of the same postconceptional age [31]. Infants with ELBW who had a cortisol level in the upper quartile at postnatal days 5–7 were found to be at risk of brain damage [32].

Multiple levels along the HPA axis might be affected in infants born preterm (**table 1**). First, several studies demonstrated that the pituitary response to exogenous CRH was impaired [33-36] when an adrenocorticotropin concentration of 9 pmol/L or more was considered as adequate [37], although this was not a universal finding [38,39]. Second, many studies showed that adrenal cortex enzymes were immature, and decreased 11β-hydroxylase activity was suggested to be the most important rate-limiting step [28,29,40-42]. Third, one study suggested that the interconversion between cortisol and cortisone favored cortisone with decreasing gestational age [43].

In infants born very preterm or with VLBW who experienced vasopressor-resistant hypotension, hydrocortisone or dexamethasone successfully enabled discontinuation of inotropics [44,45]. Prophylactic glucocorticoids for prevention of hypotension were shown to be effective too in extremely preterm infants (i.e., <28 wks of gestation) [46].

In infants born very preterm or with VLBW who experienced vasopressor-resistant hypotension, hydrocortisone or dexamethasone successfully enabled discontinuation of inotropics [44,45]. Prophylactic glucocorticoids for prevention of hypotension were shown to be effective too in extremely preterm infants (i.e., <28 wks of gestation) [46].

Similarly, extremely preterm infants exposed prenatally to synthetic glucocorticoids for fetal lung maturation had a lower likelihood of becoming hypotensive in the first days after birth [47]. In addition, in a study among preterm infants, proinsulin, insulin and C-peptide levels in cord blood remained elevated up to 48 h after the last steroid dose, in spite of a normal glucose concentration, suggestive of insulin resistance [48]. This might offer protection against neuroglycopenia. Consistent with these observations, the glucocorticoid bioactivity of a single treatment course of betamethasone was found to wear off 1–2 days after the last steroid dose [49].

From these data, it could be inferred that increased glucocorticoid bioactivity offers short-term benefits. Among infants with VLBW, the adrenocortical response to exogenous CRH rose significantly between postnatal days 7 and 14 [50]. The improvements were more marked for the group with hypotension necessitating inotropic treatment. The infants within it were also more premature and exhibited greater disease severity scores, suggesting that postnatal adversities could lead to

a sustained increase in the response to CRH. It has not been tested whether such phenomena, which are probably adaptive in nature, persist beyond the early postnatal period and, subsequently, become maladaptive.

DEVELOPMENTAL TRAJECTORIES IN VERY PRETERM SURVIVORS BEYOND THE NEONATAL PHASE: FOOTPRINTS OF INCREASED HPA AXIS ACTIVITY?

After an initial weight loss, birth weight is usually regained between the end of the 1st week and the 3rd week of life. Once birth weight is regained, the growth velocity increases to a level that approaches the intrauterine growth rate [51]. However, the rate of weight gain during hospital stay was shown to be slower in sick infants [51], which could be explained by suboptimal protein and calorie supplies for the level of illness.

After clinical improvement, CUG in weight and length is initiated. At term age, while still being smaller and lighter, absolute fat mass and abdominal fat content were greater than in full-term newborns [52]. Although the major part of CUG is completed by the age of 4 yrs, continuing CUG in height, weight and BMI was observed in late childhood and adolescence, albeit at a slower pace [51]. On average, in subjects born very preterm or with VLBW, final height was reduced by 0.5 SD compared to population-specific reference data [51]. Young adults born preterm or with VLBW, despite having a relatively normal BMI, exhibited a lower lean mass, increased fat content and centralization of fat distribution [11,14,53,54].

It is unlikely that the reduction in final height could be explained by an earlier onset or a more rapid progression of puberty. In cross-sectional studies of children aged 11–15 yrs, no differences in Tanner pubertal stages were demonstrated between boys and girls with VLBW and term-born children of the same age [55,56]. Moreover, girls with VLBW reported a similar age at menarche as their term counterparts [55-57]. However, very preterm birth and/or VLBW were associated with an earlier pubertal growth spurt [57], a more advanced bone age at adolescence [55,56] and adrenal hyperandrogenism in young adulthood [58,59].

In very preterm survivors, antecedents of the metabolic syndrome were already present in childhood. Very preterm birth was associated with a relatively high blood pressure at 7.5 yrs of age, irrespective of the presence of nephrocalcinosis [60]. Furthermore, insulin sensitivity during an intravenous glucose tolerance test was reduced 4–10 yrs after very preterm birth. This was accompanied by a compensatory increase in acute insulin release [61].

Children born very preterm or with a VLBW were found to display more internalizing problem behavior and attention problems as well as reduced scores on various cognitive tests [62]. Furthermore, imaging studies showed that very preterm birth and VLBW were associated with permanent reductions in, among other brain structures, hippocampal volume [63]. These patterns resemble those of term-born children who were exposed antenatally to higher maternal cortisol levels [64,65].

Findings from studies addressing the long-term effect of prematurity on several aspects of HPA axis activity were contradictory (**table 2**). One study found that basal cortisol was higher in adults born preterm than in normal controls [58]. Another study found that this was already evident from the corrected age of 8 mo. for children born in the extremely preterm range [66]. Evidence for sex-specific effects was provided by Walker et al. [67], who compared adults born preterm, with or without IUGR, with normal controls. In women, plasma cortisol was not different, but the total urinary cortisol metabolite excretion was lower in the preterm non-IUGR group than in the other 2 groups, suggestive of impaired metabolic clearance. In contrast, no differences in cortisol parameters were observed in men. Yet another study found that ELBW was associated with higher excretion rates of adrenal steroid metabolites at age 10 yrs [68]. Two out of 4 studies that assessed the cortisol response to psychosocial stress found that preterm birth was associated with blunted rather than enhanced responses [69-72]. Preterm birth was also associated with a higher salivary cortisol at bedtime [72] or over 24h [73], and with a greater CAR in childhood [69]. Furthermore, very preterm carriers of the 23K variant of the R23K polymorphism in the glucocorticoid receptor (*GR*) gene, which is associated with resistance to cortisol, were found to display complete CUG before the age of 1 yr, while in the noncarriers, the stature remained around the –0.5 SD line [74].

In summary, these observations suggest that very preterm survivors show features of increased HPA axis activity that persist beyond the neonatal phase and extend into adulthood, although some studies were negative, and others, especially the ones that assessed the cortisol response to psychosocial stress, reported the opposite results. Signs of increased glucocorticoid bioactivity, such as an unfavorable body composition, raised blood pressure and insulin resistance, are already present in childhood [52,60,61]. Apparently, there is a large increment in the adrenal androgen production after an age-appropriate adrenarche, contributing to an earlier initiation of the pubertal growth spurt and advancement of bone maturation at adolescent age and, hence, earlier epiphyseal closure and shorter adult stature. The growth pattern of subjects born very preterm suggests that the growth-inhibiting actions of cortisol are outweighed by the anabolic effects of excess insulin and androgen levels.

TABLE 1. Summary of studies that have assessed the HPA axis in preterm infants

First author [Ref.], year	n	Characteristics of participants	Age at assessment	Measurements	Group	Cortisol (nmol/L)		ACTH (pmol/L)		Other findings
						basal	peak	basal	peak	
No stimulation test										
Doerr [40], 1988	20	Preterm group: 33–36 wks, healthy (n = 8) Term group: healthy (n = 12)	2, 6, 12 and 24 h, 4 and 7 days	Basal cortisol, aldosterone, 17-OHP, progesterone, S, DOC, B and E	Preterm	Between 87±29[a] at 7 days and 267±107[a] at 12 h	n.a.	–	n.a.	↑ 17-OHP, aldosterone and B during first week of life in preterm compared to term group
					Term	Between 75±33[a] at 24 h and 288±71[a] at 2 h	n.a.	–	n.a.	
Lee [41], 1989	38	Preterm group: 31–35 wks, sick (n = 9) and healthy (n = 13) Term group: healthy (n = 16)	2–5 days	Basal cortisol, aldosterone, 17-OHP, 17-OH pregnenolone, S, 18-OH corticosterone, DHEA, DHEAS and androstenedione	Preterm sick	165±25[a]	n.a.	–	n.a.	↑ 17-OHP, S and aldosterone in preterm sick compared to preterm healthy group ↑ 17-OHP, 17-OH pregnenolone and DHEAS in preterm compared to term group
					Preterm healthy	190±29[a]	n.a.	–	n.a.	
					Term	171±27[a]	n.a.	–	n.a.	
Nykanen [101], 2010	67	23.6–33.1 wks, sick Group 1: <28 wks (n = 27) Group 2: ≥28 wks (n = 40)	0 and 4 days	Basal cortisol, 17-OHP, 17-OH pregnenolone, S and DHEAS	0 days	452 (61–2,704)[b]	n.a.	–	n.a.	↑ 17-OH pregnenolone and DHEAS but similar precursor ratios in group 1 compared to group 2
					4 days	277 (63–1,647)[b]	n.a.	–	n.a.	↑ cortisol, 17-OHP, 17-OH pregnenolone and DHEAS but similar precursor ratios in group 1 compared to group 2
ACTH test										
Thomas [42], 1986	52	Preterm group: 28–36 wks, sick (n = 26) and healthy (n = 15) Term group: sick (n = 11)	3–4 days	Basal and ACTH-stimulated (36 µg/kg i.m.) cortisol and 17-OHP	Preterm sick	537±94[a]	996±136[a]	–	n.a.	↑ peak 17-OHP in preterm sick compared to preterm healthy group ↑ basal 17-OHP in preterm compared to term group
					Preterm healthy	306±45[a]	631±78[a]	–	n.a.	
					Term	372±157[a]	645±101[a]	–	n.a.	
Hingre [28], 1994	25	<30 wks, sick	4 days	Basal and ACTH-stimulated (36 µg/kg) cortisol, 17-OHP, 17-OH pregnenolone and S	–	207±24[a]	–	–	n.a.	↑ cortisol precursors and S/cortisol ratio but similar cortisol compared to term reference
Kari [102], 1996	23	<30 wks, sick	13, 21 and 30 days	Basal cortisol, DHEAS, CBG and SHBG, and ACTH-stimulated (145 µg/m^2) cortisol	Dexamethasone, 13 days[g]	125[c]	607[c]	–	n.a.	–
					Placebo, 13 days	119[c]	545[c]	–	n.a.	
					Placebo, 21 days	176[c]	817[c]	–	n.a.	
					Placebo, 30 days	181[c]	1,127[c]	–	n.a.	
Korte [29], 1996	67	<32 wks, sick	2–3 days	Basal and ACTH-stimulated (0.1 or 0.2 µg/kg) cortisol, ACTH, DHEA, S and CBG	–	<414 in 76% of cases	≥414 in 36% of cases after ACTH 0.1 µg/kg and in 67% of cases after ACTH 0.2 µg/kg	range: 0–24	n.a.	↑ S/cortisol ratio when basal cortisol <414

Huysman [103], 2000	21	<30 wks, sick and healthy	4 days	Basal cortisol and 17-OHP, and ACTH-stimulated (0.5 µg) cortisol	–	277±144[d]	753±250[d]	–	n.a.	↓ cortisol and ↑ 17-OHP/cortisol ratio in ventilated compared to nonventilated infants
Bolt [43], 2002	24	<33 wks, healthy Group 1: <30 wks (n = 13) Group 2: 30–33 wks (n = 11)	5–10 days	Basal cortisol, 17-OHP and E, and ACTH-stimulated (1 µg/kg) cortisol and 17-OHP	Group 1	178±15[a]	515±33[a]	–	n.a.	↑ E and 17-OHP/cortisol ratio in group 1 compared to group 2
					Group 2	250±42[a]	733±89[a]			
Kajantie [9], 2006	44	<1,000 g, sick and healthy	1.6±1.1[d] days	Basal and ACTH-stimulated (0.06 µg/kg) cortisol	–	119 (55–300)[e]	261 (176–456)[e]	–	n.a.	–
CRH test										
Rizvi [35], 1992	10	27.3±5[d] wks, sick	7.5±9.4[d] days	Basal and CRH-stimulated (1 µg/kg) cortisol and ACTH	[g]	275[f]	No rise in 3/10 cases	–	No rise in 3/10 cases	–
Ng [38], 1997	14	<32 wks, healthy	7 and 14 days	Basal and CRH-stimulated (1 µg/kg) cortisol and ACTH	–	396±67[a]	647±62[a]	5.7±0.6[a]	11.9±2.1[a]	–
Bolt [36], 2002	29	<32 wks, healthy	7–21 days	Study 1 (n = 13): basal and CRH-stimulated (1 µg/kg) cortisol and ACTH Study 2 (n = 16): basal and CRH-stimulated (1 µg/kg vs. CRH 2 µg/kg vs. placebo) cortisol and ACTH	Study 1	350±115[d]	582±201[d]	6.9±2.1[d]	11.6±5.1[d] (<9 in 5/13 cases)	–
					Study 2: CRH 1 µg/kg	256±120[d]	509±167[d]	6.1±4.0[d]	10.5±4.4[d] (<9 in 2/5 cases)	
					Study 2: CRH 2 µg/kg	330±154[d]	815±212[d]	4.5±1.5[d]	11.3±4.3[d] (<9 in 2/6 cases)	
Ng [39], 2002	137	<1,500 g, sick and healthy	7 and 14 days	Basal and CRH-stimulated (1 µg/kg) cortisol and ACTH	7 days[g]	286[f]	513[f]	5.5[f]	10.4[f]	–
					14 days[g]	237[f]	509[f]	7.1[f]	12.7[f]	
Multiple stimulation tests										
Hanna [34], 1993	17	26.1 wks, sick	2–7 days	Basal and CRH-stimulated (1 µg/kg) cortisol and ACTH, and ACTH-stimulated cortisol	CRH test	349±58[a]	569±60[a]	6.0±1.8[a]	9.6±1.8[a]	–
					ACTH test	604±131[a,h]	883±137[a]	–	–	
Ford [33], 1997	9	25.7±1.0[d] wks, sick	19.9±8.0[d] days	Basal cortisol and ACTH; cortisol, ACTH and S after metyrapone 40 mg/kg; ACTH-stimulated (40 µg/kg) cortisol; CRH-stimulated (1 µg/kg) cortisol and ACTH	[g]	513±108[a] (<414 in 2/9 cases)	684±48[a] after ACTH	–	5.0±1.7[a] after CRH	S >7.0 µg/dL after metyrapone in 9/9 cases

17-OHP = 17-Hydroxyprogesterone; ACTH = adrenocorticotropin; B = corticosterone; CBG = cortisol-binding globulin; DOC = deoxycorticosterone; DHEA(S) = dehydroepiandrosterone (sulphate); E = cortisone; n.a. = not applicable; S = 11-deoxycortisol. [a] Mean ± SEM; [b] median (range); [c] mean; [d] mean ± SD; [e] geometric mean (interquartile range); [f] median; [g] data of infants without, or prior to, dexamethasone treatment; [h] the ACTH test was conducted 2 h after the CRH test; therefore, at the start of the ACTH test the basal cortisol level was already 604±131 nmol/L.

EXPLORING THE RELATION BETWEEN PREMATURITY AND INCREASED HPA AXIS ACTIVITY IN LATER LIFE

ANTENATAL GLUCOCORTICOID THERAPY

There is no doubt that administrating glucocorticoids to mothers with impending preterm delivery is highly efficacious for the prevention of the respiratory distress syndrome and associated complications [75]. Although numerous animal studies in various species showed that administration of synthetic glucocorticoids throughout, or during part of, gestation causes permanent metabolic perturbations, neurobehavioral alterations and dysregulation of the HPA axis in offspring [2], it remains unclear whether a single treatment course of antenatal glucocorticoids could explain at least a part of the association between preterm birth and long-term outcome. First, associations between preterm birth and DM2 were also reported in middle-aged populations born before the introduction of antenatal glucocorticoid therapy [13,15]. Second, findings from studies investigating long-term outcome after antenatal glucocorticoid therapy were highly contradictory [76-81]. However, some of these studies were restricted to infants born very preterm or with VLBW [79-81], whereas others had followed all infants, regardless of whether they were born preterm or not [76-78].

POSTNATAL GLUCOCORTICOID THERAPY

Dexamethasone has been given to prevent or treat BPD. Compared to antenatal therapy, postnatal glucocorticoids are administered for a longer time (e.g., 1–3 wks), resulting in substantially higher cumulative doses. Although dexamethasone during the first week of life was effective in the prevention of BPD, the risk of adverse outcomes, including poor growth and neurodevelopmental impairment, was increased, and therefore this therapy is not recommended [82]. However, dexamethasone could be considered after the 1st week of life to infants who cannot be weaned from the ventilator, provided that the dose and duration are kept to a minimum [83,84]. Hydrocortisone may be as effective as dexamethasone in the prevention and treatment of BPD, and it has fewer side effects, though long-term follow-up data from randomized trials are lacking [83,85]. Observational data, however, suggest that dexamethasone, when compared to hydrocortisone, for the facilitation of extubation was associated with an increased risk of adverse neurodevelopmental outcome and with reductions in cardiovascular and adrenal responses during a psychosocial stress test [86-88].

TABLE 2. SUMMARY OF STUDIES THAT HAVE ASSESSED THE HPA AXIS IN CHILDREN AND ADULTS BORN PRETERM

First author [Ref.], year	n	Characteristics of participants	Age at assessment	Measurements	Main findings
Szathmari [58], 2001	70	LBW: BW 900–2,500 g and GA ≤36 wks Controls: BW >2,500 g and GA ≥38 wks	~20 yrs	Blood specimen obtained at 7.30h after overnight fasting for determination of cortisol and androgens	♂ Higher cortisol in LBW group ♀ Higher cortisol, DHEA, DHEAS and androstenedione in LBW group
Walker [67], 2002	52	Preterm AGA: BW <2,000 g and >10th centile; mean GA 32 wks Preterm IUGR: BW <2,000 g and <10th centile; mean GA 35 wks Controls: BW >2,000 g; mean GA 39 wks	22–25 yrs	Plasma cortisol at 9.00 h after overnight fasting, 24-hour urinary cortisol metabolite excretion	♂ No differences between groups ♀ Lower cortisol metabolite excretion in preterm AGA women and similar plasma cortisol, as compared to the other 2 groups
Wust [71], 2005	102	Male twins with GA 33–43 wks and BW of 1,400–4,200 g	18.57±0.23 yrs	Salivary cortisol response during TSST	Higher cortisol response during TSST with decreasing BW and with increasing GA
Buske-Kirschbaum [69], 2007	36	Preterm: GA 26–36 wks Controls: GA 39–41 wks	8–14 yrs	Salivary cortisol response during TSST, cortisol awakening response, diurnal salivary cortisol pattern	Higher cortisol after awakening in preterm group No differences in other outcomes between groups
Grunau [66], 2007	225	ELGA: GA 23–28 wks VLGA: GA 29–32 wks Controls: GA 37–42 wks	3, 6, 8 and 18 mo. corrected age	Basal salivary cortisol	Higher cortisol at 8 and 18 months in ELGA group
Kaseva [70], 2014	94	VLBW: BW <1,500 g and GA 24–36 wks Controls: GA 38–42 wks	19–27 yrs	Salivary cortisol, and plasma ACTH, cortisol, glucose and insulin during TSST	Lower cortisol and insulin responses during TSST in VLBW group
De Graaf [73], 2014	79	NICU-treated: GA 30±3.1 wks Controls: GA 40±1.1 wks	5 yrs	Diurnal salivary cortisol pattern	Higher cortisol over 24 h in NICU-treated group
Brummelte [72], 2015	129	Preterm: GA 24–32 wks Controls: GA 38–41 wks	7 yrs	Salivary cortisol during cognitive tests, diurnal salivary cortisol pattern	Higher bedtime cortisol in preterm group No difference in cortisol during cognitive tests between groups
Gohlke [68], 2015	54	ELBW Term controls	8–11 yrs	24-hour urinary corticosteroid metabolite excretion	Higher corticosteroid metabolite excretion in ELBW group

AGA = Appropriate for gestational age; BW = birth weight; ELGA = extremely low gestational age; GA = gestational age; LBW = low birth weight; TSST = Trier Social Stress Test; VLGA = very low gestational age; ACTH = adrenocorticotropin.

EARLY POSTNATAL CARE AND STRESS

In animals, offspring subjected to maternal separation, or nonhandling, in early life exhibited dysregulation of the HPA axis, impaired cognitive capabilities, increased anxiety-like behavior and alterations in limbic structures [89]. Similarly, rat mothers that engaged in low amounts of licking and grooming with their pups had offspring that, as adults, were more responsive to stress [90]. This was associated with persistent alterations in DNA methylation and histone acetylation at the hippocampal GR 1_7 promotor, affecting nerve growth factor 1-A binding [90].

In humans, those who had experienced poor quality of parental care, such as neglect or emotional or physical maltreatment, early in childhood showed greater HPA axis activity and were at risk for mental illnesses, cardiovascular diseases and diabetes mellitus [91]. Furthermore, a study in hippocampal tissue from suicide victims showed that childhood abuse was associated with increased DNA methylation at the GR promotor and decreased expression of GR mRNA [92]. It is unknown whether these observations could be extrapolated to preterm babies, who are separated from their mothers after birth and, instead, are exposed to the stressful environment of the NICU. During admission to the NICU, exposure to stressors like invasive procedures, pain, interruption of sleep states and noise is common. Neonatal procedural pain-related stress after very preterm birth, however, has been associated with indices of cortisol production and HPA axis reactivity [72,93,94].

NEONATAL NUTRITION

There is increasing evidence to suggest that metabolic signals modify the HPA response to maternal separation [95]. In suckling mice, along with an increase in HPA axis activity, maternal separation elicited alterations in glucose, leptin and ghrelin concentrations [96]. Pharmacological manipulation of glucose or ghrelin levels attenuated the HPA response to maternal separation [96]. As adults, offspring subjected to prenatal or early postnatal malnutrition displayed greater HPA axis activity [95].

Current nutritional recommendations for preterm newborns advocate early introduction and rapid advancement of protein and energy [97]. Evidence from randomized trials and observational studies showed that strategies providing early, increased energy and protein support reduce nutritional deficits and improve neonatal growth and neurodevelopmental outcome [97]. In the intervention groups, amino acids and lipids were initiated early and rapidly increased to 3.5 – 4 g/kg per day. Nevertheless, in clinical practice, nutritional goals are rarely achieved and nutritional deficits ensue [98]. It is unknown whether this has life-long effects on the preterm human's HPA axis.

ENDOGENOUS GLUCOCORTICOID SENSITIVITY

In the challenging situation of very preterm birth, carriage of (sets of) genes that promote the response to vasopressors and/or the release of stored fuels may be beneficial. Indeed, there are some preliminary data to suggest that GR variants influencing cortisol sensitivity are related to the presence of several neonatal morbidities in babies with VLBW, albeit not consistently [99]. On the other hand, later in life, traits rendering increased glucocorticoid signaling were suggested to exacerbate the impact of antenatal glucocorticoids on the risk of developing adverse outcomes after very preterm birth, such as metabolic perturbations and poorer cognitive capabilities [81,100].

CONCLUSIONS

Glucocorticoids, given their impact on blood pressure regulation and energy homeostasis, offer resilience to potentially life-threatening conditions after very preterm birth. Adaptation of HPA axis activity to the increased requirements of unintended postnatal life seems to be an attractive coping mechanism. It is suggested that once the burdens of neonatal life are no longer present, the HPA axis continues to be upregulated, leading to alterations in growth and developmental pathways, and, ultimately, to deleterious health consequences.

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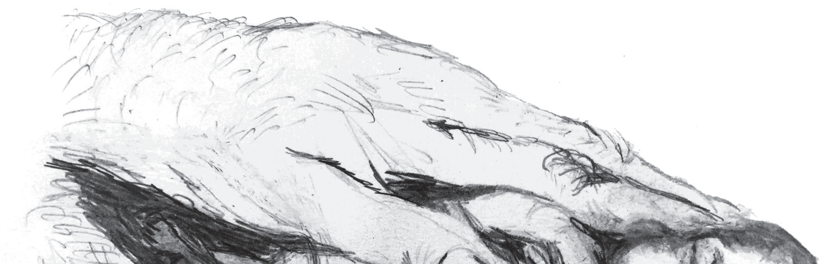

CHAPTER 2

AIM, DESIGN AND OUTLINE OF THIS THESIS

AIM

In the Netherlands in 2015, 168,773 infants were born at a gestational age (GA) of ≥ 24 wks, of whom 1,882 neonates (1.1%) were born very preterm (GA ≤ 32 wks) [1]. Worldwide, mortality rates due to prematurity and/or low birth weight are declining: in the United States these rates decreased from 4.9% to 4.2% between 2003 and 2013 [2]. Despite this improvement in survival, later in life very preterm survivors are facing problems of which some are suggestive of increased glucocorticoid bioactivity, such as an unfavorable fat distribution, raised blood pressure, insulin resistance, growth retardation, and neurodevelopmental impairments, as described in **Chapter 1**.
Although many studies described these associations, the underlying mechanisms remain to be elucidated. The aim of this thesis was therefore to explore factors, including genetic predisposition, gender, perinatal treatment and early-life feeding, that might affect HPA axis activity in very preterm newborns on the short-term and could predispose to adverse outcomes on the long-term.

DESIGN AND OUTLINE

PART I

Stress in Early Life

Assessment of HPA axis activity in very preterm newborns is challenging due to a variety of factors, including the limited availability of blood, the absence of a diurnal rhythm in the secretion of glucocorticoids, as well as immaturity of the adrenals resulting in the production of a variety of glucocorticoid metabolites that necessitates specialized assays. A matrix with the potential to minimize these difficulties is hair, although up till now unable to use for the quantification of glucocorticoid metabolites. Hair cortisol and cortisone levels reflect a longer period of time, i.e., 1 cm hair segment corresponds with 1 month of glucocorticoid exposure [3]. At this moment, studies relating infant hair glucocorticoid levels to clinical parameters are scarce [4,5] Nonetheless, measurement of hair cortisol and cortisone levels has the potential to give a retrospective view on HPA axis development in early life. In addition, neonatal hair glucocorticoid levels assessed directly postpartum might provide insights into fetal HPA axis activity and the placental transfer of maternal glucocorticoids.

In **Chapter 2,** we explored factors that influence infant hair glucocorticoid levels in early life, as well as their relation with prenatal, maternal HPA axis activity.
In **Chapter 3,** we assessed whether maternal stress experienced during gestation and/or peripartum was associated with neonatal and maternal hair glucocorticoid levels directly postpartum.

For both term and preterm neonates, human milk is recommended for its beneficial effect on growth, body composition, metabolism, neurodevelopment and long-term disease risk, all of which has been attributed to the nutritive and nonnutritive, bioactive components in human milk. Glucocorticoids are known to be present in mother's milk. Knowledge was lacking about the clinical range in which these human milk glucocorticoids were present, as well as inter- and intra-individual differences in concentrations. Therefore, the Cortisol in Mother's milk Study (COSMOS) was set-up with the aim to explore variations in human milk glucocorticoid concentrations.
We started the COSMOS project with the development of a reliable LC–MS/MS assay to determine cortisol and cortisone in human breast milk (**Chapter 4**), after which COSMOS was set up in two consecutive phases.
In COSMOS 1 (**Chapter 5**), we tested the hypothesis that human milk cortisol and cortisone concentrations during the first month postpartum were higher for mothers who delivered very prematurely (GA ≤ 32 wks) as compared to mothers who delivered at term. Subsequently, in COSMOS 2 (**Chapter 5**) we tested the hypothesis that breast-milk glucocorticoid concentrations follow a diurnal rhythm corresponding to the diurnal rhythm of the maternal HPA axis activity, by assessing multiple paired maternal salivary and breast-milk samples, collected over a 24h period by healthy lactating mothers who gave birth to a term infant.
In **Chapter 6**, we tested the effect of pasteurization on human milk glucocorticoid levels.

PART II

Later life consequences of early life stress

Adaptation to early life stress, such as very preterm birth, is suggested to result in a permanent increase in HPA axis activity with possible unfavorable disease risks later in life (Chapter 1). Moreover, gender differences in later life morbidity and mortality have been described and have been hypothesized to be partly attributable to a sexual dimorphism in HPA axis activity and reactivity. Although sex-specific differences in HPA axis activity have been postulated to emerge during puberty as a result of rises in sex steroids, already early in life a male disadvantage on the risk of premature birth [6],

as well as on short- and long-term morbidity and mortality after very preterm birth has been described [7-9].

To elucidate some potential mechanisms clarifying these associations, we assessed in **Chapter 7** on 19-year-old very preterm born subjects from the Project On Preterm and Small-for-gestational-age (POPS) birth cohort, whether antenatal glucocorticoid treatment and a genetic predisposition that influences sensitivity to glucocorticoids, were associated with neurocognitive impairments.
In **Chapter 8** we assessed HPA axis development from term age up till age 8 years by analyzing unstimulated cortisol levels in association with birth weight and postnatal growth, among children that were born very preterm and/or with a very low birth weight, i.e., <1500g (VLBW), who participated in the 'Study Towards the Effect of Postdischarge nutrition' (STEP).
In **Chapter 9** we conducted a systematic review and meta-analysis to assess whether sex-specific differences in unstimulated HPA axis activity are already present in early life in healthy children.
In **Chapter 10** we conducted a systematic review to assess whether sex-specific differences in early life in healthy children are also found in HPA axis reactivity, i.e., the response to either exogenous (e.g., pharmacological, physical or social) or endogenous stimuli (e.g., the cortisol awakening response), or as a result of diurnal rhythmicity.

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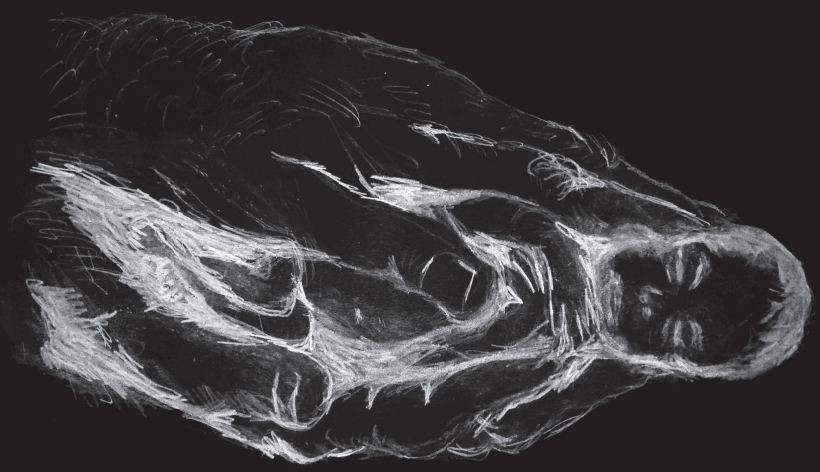
PART I
SHORT -TERM CORRELATES



CHAPTER 3

INTERPRETATION OF GLUCOCORTICOIDS IN NEONATAL HAIR: A REFLECTION OF INTRAUTERINE GLUCOCORTICOID REGULATION?

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ABSTRACT

BACKGROUND

Glucocorticoids (GCs) measured in neonatal hair might reflect intrauterine as well as postpartum GC regulation. We aimed to identify factors associated with neonatal hair GC levels in early life, and their correlation with maternal hair GCs.

METHODS

In a single-center observational study, mother-infant pairs (n=107) admitted for >72h at the maternity ward of a general hospital were included. At birth and an outpatient visit (OPV, n=72, 44±11 days postpartum), maternal and neonatal hair was analyzed for cortisol and cortisone levels by LC-MS/MS. Data were analyzed regarding: (1) neonatal GC levels postpartum and at the OPV, (2) associations of neonatal GC levels with maternal GC levels and (3) with other perinatal factors.

RESULTS

(1) Neonatal GC levels were >5 times higher than maternal levels, with a decrease of ±50% between birth and the OPV for cortisol. (2) Maternal and neonatal cortisol, but not cortisone, levels were correlated both postpartum and at the OPV. (3) Gestational age was associated with neonatal GCs postpartum (log-transformed β [95%CI]: cortisol 0.07 [0.04 to 0.10]; cortisone 0.04 [0.01 to 0.06]) and at the OPV (cortisol 0.08 [0.04 to 0.12]; cortisone 0.00 [-0.04 to 0.04]), while weaker associations were found between neonatal GCs and other perinatal and maternal factors.

CONCLUSIONS

Neonatal hair GCs mainly reflect the third trimester increase in cortisol, which might be caused by the positive feedback loop, a placenta-driven phenomenon, represented by the positive association with GA. Between birth and 1.5 months postpartum, neonatal hair cortisol concentrations decrease sharply, but still appear to reflect both intra- and extrauterine periods.

INTRODUCTION

Prenatal exposure to excessive glucocorticoids (GCs) has been associated with an increased risk of cardiovascular diseases and depressive disorders [1,2]. This might be due to permanent alterations in the settings of the fetal HPA axis, which are protective in the short term, but might pose a risk in the long term [3].

The development of the fetal HPA axis is, among other factors, influenced by the placental transfer of maternal GCs throughout pregnancy [4]. During early gestation, maternal GCs are the main supply. By the second half of gestation, the fetal adrenal starts producing its own steroids, predominantly sex steroids (which serve as a substrate for the placental production of estriol) and precursor GCs, since the adrenocortical enzymes are not fully matured yet [5]. Subsequently, during the last 6-8 wks of pregnancy, the more matured fetal adrenal produces increasing amounts of cortisol and cortisone under the control of CRH production in the placenta, which – in contrast to the negative feedback loop between cortisol and CRH under non-pregnant conditions – establishes a positive feedback loop [6]. This increase in cortisol concentration promotes maturation of the fetal lungs as well as of other organs [7].

Knowledge on the fetal HPA axis development is mainly based on animal studies [5,8], as it is difficult to measure fetal HPA axis activity in humans. Up till now, amniotic fluid GC levels and umbilical cord GC levels have been used to assess intrauterine GC regulation. Cortisol in amniotic fluid has previously been correlated with maternal cortisol levels [9] and onset of labor [10]. However, the source of amniotic fluid cortisol remains uncertain, although findings point toward fetal production [11,12]. In addition, sampling of amniotic fluid is a stressful occasion and only provides cross-sectional information. Alternatively, umbilical cord blood can be drawn non-invasively, but GC levels are influenced by delivery [13] and might not reflect normal intrauterine HPA axis activity. GCs measured in scalp hair might offer a solution, as it is used as a measure for HPA axis activity over time without the disturbing influence of the circadian rhythm. The hair GC concentrations reflect the exposure in the time frame during which the hair grows [14].

Maternal hair GC levels seem to reflect HPA axis activity during pregnancy [15-17]. Neonatal hair GC levels have also been associated with pre- and perinatal factors. A recent study by Hoffman et al. (2017) [18] has shown that gestational age as well as birth weight had a positive association with cortisol levels in neonatal hair. Neonatal hair GC levels were also significantly higher than maternal hair GC levels. This study suggests that features of the fetal adrenal development are represented in neonatal hair GC levels, although these findings are limited due to the fact that this has only

been described in one study population, cortisone levels were not taken into account and the course followed by GC levels in hair postpartum has not been studied.

Therefore, we aimed to describe cortisol and cortisone concentrations in neonatal hair, obtained directly postpartum, and their relation with maternal hair GC levels and pre- and perinatal factors. Finally, we explored the differences in hair GC concentrations between birth and an outpatient visit (OPV) at approximately 6 wks postpartum, as well as which factors are of influence on this difference.

METHODS

POPULATION

From February 2012 to August 2013, mother-infant pairs were included in the OLVG West Hospital in Amsterdam, The Netherlands. Subjects were informed regarding the study before or within 24h after delivery. The infant needed to be admitted to the hospital (maternity ward or neonatal care unit) for at least 72h for a neonatal or maternal reason, as this was an inclusion criterion for a simultaneous study [19]. Subjects were excluded for the following reasons: (1) insufficient knowledge of the Dutch or English language, (2) mental retardation of one or both parents, (3) multiple pregnancy, (4) use of illicit drugs or regular (>2 IU/week) alcohol use during the last trimester, (5) use of systemic corticosteroids during pregnancy, (6) if participating in this study would interfere with regular care, or (7) use of psychotropic medication.

The study was approved by the medical ethics committees of the OLVG West Hospital and the VUMC in Amsterdam, the Netherlands. Written informed consent was obtained from all participants.

DETERMINANTS

The mother filled in a questionnaire about demographic characteristics.
Information on perinatal characteristics and the reasons for admission to the hospital were obtained from medical records.

HAIR CORTISOL MEASUREMENTS

On the first day postpartum neonatal hair was cut from the posterior vertex of the scalp, as close as possible to the scalp, as this region shows the least variance between different strands [14]. At the OPV around 6 wks postpartum, neonatal hair was collected again. The total length of hair directly postpartum was analyzed, with the assumption

that it is an indication of GC concentrations during fetal life, while at the OPV only the centimeter of hair closest to the scalp was analyzed, with the assumption that it gives an indication of GC concentrations during the first weeks of life [20,21].

Maternal hair was also collected on the first day postpartum and at the OPV. Only the centimeter closest to the scalp of maternal hair was analyzed. As, in adults, hair grows approximately 1 cm every month [17,20,21], the hair measurement postpartum is indicative for the GC levels during the last month of pregnancy.

GC levels (cortisol and cortisone) were measured in hair as previously described [20]. In short, in the presence of deuterium-labeled GCs as internal standard, cortisol was extracted using LC-grade methanol at 25°C for 18h. These extracts were subsequently centrifuged and cleaned using solid phase extraction. GC concentrations were quantified by LC-MS/MS (Waters XEVO-TQ-S system, Waters Corporation, Milford, MA, USA). GC concentrations were reported as pg per mg hair, and 1.25mg was required for a reliable measurement.

STATISTICS

Analyses were performed with regard to:

1. Concentrations of GCs in neonatal hair directly postpartum and at the OPV. GC levels were expressed as median (range). Subsequently, GC levels were log-transformed and paired t-tests were performed.
2. The relation between maternal and neonatal (log-transformed) hair GC levels postpartum and at the OPV, was assessed using Pearson correlation coefficients and linear regression.
3. Factors associated with neonatal hair GCs directly postpartum were assessed using linear regression. Additional analyses were performed to assess the effect of the factors associated with GC levels directly postpartum on the course of GC levels (expressed as delta cortisol and cortisone) and on the GC levels at the OPV, corrected for age at the time of sampling. The following factors, based on literature [15-18], were taken into consideration:
    a. Perinatal: gestational age, birth weight (in kg and SD-score), sex, mode of delivery, perinatal infection, respiratory distress (meconium-containing amniotic fluid, respiratory insufficiency, respiratory support, PPHN [persistent pulmonary hypertension of the neonate]).
    b. Maternal: age, ethnicity, maternal smoking, parity (primi- vs. multipara), hypertensive disorders (pregnancy-induced hypertension, pre-existent hypertension, pre-eclampsia/HELLP syndrome).

Results with a P value <0.05 were considered to be statistically significant, although borderline statistically significant results (0.10 > P > 0.05) when found for both cortisol and cortisone were also further explored.

RESULTS

POPULATION

A total of 107 mother-infant pairs donated hair directly postpartum. At the OPV, 72 mother-infants pairs donated hair. The OPV took place at 44±11 days postpartum (range: 22 to 87 days). Perinatal and demographic characteristics are presented in **Table 1**.

TABLE 1. Baseline characteristics of the study population (n=107)

		Mean ± SD, median (range) or n (%)
Perinatal	Gestational age	39.5 ± 1.8, 39.5 (33.9 to 42.1)
	Birth weight	3480 ± 629, 3569 (1806 to 5290)
	Male sex	61 (55.5)
	Vaginal delivery	40 (32.5)
	Perinatal infection	34 (30.9)
	Respiratory problems	9 (8.2)
Maternal	Age	33.9 ± 4.8, 34 (21 to 44)
	Nulliparae	67 (54.5)
	Hypertensive disorders	7 (6.4)
	Non-Dutch ethnicity	52 (47.3)
	Smoking	2 (1.8)

Concentration of GCs in neonatal hair

Results are displayed in **Table 2** and **Figure 1**. Directly postpartum, the median concentration of cortisol was 169 pg/mg (range: 51 to 1294), while the median concentration of cortisone was 85 pg/mg (range: 23 to 597). Maternal GC levels were much lower than neonatal levels, with median concentrations of 5 (range: 0 to 672) and 18 (range: 2 to 87) pg/mg respectively.

Course of GC levels postpartum

Between birth and the OPV, a steep decrease in cortisol concentrations in infant hair was observed (**Table 2** and **Figure 1**). Maternal hair cortisol levels showed a subtle decrease between birth and the OPV. In contrast, infant and maternal hair cortisone levels remained stable, although a wide range of values were observed. At the OPV, both infant cortisol and cortisone concentrations were still higher than the GC levels

in maternal hair. Age of the neonate at the OPV was negatively associated with hair cortisol, but not with cortisone levels (log-transformed β [95%CI]: cortisol -0.01 [-0.02 to -0.001], p=0.03; cortisone: 0.00 [-0.01 to 0.01], p=0.70). Age of the neonate at the OPV was not associated with delta cortisol or cortisone.

TABLE 2. Concentrations of neonatal and maternal hair glucocorticoid concentrations

	Postpartum (median, range) n=107	Outpatient visit (median, range) n=72	P-value*
Infant	169, 51 to 1294	71, 2 to 479	<0.001
	85, 23 to 597	91, 30 to 346	0.99
Maternal	5, 0 to 672	4, 1 to 79	0.001
	18, 2 to 87	18, 8 to 43	0.75

Values expressed as median, range in pg/mg.
* Analyzed with a paired t-test, performed with log-transformed GC concentrations

Correlations with maternal hair GCs

Directly postpartum, maternal and neonatal hair cortisol were positively associated (n=107, r=0.336, β 0.23 [95%CI: 0.11 to 0.36], p<0.001), while no correlations were found between maternal and infant hair cortisone (p=0.66). At the OPV, the association between maternal and infant hair cortisol was stronger than directly postpartum (n=71, r=0.457, β 0.41 [95%CI: 0.22 to 0.60], p<0.001), and no correlation was found for cortisone (p=0.12).

Factors associated with neonatal hair GCs

The effect of several perinatal and maternal factors on hair GC levels measured directly postpartum was studied (**Table 3**). Gestational age was strongly associated with both cortisol and cortisone levels, as illustrated in **Figure 1**, and this association remained significant when only term-born infants (n=98) were studied. Additionally, there was a positive association for both cortisol and cortisone levels with perinatal infection (defined as the need for treatment with antibiotics for ≥7 days). Birth weight was associated with both cortisol and cortisone when expressed in kilograms, but this association was lost when birth weight was expressed as the SD-score. Moreover, delivery via caesarian section was associated with both lower cortisol and cortisone levels, while multiparity was associated with lower cortisol levels.

Next, the effect of the (borderline) significant factors on the course of GC levels was studied (**Table 4**). Gestational age was associated with a trend toward a steeper decrease in cortisol and cortisone between birth and the OPV. Additionally, perinatal infection was associated with a steeper decrease in cortisone, while and delivery via cesarian section was associated with a smaller decrease in cortisone.

TABLE 3. Associations of neonatal hair GC concentrations directly postpartum with perinatal and maternal factors

			β (95%CI)	P value
Perinatal factors	Gestational age	Cortisol	0.07 (0.04 to 0.10)	<0.001
		Cortisone	0.04 (0.01 to 0.06)	0.004
	Gestational age (only term pregnancies)	Cortisol	0.11 (0.07 to 0.16)	<0.001
		Cortisone	0.04 (-0.001 to 0.08)	0.06
	Birth weight (kg)	Cortisol	0.09 (-0.003 to 0.17)	0.06
		Cortisone	0.10 (0.03 to 0.17)	0.008
	Birth weight (SD)	Cortisol	0.01 (-0.05 to 0.06)	0.79
		Cortisone	0.03 (-0.02 to 0.07)	0.23
	Male sex	Cortisol	0.10 (-0.02 to 0.21)	0.09
		Cortisone	0.08 (-0.01 to 0.18)	0.07
	Delivery via caesarian section	Cortisol	-0.14 (-0.26 to -0.03)	0.015
		Cortisone	-0.14 (-0.24 to -0.05)	0.003
	Perinatal infection (≥7 days antibiotics)	Cortisol	0.17 (0.06 to 0.29)	0.003
		Cortisone	0.22 (0.13 to 0.31)	<0.001
	Respiratory problems	Cortisol	-0.07 (-0.28 to 0.13)	0.47
		Cortisone	-0.06 (-0.22 to 0.11)	0.52
Maternal factors	Age	Cortisol	-0.01 (-0.02 to 0.01)	0.31
		Cortisone	0.00 (-0.01 to 0.01)	0.82
	Ethnicity	Cortisol	-0.04 (-0.15 to 0.08)	0.55
		Cortisone	-0.09 (-0.16 to 0.01)	0.07
	Maternal smoking	Cortisol	-0.09 (-0.18 to 0.003)	0.06
		Cortisone	-0.09 (-0.43 to 0.26)	0.62
	Parity	Cortisol	-0.26 (-0.36 to -0.16)	<0.001
		Cortisone	-0.07 (-0.17 to 0.02)	0.12
	Hypertensive disorders	Cortisol	-0.02 (-0.25 to 0.21)	0.85
		Cortisone	-0.15 (-0.33 to 0.04)	0.12

Values represent log-transformed β (95% confidence interval) as calculated with linear regression

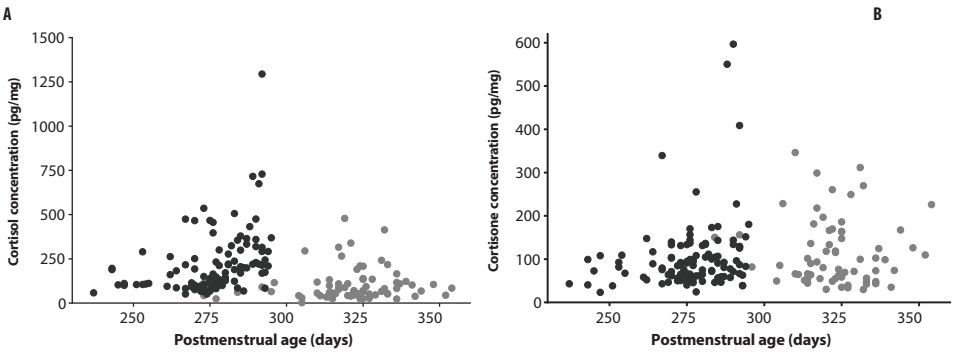

FIGURE 1. Neonatal hair cortisol (A) and cortisone (B) levels measured directly postpartum (●) and at the OPV (●)

Finally, the effect of these factors on the GC levels at the OPV was analyzed (**Table 4**). Gestational age was still positively associated with infant hair cortisol levels, but not with cortisone. Additionally, males had higher cortisol levels in hair at the OPV, but no association was found with cortisone. The other factors were not associated with OPV hair GC levels.

TABLE 4. Associations of the course of neonatal hair GC concentrations with perinatal and maternal factors

			Effect on delta		Effect on OPV values	
			β (95%CI)	P value	β (95%CI)	P value
Perinatal factors	Gestational age	Cortisol	-0.02 (-0.05 to 0.00)	0.08	0.08 (0.04 to 0.12)	<0.001
		Cortisone	-0.006 (-0.013 to 0.001)	0.08	0.00 (-0.04 to 0.04)	0.87
	Birth weight (kg)	Cortisol	-0.05 (-0.12 to 0.02)	0.19	0.13 (0.003 to 0.26)	0.05
		Cortisone	-0.02 (-0.03 to 0.01)	0.15	0.00 (-0.12 to 0.12)	0.96
	Male sex	Cortisol	0.02 (-0.07 to 0.11)	0.68	0.19 (0.02 to 0.36)	0.03
		Cortisone	-0.01 (-0.03 to 0.02)	0.46	0.00 (-0.14 to 0.13)	0.99
	Delivery via caesarian section	Cortisol	0.06 (-0.04 to 0.16)	0.24	-0.06 (-0.25 to 0.13)	0.55
		Cortisone	0.03 (0.01 to 0.05)	0.02	-0.08 (-0.23 to 0.08)	0.33
	Perinatal infection (≥7 days antibiotics)	Cortisol	-0.06 (-0.15 to 0.04)	0.25	-0.04 (-0.22 to 0.14)	0.66
		Cortisone	-0.03 (-0.05 to -0.004)	0.02	0.12 (-0.03 to 0.27)	0.11
Maternal factors	Parity	Cortisol	0.07 (-0.02 to 0.16)	0.11	-0.11 (-0.28 to 0.05)	0.18
		Cortisone	0.01 (-0.01 to 0.03)	0.32	0.01 (-0.13 to 0.14)	0.91

Values represent log-transformed β (95% confidence interval) as calculated with linear regression.
All associations were corrected for age at the OPV.

DISCUSSION

In this study, we have described the levels of cortisol and cortisone in neonatal hair, both directly postpartum, as well as at an OPV at 44±11 days postpartum. GC levels in neonatal hair directly postpartum seem to reflect intrauterine GC exposure, they are much higher than maternal levels and appear to be influenced mainly by gestational age, possibly reflecting the normal prenatal increase in endogenous fetal cortisol. After birth, cortisol levels decrease sharply, although at the OPV neonatal levels are still much higher compared to maternal levels. This suggests that at that time point GC levels represent both the intra- and extrauterine period, since GC levels in infants are not markedly different from maternal cortisol levels [22,23]. Additionally, at birth,

neonatal hair GC levels are associated with other perinatal factors such as perinatal infection, although to a lesser degree than gestational age.

In our study, we could confirm the association described by Hoffmann et al. [18] between neonatal hair cortisol levels and both gestational age and birth weight directly postpartum. However, we did not find an association with birth weight SDS. Since birth weight and gestational age are correlated, the association with birth weight probably reflects the effect of gestational age rather than of intrauterine growth.

The association with gestational age might be indicative of several mechanisms. First, adrenal maturation occurs throughout pregnancy, resulting in increased cortisol production by the fetal adrenal [5]. A higher concentration of GCs in hair might therefore reflect a longer exposure to the maturing HPA axis. However, since the association between gestational age and neonatal hair GCs is also still present in term neonates, another mechanism appears to be present as well. Induction of labor has been suggested to be partly due to an increase in cortisol, which promotes fetal organ maturation [7,24]. This increase in cortisol is thought to originate from a positive feedback loop established between placenta-derived CRH and cortisol originating from the fetal adrenals [6,24], which can only be broken by the severance of the umbilical cord. Fetal distress may accelerate this feedback loop [8], which might explain the increased hair GC levels in neonates who are treated for a perinatal infection.

It is as of yet unknown whether neonatal hair GC levels fully result from fetal cortisol production or whether the transplacental supply of cortisol might also contribute to neonatal GC levels in hair. Previous research has suggested that cortisol is transferred via the placenta to the fetus, although most cortisol is inactivated to cortisone by placental 11βHSD type 2 [8]. However, as maternal serum cortisol levels are 10 times higher than fetal serum levels, even small amounts of cortisol could account for about 40% of the variance in fetal concentrations [4]. In our study, we found a positive correlation between maternal and neonatal hair cortisol levels, but not with cortisone. The positive correlation between neonatal and maternal hair cortisol levels might therefore be a reflection of placental transfer. However, this does not explain why the neonatal hair GC levels were much higher compared to maternal levels. We speculate that this may be due to differences in hair growth and structure between the fetus and its mother.

While it is feasible that cortisol in neonatal hair is derived from hair follicles, where it is incorporated after diffusion from blood [14], cortisol in amniotic fluid might contribute to the GC concentrations measured in hair. Moreover, although hair growth in utero is roughly known, the specifics are still unclear. The first stage of hair growth starts during the 15th week of gestation, and by week 18 to 20 the entire scalp is covered with hair in the primary, anagen stage. Next, between week 24 and

28, the anagen hair converts to telogen hair via a catagen phase [26]. Hair growth, as well as the conversion to more mature hair, is region-specific, and dependent on several biochemical and individual variations [26-28]. Whether hair in the anagen phase already contains GCs, or whether the accumulation of GCs occurs at a later phase, is unknown. Therefore, although it is thought that neonatal hair reflects at least the third trimester of pregnancy [26], the true time window which is represented by GCs measured in hair is not known. Since perinatal infection and mode of delivery also appear to influence hair GC levels, it is likely that the last stages of pregnancy have a significant contribution to GC levels measured in hair. Future studies should include measurements of growth velocity of neonatal hair.

Our study showed significantly increased GC levels in neonatal hair compared to maternal hair at the OPV (44±11 days postpartum), although a decrease between birth and the OPV was observed in cortisol levels. This suggests that GC levels at the OPV represent a combination of intra- and extrauterine influences, supported by our finding that GC levels at the OPV are still associated with several perinatal factors. However, due to the biochemical and individual variations in hair growth [26-28], and since hair was only measured twice in this study, the contribution of intrauterine and extrauterine influences on hair GC levels at the OPV is unknown. We recommend to assess at which point in time intra-uterine factors are no longer related to hair GC levels, since this might provide a clear view of early life influences on HPA axis development. Since hair GC levels appear to be moderately stable in the second half of the first year of life [28], intrauterine influences on hair GC levels most likely disappear within the first 6 months.

Our study has several strengths and limitations. First, GC analyses were performed using LC-MS/MS, which has high sensitivity. Hoffman et al. [18] measured hair cortisol levels with an immunoassay, which might explain the fact that they did not find an association between maternal and neonatal hair cortisol levels, and reported maternal and neonatal cortisol levels which were much higher compared to our results, since immunoassay are more sensitive to cross-reactivity than LC-MS/MS [30,31]. Cross-reactivity is particularly important to take into account when researching newborns, as they have high concentrations of (precursors of) sex steroids and GCs [5], which are partly of maternal origin. Additionally, we measured cortisone as well as cortisol, which providess valuable knowledge due to the conversion of cortisol to cortisone by placental 11βHSD2 [8]. Our database also allowed us to analyze a wide range of pre- and perinatal factors. One of the limitations of our study is that the participants might not represent a normal population, since the participants in our study had to be hospitalized >72h. Additionally, there might be selection bias at the OPV measurements due to losses to follow-up, although the number of participants

was relatively high (67%). Finally, there was a discrepancy between mother and child in the time frame that the hair measurements represented. In mothers, only the last centimeter of hair was analyzed. As adult hair grows with approximately 1cm per month [20,21], these analyses are representative of only the last month of pregnancy. Neonatal hair was analyzed in its entirety, and is therefore representative of the intrauterine period during which cortisol can be incorporated in hair. Correlations between GCs in maternal and neonatal hair should therefore be interpreted in this context.

In conclusion, our findings suggest that infant hair GCs reflect the third trimester increase in cortisol, which might be caused by the positive feedback loop, a placenta-driven phenomenon, represented by a positive association with GA. Between birth and 1.5 months postpartum, cortisol concentrations decrease sharply. At this time point, GC levels appear to reflect both the intra- and extrauterine period, since neonatal levels are significantly higher than maternal GC levels. Perinatal complications and maternal HPA axis activity had minor influences on infant hair GCs.

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CHAPTER 4

MATERNAL STRESS DURING PREGNANCY IS ASSOCIATED WITH DECREASED CORTISOL AND CORTISONE LEVELS IN NEONATAL HAIR, PLAUSIBLY THROUGH ELEVATION OF MATERNAL HPA AXIS ACTIVITY

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ABSTRACT

BACKGROUND

Maternal stress during pregnancy has been associated with unfavorable infant neurodevelopment, possibly through an increased placental transfer of maternal glucocorticoids (GCs). Hair GC levels offer a retrospective view on chronic GC exposure. Since neonatal hair GCs might reflect intra-uterine GC regulation, we assessed whether maternal stress experienced pre- and perinatally is associated with neonatal and maternal hair GCs postpartum (pp).

METHODS

A total of 169 mother-infant pairs donated hair on the first day pp. Of this group, 66 pairs had consulted a center of expertise for pregnancy and psychiatric disorders, where maternal stress was scored on the Hospital Anxiety and Depression Scale (HADS) during the first or second (n=45) and third trimester (n=57). In addition, all mothers filled in the HADS directly pp. Hair cortisol and cortisone levels were determined by LC-MS/MS. The influence of maternal pre- and postnatal HADS-scores, anxiety (HAS) and depression (HDS) subscores, as well as antidepressant use, on maternal and neonatal hair GC levels was analyzed with linear regression, and expressed as log 10 (β [95%CI]).

RESULTS

Neonatal hair glucocorticoids were negatively associated with elevated HAS-scores during the first or second trimester (cortisol -0.19 [-0.39 to 0.02], p=0.07 and cortisone -0.10 [-0.25 to 0.05], p=0.17), third trimester (cortisol -0.17 [-0.33 to 0.00] p=0.05 and cortisone -0.17 [-0.28 to -0.05] p=0.01), and pp (cortisol -0.14 [-0.25 to -0.02] p=0.02 and cortisone -0.07 [-0.16 to 0.02] p=0.10). A similar pattern was observed for elevated HDS-scores. Elevated HAS- and HDS-scores throughout the entire pregnancy showed the strongest negative associations. Maternal hair GCs were positively associated with elevated HAS-scores pp (cortisol 0.17 [0.01 to 0.32] p=0.04, cortisone 0.18 [0.06 to 0.31] p=0.01), but not with elevated HDS-scores pp or prenatally. Antidepressant use was associated with elevated maternal hair GCs ($p \leq 0.05$), but did not affect neonatal hair GCs.

CONCLUSION

Maternal stress during pregnancy seems associated with suppressed fetal HPA axis activity, resulting from increased maternal HPA axis activity. Persistent maternal stress throughout pregnancy was associated with the largest decrease in neonatal hair GCs. Further studies are needed to estimate the clinical relevance of these findings on neurodevelopment, and mental and physical health.

INTRODUCTION

Anxiety or depressive disorders are associated with alterations in HPA axis activity [1,2], which, when present during pregnancy, have been associated with permanent alterations in offspring's HPA axis activity and neurocognitive development [3-5]. During pregnancy, anxiety and depressive disorders are frequent, with numbers ranging from 1 in 10 to 1 in 5 pregnant women [6-8]. Although many observational studies described associations between prenatal stress and unfavorable infant outcomes, caution must be exercised in the interpretation of these findings due to the use of subjective measures of stress, while indices of HPA axis activity are lacking [4,9]. As part of physiological changes during pregnancy, both maternal and fetal glucocorticoids exert a positive feedback effect on the placenta by stimulating the synthesis of placental CRH. Due to this physiological feed forward response, maternal cortisol increases during gestation [3]. In addition, pregnancy is also characterized by high levels of estrogens, which have a well-known stimulating effect on CBG [10]. CBG, in turn, binds the majority of free cortisol. Moreover, placental 11βHSD type 2 converts maternal cortisol to inert cortisone. Accordingly, the fetus is protected from overexposure to maternal cortisol [11].

Hair cortisol and cortisone levels are indices of long-term glucocorticoid (GC) exposure, at least in adults and children above the age of 4 years [12-14]. Newborn hair GC levels are thought to offer a retrospective view on GC exposure during the last part of pregnancy [15,16], although various other factors could explain inter- and intra-individual differences [17,18]. Kapoor et al. studied hair GC levels in the offspring of rhesus monkeys that were randomized to receive exposure to frightening noises while being in a darkened room for 10 minutes a day 5 days a week during 20% of their pregnancies, and found decreased hair cortisol in prenatally exposed offspring, but no difference in hair cortisone [19]. In humans, Hoffman et al. studied neonatal and maternal hair cortisol directly postpartum (pp), and showed that neonatal cortisol increased with advancing gestational age and birth weight [20]. Unfortunately, in this study stress exposure was not taken into account, and hair cortisone levels were not measured. Nonetheless, these data suggest that determination of neonatal hair GCs might offer valuable insights into intra-uterine GC regulation.

Therefore, in the present study we assessed whether exposure to maternal stress pre- and postnatally is associated with neonatal and maternal hair cortisol and cortisone levels directly pp. To study this, we used data from a cohort in which women with severe stress during pregnancy were overrepresented.

METHODS

STUDY DESIGN AND PARTICIPANTS

The present study was part of a prospective cohort study that aimed to explore biomarkers (neonatal hair glucocorticoids [GCs] and urinary 5-hydroxyindoleacetid acid levels) of poor neonatal adaptation after exposure to selective antidepressants (SADs) or experienced maternal stress in utero [21,22]. Therefore, this cohort included an overrepresentation of mothers who experienced severe stress during their pregnancies.

A total of 169 mother-infant pairs were recruited at the maternity department, as well as at the psychiatric-obstetric-pediatric (POP) clinic of the OLVG-west Hospital, Amsterdam, The Netherlands, which offers consultation to women with psychiatric disorders before, during and after pregnancy, on an outpatient basis. Approximately one third (n=66) of the total sample consisted of patients from the POP clinic. The other part of the sample (n=103) consisted of mothers admitted for other neonatal or maternal medical reasons.

Mother-infant pairs with an expected hospital stay of ≥72h after delivery, and who were willing to donate hair and complete the Hospital Anxiety and Depression Scale (HADS) questionnaire were eligible for inclusion. Exclusion criteria were: use of psychotropic medication other than SADs, use of systemic corticosteroids, use of non-pharmacologic drugs, alcohol or nicotine smoking during the third trimester of pregnancy, insufficient knowledge of the Dutch or English language, mental impairment of one or both parents, and multiple pregnancies. Parents were informed and written informed consent was obtained within 24h after delivery. The study was approved by the Medical Ethics Committees of the OLVG-west Hospital and the VUMC.

PRE- AND PERINATAL MATERNAL STRESS

As part of standard care at the POP clinic, the HADS [23] was used as a screening instrument for clinically elevated stress levels experienced in the previous week, with a retest reliability that correlates good with the previous 6 weeks [24]. The HADS consists of 14 questions, seven for anxiety and seven for depression. Anxiety (HAS) and depression (HDS) subscores can be calculated and interpreted as independent, clinically relevant dimensions of the HADS. The HAS and HDS can be measured both as continuous and dichotomized variables, with ≥8 out of 21 points per subscale as cut-off for clinically elevated stress scores [23,24].

During the first or second trimester, and/or during the third trimester, the HADS was

scored for mothers who sought advice at the POP clinic. In addition, within 12 - 36h pp the HADS was scored for all participating mothers.

USE OF SELECTIVE ANTIDEPRESSANTS (SADS)

When admitted to the maternity ward pp, mothers were asked whether they used SADs during at least the last two weeks of pregnancy. In our cohort, 65 mothers (39%) reported using either selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), noradrenergic and specific serotonin antidepressants (NaSSAs), or a combination of these.

HAIR GLUCOCORTICOID LEVELS

Mother-infant pairs donated hair on the first day pp. A lock of hair was cut from the posterior vertex as close as possible to the scalp. A minimum of 1.25mg of hair was needed for a reliable measurement. Fetal hair growth velocity and the timing of transition from lanugo via vellus into terminal hair strands varies significantly between infants [25]. Therefore, the total length of neonatal hair was analyzed and mean cortisol and cortisone levels, per mg hair, were calculated. Of maternal hair, the centimeter closest to the scalp was analyzed, representing mean cortisol and cortisone levels during the last month of pregnancy, as adult hair grows approximately 1 centimeter per month [16,26].

Hair cortisol and cortisone levels were measured as described previously by Noppe and De Rijke et al. [13]. In short, hair was washed with isopropanol and hair GCs were extracted using methanol and solid-phase extraction. Subsequently, cortisol and cortisone concentrations were quantified by LC-MS/MS (Waters XEVO-TQ-S system, Waters Corporation, Milford, MA, USA), with positive electrospray ionization, and reported in pg/mg hair.

DATA ANALYSIS

Maternal and neonatal characteristics were compared between patients from the POP clinic and mothers admitted for other neonatal or maternal medical reasons, using independent t-test, Chi Square or Fisher exact tests.

Hair cortisol and cortisone levels were skewed to the right and therefore logarithmically transformed prior to analysis. Linear regression was used to assess the association between HADS-scores and neonatal or maternal hair GC levels. The influence of maternal stress was assessed with hair GC levels as dependent factor, and HAS or HDS subscores as independent factor, continuously or dichotomously, with a score of ≥8 points as cut-off for elevated stress [23]. When significant associations

were found between prenatal maternal stress scores and hair GC levels, we tested the relative contributions of prenatal and postnatal stress by analyzing combinations of (1) low pre- and low postnatal (reference), (2) low pre- and high postnatal, (3) high pre- and low postnatal or (4) high pre- and high postnatal stress. In addition, the influence of maternal SAD use was analyzed with hair GC levels as dependent factor, and SAD use as dichotomous (no medication vs. SAD use) or categorical independent variable (no medication use vs. use of SSRi, SNRI and/or NaSSA).

Confounders were selected a priori, based on the literature [19-21,27]. Gender, birth weight (percentile) and primiparity were added to the multivariable model, one by one. Subsequently, based on statistical impact (>10% change in beta) the final model was created. When confounders were found to have a statistical impact on more than 50% of the associations being analyzed, we also explored their univariate influence on the outcome afterwards.

RESULTS

Characteristics of all subjects who donated hair pp are shown in **Table 1**. Of these, a total of 66 women had consulted the POP center of expertise for pregnancy and psychiatric disorders, of whom 96% reported SAD use, 29% had an elevated HAS-subscore, and 17% had an elevated HDS-subscore. For mothers admitted for strictly medical reasons (n=103), these numbers (2%, 10%, and 8%, respectively) were similar to previously reported prevalence rates in normal populations [6-8,28]. Descriptive statistics for gender, gestational age, birth weight, primiparity, maternal age and ethnicity did not differ between the two subgroups.

TABLE 1. Characteristics of mother-infant pairs (n = 169)

Neonatal	**Males**		92	(54%)
	Gestational age	wks	39.4	± 1.7
	Birth weight	g	3,446.5	± 585.8
		percentile	53.9	± 26.4
	Hair cortisol pp	pg/mg hair	156.3	(101.3 to 227.5)
	Hair cortisone pp	pg/mg hair	80.8	(61.5 to 108.8)
Maternal	**Primiparous**		84	(50%)
	Age	yr	33.8	± 4.7
	Ethnicity	Caucasian, non-Dutch	16	(9%)
		non- Caucasian	59	(35%)
	Antidepressants	SSRI	44	(26%)
		SNRI	7	(4 %)
		NaSSA	9	(5%)
		Combination*	5	(3%)
	HADS score pp	HAS-score ≥ 8	29	(17%)
		HDS-score ≥ 8	19	(11%)
	Hair cortisol pp	pg/mg hair	5.3	(3.6 to 10.6)
	Hair cortisone pp	pg/mg hair	19.5	(14.5 to 32.7)

Data are expressed as mean ± SD, median (interquartile range) or n (%). Abbreviations: pp = postpartum; HADS = Hospital Anxiety and Depression Scale; HAS = Hospital Anxiety Scale; HDS = Hospital Depression Scale. *These subjects were treated with a combination of SSRI with NaSSA (*n*=4), or NaSSA with SNRI (*n*=1)

THE INFLUENCE OF MATERNAL STRESS ON NEONATAL HAIR GCS

Prenatal HADS scores were known for the mothers who had consulted the POP clinic during pregnancy: n=45 during the first or second trimester, and n=57 during the third trimester.

Neonatal hair cortisol levels were negatively associated with maternal anxiety and depression experienced pre- and/or postnatally (**Table 2**). Neonatal hair cortisone levels showed similar, but weaker, associations. Correction for gender or birth weight percentiles did not change these associations. Correction for primiparity strengthened the associations with first or second trimester stress scores, but weakened the associations with third trimester and pp stress scores (**Table 2**).

When tested univariably, primiparity was positively associated with neonatal hair cortisol and cortisone levels (0.24 [0.16 to 0.32] $p < 0.01$, and 0.10 [0.04 to 0.17] $p < 0.01$, respectively).

Consistently elevated stress scores both pre- and postnatally, had the greatest impact on neonatal hair GC levels, as compared to consistently low stress scores in the subgroup of mothers who were seen at the POP clinic during pregnancy (**Table 3**).

TABLE 2. Maternal stress in association with neonatal hair cortisol and cortisone levels

			Postpartum *n*= 169		**3rd trimester** *n*= 57		**1st – 2nd trimester** *n*= 45	
			raw	adjusted	raw	adjusted	raw	adjusted
Cortisol	**Anxiety**	HAS score	-0.01 (-0.03 to 0.00) *	-0.01 (-0.02 to 0.00)	-0.02 (-0.03 to 0.00) *	-0.01 (-0.03 to 0.00)	-0.02 (-0.04 to 0.00) *	-0.03 (-0.04 to 0.01) *
		Elevated HAS	-0.14 (-0.25 to -0.02) *	-0.10 (-0.20 to 0.01) †	-0.17 (-0.33 to 0.00) *	-0.10 (-0.26 to 0.07)	-0.19 (-0.39 to 0.02) †	-0.22 (-0.40 to -0.03) *
	Depression	HDS score	-0.01 (-0.03 to 0.00) †	-0.01 (-0.02 to 0.00)	-0.01 (-0.03 to 0.00) †	-0.01 (-0.03 to 0.01)	-0.03 (-0.05 to -0. 01) *	-0.03 (-0.05 to -0. 01) *
		Elevated HDS	-0.11 (-0.25 to 0.03)	-0.09 (-0.22 to 0.03)	-0.19 (-0.38 to -0.01) *	-0.12 (-0.31 to 0.07)	-0.35 (-0.57 to -0. 12) *	-0.32 (-0.53 to -0. 11) *
Cortisone	**Anxiety**	HAS score	-0.01 (-0.02 to 0.00) *	-0.01 (-0.02 to 0.00)	-0.01 (-0.03 to 0.00) *	-0.01 (-0.02 to 0.01)	-0.01 (-0.03 to 0.01)	-0.01 (-0.03 to 0.00) †
		Elevated HAS	-0.07 (-0.16 to 0.02)	-0.05 (-0.14 to 0.03)	-0.17 (-0.28 to -0.05) *	-0.12 (-0.23 to 0.00) †	-0.10 (-0.25 to 0.05)	-0.12 (-0.26 to 0.01) *
	Depression	HDS score	-0.01 (-0.02 to 0.00) *	-0.01 (-0.02 to 0.00) *	-0.01 (-0.02 to 0.00)	-0.01 (-0.02 to 0.01)	-0.01 (-0.02 to 0.01)	-0.01 (-0.02 to 0.01)
		Elevated HDS	-0.09 (-0.20 to 0.01) †	-0.08 (-0.19 to 0.02)	-0.17 (-0.31 to -0.03) *	-0.11 (-0.25 to 0.03)	-0.09 (-0.27 to 0.09)	-0.07 (-0.24 to 0.10)

Data are presented as Log10 transformed β (95%CI). The adjusted model is corrected for primiparity. Cortisol and cortisone levels are reported in pg/mg hair.
* p<0.05 † p<0.10 Abbreviations: HAS = Hospital Anxiety Scale; Elevated HAS = score ≥8; HDS= Hospital Depression Scale; Elevated HDS = score ≥8

TABLE 3. The course of HADS-scores over time from mothers who were seen at the POP clinic during pregnancy, in association with neonatal hair cortisol and cortisone levels

	Category		Cortisol		Cortisone	
		n	Beta	(95%CI)	Beta	(95%CI)
HAS	Low pre- & low postnatally	36	ref		ref	
	High pre- & low postnatally	6	-0.03	(-0.29; 0.23)	0.01	(-0.18; 0.20)
	Low pre- & high postnatally	10	-0.09	(-0.30; 0.12)	-0.14	(-0.29; 0.02) †
	High pre- & high postnatally	12	-0.19	(-0.39; 0.01) †	-0.15	(-0.29;- 0.01) *
HDS	Low pre- & low postnatally	49	ref			
	High pre- & low postnatally	2	-0.13	(-0.55 to 0.29)	-0.14	(-0.45 to 0.17)
	Low pre- & high postnatally	5	-0.09	(-0.36 to 0.19)	-0.18	(-0.38 to 0.03) †
	High pre- & high postnatally	8	-0.27	(-0.49 to- 0.05) *	-0.17	(-0.33 to 0.00) *

Data are presented as Log10 transformed β (95%CI). Cortisol and cortisone levels are reported in pg/mg hair.
Prenatal stress is scored in either the first, second, or third trimester. * p<0.05 † p<0.10 Abbreviations: HAS = Hospital Anxiety Scale score ≥8; HDS= Hospital Depression Scale score ≥8

THE INFLUENCE OF MATERNAL STRESS ON MATERNAL HAIR GCS

Maternal hair cortisol and cortisone levels were positively associated with maternal anxiety experienced directly pp, but not associated with HADS-scores measured prenatally (**Table 4**). Correction for gender, birth weight percentiles or primiparity did not significantly change these associations.

THE INFLUENCE OF SAD USE ON NEONATAL AND MATERNAL HAIR GLUCOCORTICOID LEVELS

In total, 65 mothers (38%) reported using SADs: 44 SSRIs, 7 SNRIs, 9 NaSSAs and 5 a combination of SADs (4 SSRI with NaSSA, and 1 NaSSA with SNRI).

When analyzed dichotomously, maternal SAD use was not associated with neonatal hair GC levels, and positively associated with maternal hair cortisol and cortisone levels (0.13 [0.00, 0.26] p=0.05 and 0.18 [0.09 to 0.28] p< 0.01, respectively). When analyzed categorically, neonatal hair cortisol and cortisone was not associated with SSRI, SNRI, or NaSSA use. However neonatal hair cortisol and cortisone was negatively associated with the use of a combination of SADs, as compared to neonates that were not exposed to SADs in utero (-0.32 [-0.57 to -0.06] p=0.02 and -0.22 [-0.42 to -0.02] p=0.04, respectively). Maternal hair cortisol and cortisone levels were positively associated with SSRI-use, as compared to no SAD use (0.16 [0.01 to 0.30] p=0.03 and 0.24 [0.14 to 0.35] p<0.01, respectively), but not with SNRI or NaSSa use. Maternal hair cortisol levels showed a trend towards a positive association with the use of a combination of SADs (0.33 [-0.04 to 0.70] p=0.08), as compared to no SAD use, maternal hair cortisone levels were not associated.

TABLE 4. Maternal stress in association with maternal hair cortisol and cortisone levels

			Postpartum *n*= 169		**3rd trimester** *n*= 57		**1st – 2nd trimester** *n*= 46	
			raw	adjusted	raw	adjusted	raw	adjusted
Cortisol	**Anxiety**	HAS score	0.01 (-0.01 to 0.03)	0.02 (0.00 to 0.03) †	-0.01 (-0.04 to 0.01)	-0.01 (-0.03 to 0.01)	0.00 (-0.03 to 0.03)	0.00 (-0.03 to 0.03)
		Elevated HAS	0.17 (0.01 to 0.32) *	0.19 (0.04 to 0.35) *	-0.14 (-0.36 to 0.08)	-0.08 (-0.30 to 0.15)	-0.03 (-0.30 to 0.25)	-0.05 (-0.33 to 0.22)
	Depression	HDS score	0.00 (-0.02 to 0.02)	0.00 (-0.02 to 0.02)	0.00 (-0.03 to 0.02)	0.00 (-0.02 to 0.02)	0.01 (-0.03 to 0.04)	0.01 (-0.02 to 0.04)
		Elevated HDS	0.02 (-0.17 to 0.21)	0.03 (-0.15 to 0.21)	-0.08 (-0.33 to 0.17)	-0.01 (-0.26 to 0.25)	0.18 (-0.14 to 0.50)	0.21 (-0.11 to 0.53)
Cortisone	**Anxiety**	HAS score	0.02 (0.00 to 0.03)*	0.02 (0.01 to 0.03)*	-0.01 (-0.03 to 0.01)	-0.01 (-0.03 to 0.02)	0.00 (-0.02 to 0.03)	0.00 (-0.02 to 0.03)
		Elevated HAS	0.18 (0.06 to 0.31)*	0.20 (0.08 to 0.33)*	-0.15 (-0.35 to 0.04)	-0.11 (-0.31 to 0.10)	-0.03 (-0.26 to 0.20)	-0.04 (-0.27 to 0.19)
	Depression	HDS score	0.01 (-0.01 to 0.02)	0.01 (-0.01 to 0.02)	0.00 (-0.02 to 0.02)	0.00 (-0.02 to 0.02)	0.00 (-0.03 to 0.03)	0.00 (-0.02 to 0.03)
		Elevated HDS	0.10 (-0.06 to 0.25)	0.11 (-0.05 to 0.25)	-0.01 (-0.23 to 0.21)	0.06 (-0.17 to 0.29)	0.11 (-0.17 to 0.38)	0.12 (-0.15 to 0.40)

Data are presented as Log10 transformed β (95%CI). The adjusted model includes primiparity as confounding factor. Cortisol and cortisone levels are reported in pg/mg hair.
* p<0.05 † p<0.10 Abbreviations: HAS = Hospital Anxiety Scale; Elevated HAS = score ≥8; HDS= Hospital Depression Scale; Elevated HDS = score ≥8

A trend towards a higher prevalence of elevated HAS subscores pp was seen in mothers who used SSRIs, as compared to mothers who did not use SADs (26.1% vs. 13.4%, p = 0.07). Mothers who used a combination of SADs had significantly (p <0.01) more often clinically elevated HAS and HDS subscores, as compared to mothers who did not use SADs (80.0% vs. 13.4% and 80.0% vs. 7.6%, respectively).

DISCUSSION

In this study, exposure to excessive pre- and perinatal maternal stress was associated with a decrease in neonatal hair GC levels, with the largest decrease seen in children of mothers with persistent stress throughout pregnancy. In addition, elevated maternal stress-scores around birth were associated with increased maternal hair GC levels, while elevated stress-scores earlier in gestation had no effect on maternal hair GCs. These associations could not be explained by SAD use during pregnancy.

One possible explanation for the discrepancy in the association between maternal stress with maternal vs. neonatal hair GCs could lie in the difference in growth rate between fetal and maternal hair. Whereas adult hair is known to grow one centimeter per month [16,26], fetal hair growth velocity and the timing of transition into terminal hair strands is thought to vary significantly between and within neonates [18,25,29]. In an attempt to minimize the influences of these inter- and intra-individual variations, we chose to analyze the entire hair instead of the most proximal centimeter. Accordingly, it is possible that the analyzed hair segments of mother and child reflected different windows, i.e., the last month vs. a substantial part of the second half of pregnancy [17,18]. Presumably as a result, similar to that what was seen in rhesus monkeys exposed to prenatal stress [19], stressful events early in pregnancy were not reflected in the most proximal maternal hair segment while they were in the neonatal hair segment.

In addition, the negative association between maternal stress and neonatal hair GC levels might be interpreted as a suppression of fetal HPA axis activity due to overexposure to maternal GCs. In adults, it has previously been shown that exposure to supraphysiological levels of GCs is indeed associated with suppressed hair GCs [30]. The supply of glucocorticoids to the fetus comes primarily from the placental transfer of maternal glucocorticoids, and in addition, from the fetal adrenal itself, with the latter contribution expanding throughout gestation, due to maturation of the fetal adrenal. Because evidence is lacking concerning which part of intra-uterine GC regulation is reflected in neonatal hair GCs, it has been hypothesized that neonatal hair GC levels might reflect amniotic fluid GC levels [19]. This seems unlikely, since in our study neonatal hair cortisol was higher than cortisone, whereas due to conversion of

cortisol to inert cortisone by placental 11βHSD2, amniotic fluid cortisone was higher than cortisol [31]. In addition, evidence from studies in adults suggests that hair GCs reflect the body's HPA axis activity, with hair GCs being associated with long-term integrated salivary cortisol [16]. Thus, neonatal hair GCs plausibly reflect a combination of exposure to maternal GCs, placental CRH, and fetal adrenal maturation.

Exposure to excessive pre- and perinatal distress has been associated with permanent impairments in offspring's neurocognitive development [3-5]. Although the mechanisms behind these associations are not fully elucidated yet, there is some evidence suggesting that disturbances in maternal HPA axis activity during pregnancy could partly explain these effects [9,32]. Therefore, long-term follow-up of our cohort is warranted to explore effects on various aspects of HPA axis development, including stress reactivity and establishment of a diurnal rhythm, as well as neurodevelopmental and cardiometabolic outcome [10].

The major strength of this study is the unique sample with a wide range of pre- and postnatally experienced stress levels, including an overrepresentation of severely stressed mother-infant pairs. In addition, to the best of our knowledge, this is the first translational study that measured hair GC levels in both mothers and infants who experienced a reliably quantified amount of distress during pregnancy [33].

A limitation of our study is that prenatal HADS-scores were only known for the subgroup of mothers who sought consultation at the POP clinic during their pregnancies. Although the strength of our population was the overrepresentation of severely stressed subjects, their vulnerability made us choose for non-invasive measures of HPA axis activity. Ideally, various aspects of HPA axis activity, such as reactivity and rhythmicity, should have been included too [34]. Moreover, this study was not designed to assess the influence of SAD use. Nonetheless, our data strongly suggested that elevated maternal stress-scores explained the associations between SAD use and maternal hair GCs.

In conclusion, we found that pre- and perinatal exposure to excessive maternal stress was associated with a decrease in neonatal hair GC levels, with the largest decrease seen in children of mothers with persistent stress throughout pregnancy. In addition, elevated maternal stress-scores around birth were associated with increased maternal hair GC levels, while elevated stress-scores earlier in gestation had no effect on maternal hair GCs. Accordingly, maternal stress during pregnancy might have increased intra-uterine GC exposure, thereby suppressing fetal HPA axis activity.

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CHAPTER 5

DETERMINATION OF CORTISOL AND CORTISONE IN HUMAN MOTHER'S MILK

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Human mother's milk is recommended as the standard nutrition for neonates, due to its widely acknowledged benefits. Besides being a source of nutrients, milk contains a variety of non-nutritive bioactive and immunomodulatory components [1]. Glucocorticoids are found in mother's milk, bound mainly to CBG and albumin [2]. Glucocorticoids are suggested to induce proliferation and differentiation of glandular cells in the mammary gland, and also to influence the neonate via the mother's milk [2,3]. Despite efforts to optimize the contents of formula feeding, human milk is still recommended for its widely acknowledged benefits. Maternal glucocorticoids might be one of the factors contributing to the advantages of breast milk over formula feeding and are therefore worthwhile to investigate.
In order to be able to measure glucocorticoids in human milk we developed a reliable LC-MS/MS method to determine cortisol and cortisone in mother's milk. In this letter, we describe this method and the results of the validation of our assay, including experiments that investigated the stability of cortisol and cortisone in human milk.

Donor mother's milk of 13 healthy mothers, donated 8 to 28 wks postpartum to the Dutch Human Milk Bank of the VUMC, Amsterdam was used. All samples were stored in polypropylene vials at −20°C. Preparation of the samples was initiated by the addition of $^{2}H_{4}$ labeled cortisol (Cambridge Isotope Laboratories) and $^{2}H_{8}$ labeled cortisone (CDN Isotopes Inc.), both serving as internal standards, to 200 µL thawed milk and thorough mixing. To remove undesired lipids, the milk was washed by the addition of 2 mL hexane [2]. Capped tubes were mixed for 2 minutes in a multivortexer and centrifuged for 2 minutes, at 19°C, 1,900 g, resulting in the separation of the lipid layer from the aqueous layer. Thereafter the sample was frozen in a −60°C CO_2 ice bath, which enabled the liquid hexane to be decanted from the frozen milk. This washing procedure was completed three times, in total.
Forty µL of the washed milk was injected onto a Symbiosis online solid phase extraction (SPE) system (Spark Holland, Emmen, The Netherlands). Online SPE with C8 cartridges (Spark Holland) was performed for further purification of the samples. The analyte was eluted from the cartridge with methanol-water and focused on the Synergi Hydro RP column (Phenomenex, Utrecht, The Netherlands), which was equipped with a C18 guard column (Phenomenex). A linear binary gradient from 55 to 61% methanol containing 0.1% formic acid and 2 mmol/L ammonium acetate was applied, after which the methanol content was increased to 100%. Cortisol, cortisone and their internal standards eluted at retention times of 5.2 and 4.8 minutes, respectively. The total run time was 7.5 minutes. A Quattro Premier XE tandem mass spectrometer (Waters Corp., Milford, MA), which operated in the electrospray positive ionization mode, served as detection instrument which operated in the electrospray

positive ionization mode. Capillary voltage was 0.5 kV and the source temperature 120 °C. Argon was used as collision gas. Cortisol and [$^2H_4$]cortisol were measured using the transitions (Q1>Q3)m/z 363.2 à 121.1 and m/z 367.2 à 121.1, respectively. Cortisone and [$^2H_8$]cortisone were measured using the transitions (Q1>Q3)m/z 361.2 à 163.1 and m/z 369.2 à 124.1, respectively.

TABLE 1. Intra- and interassay coefficients of variations (CVs) of cortisol and cortisone in human milk

Intra-assay variation

Mean cortisol concentration*	n	Intra-assay CV %	Mean cortisone concentration*	n	Intra-assay CV %
0.56	13	13%	3.1	10	7%
3.3	38	8%	7.7	8	5%
7.0	77	4%	15.0	36	6%
23.0	48	5%	33.0	74	5%

Inter-assay variation

Mean cortisol concentration*	n	Inter-assay CV %	Mean cortisone concentration*	n	Inter-assay CV %
6.3	18	9%	31.9	16	9%
22.0	5	4%			

*concentrations are reported in nmol/L

Table 1 shows that the intra coefficients of variation (CV%) were 4% and 5% at a level of 7 and 23 nmol/L, respectively, for cortisol and 5% at a level of 8 and 33 nmol/L for cortisone and that the inter CV% was <9% for both cortisol and cortisone. Recovery, as judged by the recovery of spiked analytes, was 97 to 102% for cortisol and 98 to 106% for cortisone. Linearity was shown by 2-, 4- and 8-fold dilution. The difference between expected and observed values varied between 93 and 106%. The Lower Limit of Quantitation (LLOQ) with a total allowable intra-assay CV of 15% was 0.5 nmol/L for cortisol and 0.25 nmol/L for cortison, determined using diluted milk samples.

In addition, the stability of cortisol and cortisone in human milk under various conditions was evaluated. We determined the stability of cortisol and cortisone in milk stored at room temperature by aliquoting one sample of fresh human milk and storing these for 0, 2, 4, 6, 8, 10, 12, 24 and 36 hours at 4°C and 20°C. After these time periods all aliquots were directly stored at −20°C. Subsequently, all samples were measured in one run. The analysis showed that both cortisol and cortisone were stable (<10% decrease) during 36h storage at 4°C and 20°C in comparison to direct storage at −20°C.

Finally, we evaluated the influence of freeze-thaw cycles and saw that at least six cycles did not influence (<5% decrease) cortisol and cortisone concentrations in human milk (n = 4).

In order to determine reliable reference values a larger sample size should be used for measurements. The number of days postpartum and time of the day might influence milk cortisol concentration and should be taken into account [2]. In our study, based on 13 samples of healthy mothers collected at different moments during the day and on varying days up till 28 wks postpartum, cortisol concentrations ranged from 4 to 23 nmol/L, cortisone concentrations ranged from 11 to 33 nmol/L and the cortisol/cortisone ratio ranged from 0.2 to 0.6.

In the literature, a wide range of human milk cortisol concentrations is reported, from 0 to 1700 nmol/L (**table 2**). This might have been caused by the variety of methods, including the potential cross reactivity with other steroids in the immunoassays, used in 4 out of 7 reported studies [2,4-6]. Nowadays, for reliable steroid hormone analysis LC–MS/MS is preferred, due to its superiority in specificity compared to immunoassays. Moreover the variety in reported concentrations might have been caused by the use of enzymatic conjugates, in 3 out of 7 reported studies [4,7,8]. Conjugated steroids are biologically inactive and need to be hydrolyzed by sulfatases and glucuronidases to become reactivated and absorbed by the intestinal mucosal cells [9]. These enzymes are absent at birth in the human gut and are increasingly present with age due to bacterial colonization [9]. Therefore incubating the milk with conjugate enzymes could give falsely high levels, not reflecting the clinically relevant, biologically available glucocorticoid compound to the neonate.

TABLE 2. Literature overview of used methods and reported concentrations of human milk cortisol and cortisone.

Reference	Subjects	Method	Concentration cortisol *	Concentration cortisone *
Grey et al. [4]	52 full term mothers; 3 mo. pp	Chemiluminescent immunoassay after enzymatic deconjugation	Mean 6.1	-
Xu et al. [7]	8 full term mothers; 0-3 days pp	LC-MS/MS after enzymatic deconjugation	Mean ± SD 3.4 ± 1.7	Mean ± SD 28 ± 9.4
Kulski & Hartmann[2]	11 full term mothers; 1 mo. before birth -13 mo. pp	Radioimmunoassay	Postpartum: Range: 0.6 – 88	-
Hart et al.[5]	40 full term mothers; 1 wk pp	Fluorometric immunoassay	Mean ± SD 13.2 ± 13.0 Range 0 – 38.6	-
Sahlberg & Axelson [8]	6 full term mothers; <1 mo. pp	GC-MS after enzymatic deconjugation	Range 0 - 0.4	Range 0 - 17
Groer et al. [6]	34 preterm and 29 full term mothers; 5 days pp	Radioimmunoassay	Preterm milk Mean 1700 Full term milk Mean 1600	-
Ost et al. [10]	6 healthy women; 4-6 days pp	HPLC	Range ≤ 41	
Van der Voorn et al. (Current study)	13 full term mothers; 8-28 wks pp	LC-MS/MS	Range 4 - 23	Range 11 - 33

*concentrations are reported in nmol/L. GC-MS = Gas chromatography–mass spectrometry; HPLC = High-performance liquid chromatography; LC-MS/MS = liquid chromatography - tandem mass spectrometry

Furthermore, maternal, perinatal stress experienced in the first days postpartum could have caused higher milk cortisol concentrations. However, in relation to reported cortisol concentrations in other studies who used immunoassays with [2] or without [4,5] enzymatic deconjugation and studies who used samples collected during the first days postpartum [5,7] the extremely high concentrations (~1700 nmol/L) reported by Groer et al. [6] cannot be explained.

In conclusion, in this study we present a reliable LC-MS/MS method to measure cortisol and cortisone in human milk, using a relatively easy sample work-up which requires only a small amount of milk. Moreover, we showed that cortisol and cortisone in human milk are stable during 36h of storage at room temperature and during at least six freeze-thaw cycles. In addition, an overview of human milk cortisol and cortisone concentrations reported in literature is presented.

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CHAPTER 6

BREAST-MILK CORTISOL AND CORTISONE CONCENTRATIONS FOLLOW THE DIURNAL RHYTHM OF MATERNAL HYPOTHALAMUS-PITUITARY-ADRENAL AXIS ACTIVITY

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ABSTRACT

BACKGROUND

Very preterm infants often receive donor milk from mothers who deliver at term, but its composition differs from that of their own mothers milk. Because breast-milk glucocorticoids can support developing neonates, we explored concentration variability within and between mothers. We hypothesized that breast-milk glucocorticoid concentrations would be higher after very preterm delivery (GA <32 wks; study 1) and would follow the diurnal rhythm of maternal adrenocortical activity (study 2).

METHODS

Study 1 assessed differences in milk cortisol, cortisone, and the cortisone-to-(cortisol+cortisone) ratio of mothers who delivered at (median) GA: 28.6 wk or at term weekly during the first month postpartum.
Study 2 assessed variations in milk cortisol, cortisone, and the cortisone-to-(cortisol+cortisone) ratio over 24 h, and tested Pearson correlations between milk and salivary concentrations in mothers who delivered at term (median GA: 38.9 wk) during week 4 postpartum.
In these studies, foremilk glucocorticoids were measured by LC-MS/MS. Associations of milk cortisol, milk cortisone, and the milk cortisone-to-(cortisol+cortisone) ratio with prematurity (study 1) or collection time (study 2) were studied with longitudinal data analyses.

RESULTS

In study 1, giving birth to a very preterm infant was associated with reductions in milk cortisol and cortisone concentrations of 50% (β: 0.50; 95%CI: 0.26, 0.99; P = 0.05) and 53%(β: 0.53; 95%CI: 0.30, 0.93; P = 0.03), respectively, when adjusted for collection time.
In study 2, concentrations of milk cortisol and cortisone were associated with collection time (both P < 0.01), peaking at ~0700.Milk and salivary concentrations of cortisol (r = 0.92, P < 0.01) and cortisone (r = 0.93, P < 0.01) as well as the cortisone-to-(cortisol+cortisone) ratio (r = 0.64, P < 0.01) were correlated with one another.

CONCLUSIONS

Breast-milk glucocorticoid concentrations follow the diurnal rhythm of maternal HPA axis activity and are lower in mothers who deliver very preterm.

INTRODUCTION

In term infants, human milk is recommended for its beneficial effect on growth, neurodevelopment, the immune system, and cardiovascular health, all of which are attributable to nutritional and nonnutritive, bioactive components [1-3]. Similar benefits of human milk are observed in very preterm infants [4-7]. Therefore, when breast milk is not yet available after very preterm delivery, donated human milk is recommended [8,9]. Human milk is known for its dynamic composition, often filling neonatal requirements [1]. Its nutritional composition is determined by stage of lactation, maternal diet, body composition, and genetics [10]. Human milk has been associated with postnatal growth restriction in very preterm infants, because it does not meet the very preterm (GA <32 wks) infants nutritional requirements [4]. Still, compared with formula feeding, donor milk reduces morbidity, likely by delivering protective factors to the immature gut mucosa [1,9,11]. However, little is known about factors that could influence the composition of nonnutritive, bioactive components (e.g., hormones).

Many ill, very preterm newborns have inappropriately low cortisol concentrations. This might result in increased vulnerability to potentially life-threatening illnesses [12,13]. Glucocorticoids in breast milk [1,2] could provide resilience against these illnesses. Therefore, we conducted 2 studies with the aim of exploring variations in glucocorticoid concentrations in human milk. First, we studied whether breast-milk cortisol and cortisone concentrations in the first month postpartum differed between mothers who delivered very preterm and those who delivered at term. We hypothesized that very preterm delivery would be associated with higher breast-milk glucocorticoid concentrations. Results from this study showed that breast-milk cortisol and cortisone concentrations vary widely, not only between mothers but also within mothers. In a post hoc analysis, milk cortisol and cortisone concentrations were found to be dependent on time of collection. Subsequently, in a second study, we explored whether breast-milk glucocorticoid concentrations follow a diurnal rhythm, and whether they correspond to the circadian rhythm of maternal HPA axis activity.

METHODS

PARTICIPANTS

For both studies, mothers were recruited at the maternity department of VUMC in Amsterdam, Netherlands. Both studies were approved by the Medical Ethics

Committee of the VUMC (protocol METc VUMC 2013/80 and 15/001), and written informed consent was obtained from all participants.

Study 1. We recruited 10 mothers who delivered at term and 10 who delivered very prematurely within the first week postpartum. Mothers who breastfed their very preterm infant (GA <32 wks) or full-term infant (GA ≥ 37 wks) were eligible for inclusion. Exclusion criteria were multiple pregnancy, breast surgery, HELLP syndrome, pre-existing or gestational diabetes, autoimmune disease, life expectancy of the neonate of <72h, and/or major congenital anomalies of the neonate.

Study 2. We recruited 10 mothers who delivered at term within the first month postpartum. Healthy mothers who breastfed their full-term infants were eligible for inclusion. To minimize confounding influences such as lifestyle and psychological factors, we excluded mothers with the following: a prepregnancy BMI (in kg/m^2) <18.5 or >30 [14]; excess pregnancy weight gain of >16 kg for women with a prepregnancy BMI of 18.5 to 25 or >11 kg for women with a prepregnancy BMI of 25 to 30, according to 2009 Institute of Medicine/NRC guidelines [15]; medication use; alcohol consumption of >7 IU/wk (1 IU of beer = ~12 ounces, 1 IU of wine = ~4 ounces) [16]; fever > 38.5 ^{0}C [17]; and/or an Edinburgh Postnatal Depression Scale score >10 (of 30) in the third week postpartum [18-20].

SAMPLE COLLECTION

Study 1. Milk samples were obtained weekly during the first month postpartum, at the same time of day, to avoid bias related to the timing of collection. Weekly collections within the first month postpartum were chosen, because previous studies showed that macronutrient milk composition differed between term and very preterm milk, especially in the early stages of lactation [21,22]. One milliliter of foremilk was collected with a breast pump just before every feeding occasion, although cortisol concentrations were found to be equal in fore- and hindmilk, irrespective of lactation stage [23]. Samples were stored in polypropylene vials at –20 ^{0}C until analysis.

Study 2. Paired foremilk and saliva samples were collected in the fourth week postpartum, over a 24-h period. Samples were collected at each feeding occasion (7 to 8 times/24h). Mothers breastfed their children on demand and were therefore asked to write down the exact time of sample collection. In addition, we asked mothers to collect 2 additional samples early in the morning (between 0600 and 1200) to obtain a more accurate assessment of peak cortisol and cortisone (see **Figure 1**).

At each sampling occasion, 1 mL foremilk was collected according to the previously mentioned procedure. Simultaneously, with every milk sample, a saliva sample was

collected with a Salivette (Sarstedt). Participants were instructed to rinse their mouth with tap water to remove potential food residues, before saliva collection at home, which they did ≥30 min after brushing their teeth.

FIGURE 1. Study design of milk and saliva collections over 24h by healthy mothers who delivered at term. Mothers were asked to collect samples at each feeding occasion, every 3h on average (○). In addition, we asked them to collect 2 extra samples in between the morning feedings (☆). Mothers breastfed their children on demand; therefore times shown in the figure are only representative *(Study 2)*.

DETERMINATION OF CORTISOL AND CORTISONE CONCENTRATIONS IN BREAST MILK AND SALIVA

We used 0.5 mL of mother's milk to assess cortisol and cortisone concentrations with the use of an isotope dilution LC-MS/MS as described previously [24]. In short, internal standards ($^2H_4$-labeled cortisol and $^2H_8$-labeled cortisone) were added to the samples. Lipids were removed by washing the milk 3 times with 2 mL hexane. Samples were extracted and analyzed by online solid phase extraction coupled to LC-MS/MS (XLC-tandem MS [Spark Holland, Emmen, Netherlands]), coupled to a Quattro Premier XE tandem MS (Waters Corporation). The lower limit of quantitation was 0.5 nmol/L for cortisol and 0.3 nmol/L for cortisone. The intra-assay CVs were 4% and 5%at cortisol concentrations of 7 and 23 nmol/L and 5% at cortisone concentrations of 8 and 33 nmol/L, respectively. The interassay CVs were <9% for cortisol and cortisone. Cortisol and cortisone concentrations in saliva were determined with the same method as that for breast milk, but without the hexane-washing procedure.

DATA ANALYSIS

Continuous data were compared by using Mann-Whitney U tests, and dichotomous data were compared by using Fisher's exact tests. Generalized estimating equations (GEEs) were used for analyses of longitudinal data. GEEs adjust for grouped samples, collected from the same participant at different times, by using a correlation structure. For our data analyses, we chose an exchangeable correlation structure, in which 1 average within subject correlation between samples over time is assumed. Cortisol and cortisone concentrations were skewed to the right and logarithmically transformed before analysis. Therefore, GEE results are presented as exponentiated βs (95% CIs). $P < 0.05$ was defined as being significant.

Study 1. Stepwise GEEs were performed. First, the influence of very preterm delivery (as a dichotomous factor) on milk cortisol concentration was investigated. Second, the influence of time of collection (as a continuous factor) on milk cortisol concentration was investigated. Third, the influence of very preterm delivery on milk cortisol concentration, adjusted for time of collection, was investigated. Analyses were repeated for milk cortisone concentration and the cortisone-to-(cortisol + cortisone) ratio.

Study 2. Stepwise GEEs were performed to assess the diurnal pattern of milk cortisol concentrations. First, the influence of time of collection (as a continuous factor) on milk cortisol concentration over time was assessed in a linear model. Second, linear modeling was repeated after dividing the day into 2 parts on the basis of visual peak identification: the increase (i.e., 0000 to 0700) and the decline (i.e., 0701 to 2359). Third, the influence of time of collection (as a continuous factor) on milk cortisol concentration over time was assessed in a quadratic model (i.e., $y = a + bx + cx^2$, with y representing milk cortisol concentration and x the time of collection). Fourth, the influence of time of collection (as a continuous factor) on milk cortisol concentration over time was assessed in a cubic model (i.e., $y = a + bx + cx^2 + dx^3$). Analyses were repeated for milk cortisone concentration and the cortisone-to-(cortisol + cortisone) ratio. Associations between milk and salivary concentrations were tested by using Pearson correlation and linear regression analysis.

RESULTS

Study 1. **Table 1** presents baseline characteristics of the mothers who delivered very preterm compared with those who delivered full-term infants. All of the children had an appropriate size for GA.

TABLE 1. Characteristics of participants: Study 1.

	Mothers who delivered Full-term	Mothers who delivered Preterm
Age Mother, yr	33 (29 to 39)	33 (24 to 40)
Parity	2 (1 to 4)	1 (1 to 3)
Vaginal delivery	6 (60 %)	7 (70 %)
Gestational age, wks	39.0* (37.6 to 41.3)	28.7 (24.7 to 30.9)
Birth weight, g	3392* (2155 to 4064)	1058 (660 to 1845)
Glucocorticoid treatment for impending preterm	1* (10 %)	8 (80 %)

Values are median (ranges) or frequencies (%), n= 10 vs. 10 * P<0.05 between groups.

Table 2 shows the breast-milk cortisol and cortisone concentrations for both groups. During the first month postpartum, the median concentrations (ranges) in milk from mothers who delivered at term were 4.3 nmol/L (1.7 to 12.5 nmol/L) for cortisol, 29.6 nmol/L (15.9 to 44.0 nmol/L) or cortisone, and 0.86 (0.82 to 0.91) for the cortisone-to-(cortisol + cortisone) ratio. In milk from mothers who delivered very preterm the corresponding values were 3.2 nmol/L (1.1 to 6.1 nmol/L) for cortisol, 20.5 nmol/L (7.5 to 35.3 nmol/L) for cortisone, and 0.87 (0.82 to 0.91) for the cortisone-to-(cortisol + cortisone) ratio.

No differences were found between very preterm and term milk samples in cortisol (β: 0.59; 95% CI: 0.25 to 1.40; P = 0.23), cortisone (β: 0.60; 95% CI: 0.30 to 1.18; P = 0.14), or the cortisone-to-(cortisol + cortisone) ratio (β: 1.02; 95% CI: 0.96 to 1.08; P = 0.57) when assessed by GEE over time. When visually plotting the milk cortisol and cortisone concentrations with collection time, regardless of postnatal age, we found a pattern suggestive of diurnal rhythmicity (data not shown). Collection time was strongly associated with milk cortisol concentration (β: 0.90; 95% CI: 0.86 to 0.95), milk cortisone concentration (β: 0.94; 95% CI: 0.91 to 0.98), and the milk cortisone-to-(cortisol + cortisone) ratio (β: 1.01; 95% CI: 1.00 to 1.01) (all P < 0.01), when assessed by GEE. When adjusted for collection time, giving birth to a very preterm infant was associated with reductions in milk cortisol and cortisone concentrations of 50% (β: 0.50; 95% CI: 0.26 to 0.99; P = 0.05) and 53% (β: 0.53; 95% CI: 0.30 to 0.93; P = 0.03), respectively. The milk cortisone-to-(cortisol + cortisone) ratio was not related to prematurity.

TABLE 2. Glucocorticoid concentrations in breast milk of mothers who delivered very preterm vs. those who delivered at term: Study 1

	1wk postpartum		2wk postpartum		3wk postpartum		4wk postpartum	
	Preterm	Term	Preterm	Term	Preterm	Term	Preterm	Term
	n = 10	n = 10	n = 10	n = 10	n = 10	n = 7	n = 9	n = 9
Cortisol	1.2 (0.4 to 5.1)	2.5 (1.3 to 12.9)	3.2 (1.1 to 4.8)	4.3 (1.3 to 10.9)	4.0 (1.0 to 8.5)	5.2 (2.1 to 11.2)	5.6 (2.4 to 14.8)	4.6 (1.7 to 15.6)
Cortisone	15.5 (3.0 to 35.2)	31.2 (14.4 to 58.3)	22.8 (8.3 to 33.5)	27.9 (16.8 to 48.8)	22.0 (6.0 to 38.0)	38.5 (15.5 to 40.0)	20.5 (11.6 to 39.3)	29.8 (11.3 to 43.2)
Ratio	0.92 (0.89 to 0.93)	0.91 (0.83 to 0.94)	0.87 (0.85 to 0.90)	0.87 (0.82 to 0.90)	0.86 (0.82 to 0.88)	0.87 (0.78 to 0.92)	0.80 (0.72 to 0.85)	0.82 (0.73 to 0.88)

Values are median (IQRs). Unit of measurement is nmol/L. Ratio is calculated as Cortisone/ (Cortisol + Cortisone) No significant differences over time between preterm and term samples in milk cortisol, milk cortisone, or the cortisone-to-(cortisol+cortisone) ratio were observed.

Study 2. Table 3 presents the baseline characteristics of the mothers who delivered at term. The mean 6 SD interval between sample collections was 3:21 6 1:23 h. **Figure 2** shows diurnal variations in milk cortisol and cortisone concentrations and those of the cortisone-to-(cortisol + cortisone) ratio during week 4 postpartum. Peak milk cortisol and cortisone concentrations were reached at ~0700. At the same time, a nadir was observed for the milk cortisone-to-(cortisol + cortisone) ratio.

At ~0700 (0600 to 0800), milk cortisol was 10.3 nmol/L (4.2 to 46.4 nmol/L), milk cortisone was 38.6 nmol/L (23.1 to 52.2 nmol/L), and the milk cortisone-to-(cortisol + cortisone) ratio was 0.75 (0.46 to 0.89). In saliva obtained at the same time, cortisol was 6.1 nmol/L (2.2 to 19.9 nmol/L), cortisone was 24.7 nmol/L (13.2 to 50.1 nmol/L), and the cortisone-to-(cortisol + cortisone) ratio was 0.80 (0.69 to 0.87).

TABLE 3. Baseline characteristics of participants: Study 2

Mother's age, yr	33 (28 to 37)
Gestational age, wks	39 (37.1 to 41.3)
Prepregnancy BMI, kg/m²	22 (20 to 30)
Pregnancy weight gain, kg	12 (6 to 16)
EPDS score	4 (1 to 10)

Values are median (ranges), n= 10. EPDS = Edinburgh Postnatal Depression Scale

Table 4 presents the results of the GEEs. When analyzed linearly, time of collection was significantly associated with milk cortisol and cortisone concentrations, as well as the milk cortisone-to-(cortisol + cortisone) ratio. When splitting the day into 2 parts, a significant increase in milk cortisol and cortisone concentrations was observed before the peak at ~0700 and a significant decrease was observed after the peak. A reverse pattern was observed for the milk cortisone-to-(cortisol + cortisone) ratio (**Table 4, Figure 2**) Cubic modeling provided the best fit ($P < 0.01$) to describe the diurnal rhythm of milk cortisol, milk cortisone, and the milk cortisone-to-(cortisol + cortisone) ratio (**Supplemental Table 1, Supplemental Figure 1**).

TABLE 4. GEE-analysis of the association between time of collection and cortisol and cortisone levels, and the cortisone/(cortisol + cortisone) ratio in breast milk: Study 2

Dependent variable	Independent variable		ß (95% CI)	*P*-value
Cortisol	Time of collection		0.92 (0.89 ; 0.95)	<0.01
		0.00 - 7.00h	1.77 (1.55 ; 2.02)	<0.01
		7.00 - 23.59h	0.85 (0.83 ; 0.87)	<0.01
Cortisone	Time of collection		0.96 (0.93 ; 0.98)	0.01
		0.00 - 7.00h	1.35 (1.27 ; 1.44)	<0.01
		7.00 - 23.59h	0.91 (0.90 ; 0.92)	<0.01
Ratio	Time of collection		1.01 (1.00 ; 1.01)	<0.01
		0.00 - 7.00h	0.97 (0.95 ; 0.98)	<0.01
		7.00 - 23.59h	1.01 (1.01 ; 1.01)	<0.01

Values are exponentiated betas (95% CIs), n=10 mothers, who collected 92 milk samples in total. Cortisol and cortisone levels are measured in nmol/L

Milk and salivary concentrations of cortisol ($r = 0.92$, SEE = 2.8 nmol/L; $P < 0.01$) and cortisone ($r = 0.93$, SEE = 4.4 nmol/L; $P < 0.01$) as well as the cortisone-to-(cortisol + cortisone) ratio ($r = 0.64$, SEE = 0.06; $P < 0.01$) were correlated with one another. For cortisol, the increase in breast-milk concentration seemed to be disproportionate to the higher range of salivary concentrations (**Figure 3**).

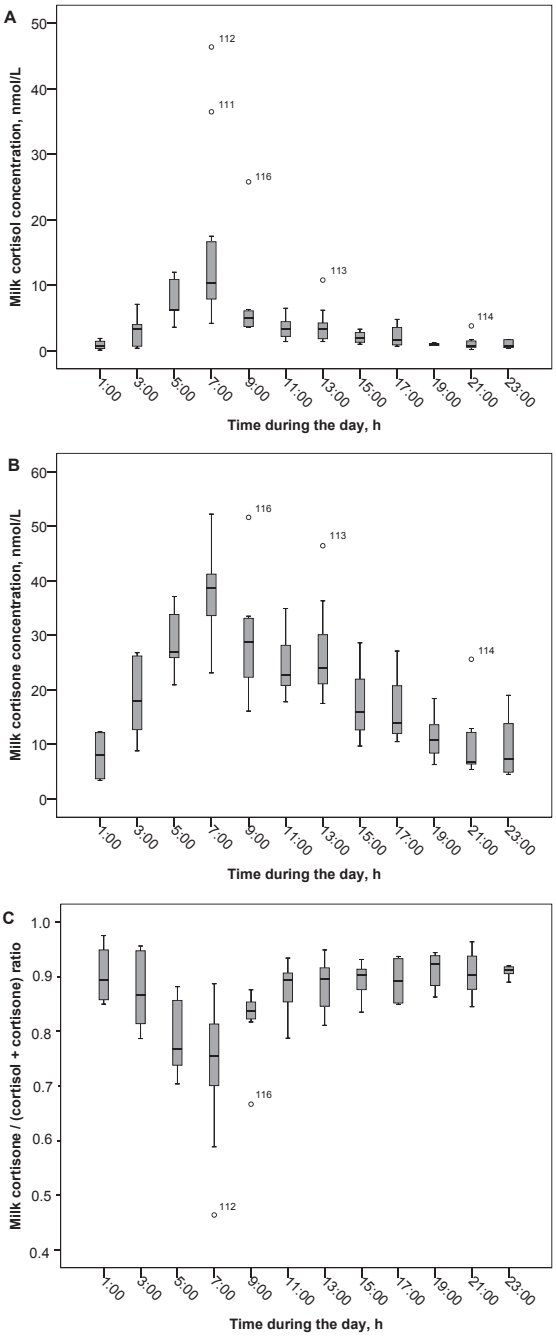

FIGURE 2. Boxplots showing medians and ranges of concentrations of milk cortisol (A) and milk cortisone (B) and the cortisone-to-(cortisol + cortisone) ratio (C) over 24 h during week 4 postpartum (Study 2). The circles represent outliers and are labeled with their anonymized study number. n = 10 mothers and 92 milk samples.

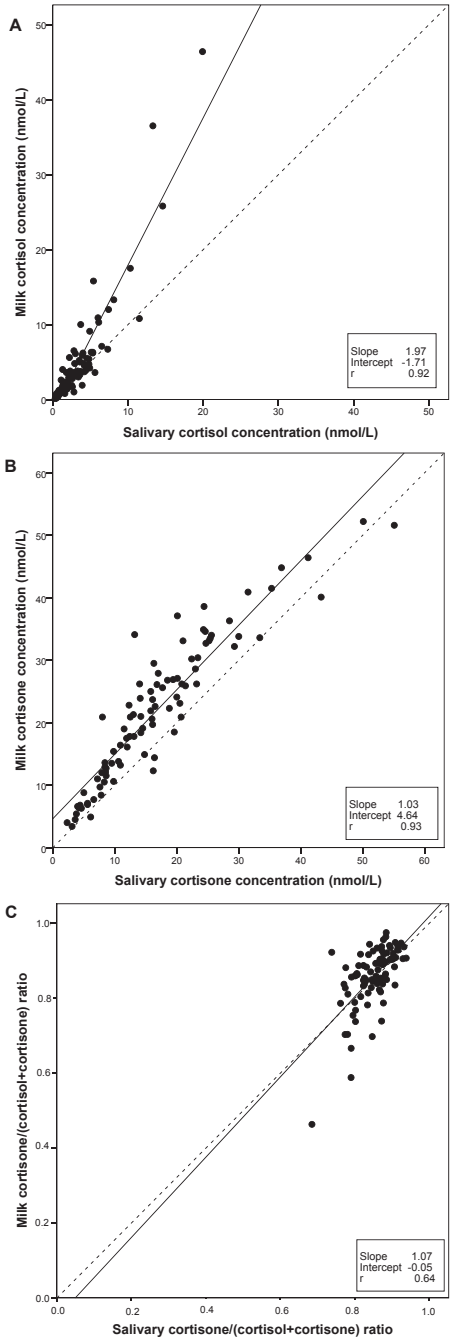

FIGURE 3. Comparison of concentrations of cortisol (A) and cortisone (B) and the cortisone-to-(cortisol + cortisone) ratio (C) in paired salivary and milk samples collected during week 4 postpartum. The dashed lines represent the line of equality, and the solid lines the line that best fits the data (Study 2). n = 92 paired samples.

DISCUSSION

To the best of our knowledge, the present study is the first to show diurnal rhythmicity of breast-milk cortisol and cortisone concentrations, with a peak at ~0700. Glucocorticoid concentrations in breastmilk and saliva were strongly correlated, with cortisone being higher than cortisol in both fluids. Giving birth to a very preterm infant was associated with a reduction in breast-milk glucocorticoid concentrations when adjusting for time of collection.

In our studies, breast-milk cortisol concentrations ranged between 0.1 and 97.4 nmol/L, and cortisone between 1.7 and 95.0 nmol/L. A similar variability was found in other studies, with the exception of one study that reported a mean milk cortisol concentration >20-fold higher [24], possibly due to blood contamination. Comparison between studies is difficult, because several studies used immunoassays, which are prone to cross-reactivity when measuring glucocorticoids. Due to its superior specificity, LC–MS/MS is the method of choice for steroid hormone analysis [25,26]. In addition, none of the previous studies took time of collection into account. In our first study, we asked participants to collect all samples at the same time of the day, although not specifically in the morning. However, time of collection was found to explain a large part of inter-individual variation in glucocorticoid concentrations, which was confirmed by our second study.

We expected that the stress surrounding very preterm birth would increase the breast-milk glucocorticoid concentrations of mothers. Instead, breast milk of mothers who delivered very preterm was found to contain lower glucocorticoid concentrations than milk of mothers who delivered at term, when corrected for time of collection. This can possibly be explained by pregnancy-induced maturational changes in the mammary gland. Previously, Tucker and Schwalm showed that the majority of the glucocorticoid molecules are bound to high affinity glucocorticoid binding sites in mammary tissue during lactation, whereas only a fraction of the circulating glucocorticoids are released into milk [27]. In addition, Flaxman showed that a combination of prolactin, insulin, and hydrocortisone enhanced the proliferation of lactating cells *in vitro* [28].

The lower milk cortisol and cortisone concentrations could also be explained by the nutritional composition of breast milk, because protein and fat contents are higher in very preterm milk [21,22]. These nutrients are tightly correlated with milk cortisol, at least in nonhuman primates[29]. However, protein and fat contents were not assessed

in our milk samples. Furthermore, the maternal HPA axis could be suppressed by synthetic glucocorticoids. These were administered to the majority of mothers who delivered very preterm for acceleration of fetal lung maturation, whereas only one mother who delivered at term received synthetic glucocorticoids. However, this short course is unlikely to result in long-lasting HPA axis suppression [30].
During early development, cortisone concentrations are much higher than cortisol concentrations in the majority of tissues [31]. After birth, tissue concentrations of cortisol gradually increase, which could either be attributed to an increase in 11βHSD type 1 activity or a decrease in 11βHSD type 2 activity [31]. In our study samples, similar to saliva but contrasting with serum, breast-milk cortisone concentrations exceeded those of milk cortisol. It is unknown whether this reflects increased 11βHSD type 2 activity in the mammary gland or better penetrance of serum cortisone into breast milk relative to cortisol.

Animal studies indicate that hormones present in breast milk can be absorbed by the neonatal gut, which is accompanied by an attenuated gastric acid secretion [32]. Locally [32], as well as after absorption in the systemic circulation [33], milk-borne hormones such as glucocorticoids are postulated to play a role in the proliferation and differentiation of the intestinal epithelium. This might be an explanation for the reduction in necrotizing enterocolitis and late-onset sepsis in breastfed, compared with formula fed, very preterm infants [4-6]. In our laboratory, milk cortisol and cortisone concentrations were below the lower limit of quantitation in standard infant formulas (unpublished observations of our research group, 2015). Breast-milk glucocorticoids have been suggested to affect neurodevelopment in primate offspring [29,34]. Studies in humans are limited but suggest similar effects [35,36]. If these findings in animals apply to humans, there might be implications for donor milk banks. Milk banks often provide very preterm newborns with milk from mothers who delivered at term, which we have shown to be richer in glucocorticoids. Heating during pasteurization has the potential to affect the concentrations and activities of hormones such as insulin and leptin [8]. Whether this also applies to steroid hormones is not exactly known, although they tend to be more stable.

The major strength of this study is the detailed assessment of the diurnal variation and the use of our recently developed, reliable isotope dilution LC–MS/MS assay[24]. However, a limitation of our study is the small sample size. Nevertheless, our study was adequately powered and significant results were found. In addition, our findings can be used to design a larger study, aimed at exploring whether the diurnal rhythm in breastmilk glucocorticoids exerts biologically relevant effects on the developing

newborn. This has also been suggested for other chronobiotic signaling factors in human milk, such as melatonin, which might guide the development of a sleep-wake rhythm in infants [37].

In conclusion, in mothers who gave birth to a very preterm infant, breast-milk glucocorticoid concentrations were lower. In addition, we showed that they follow a diurnal rhythm, highly correlated to the circadian rhythm of HPA axis activity. Little is known about the effect this has on the developing newborn, so future studies are needed to elucidate this.

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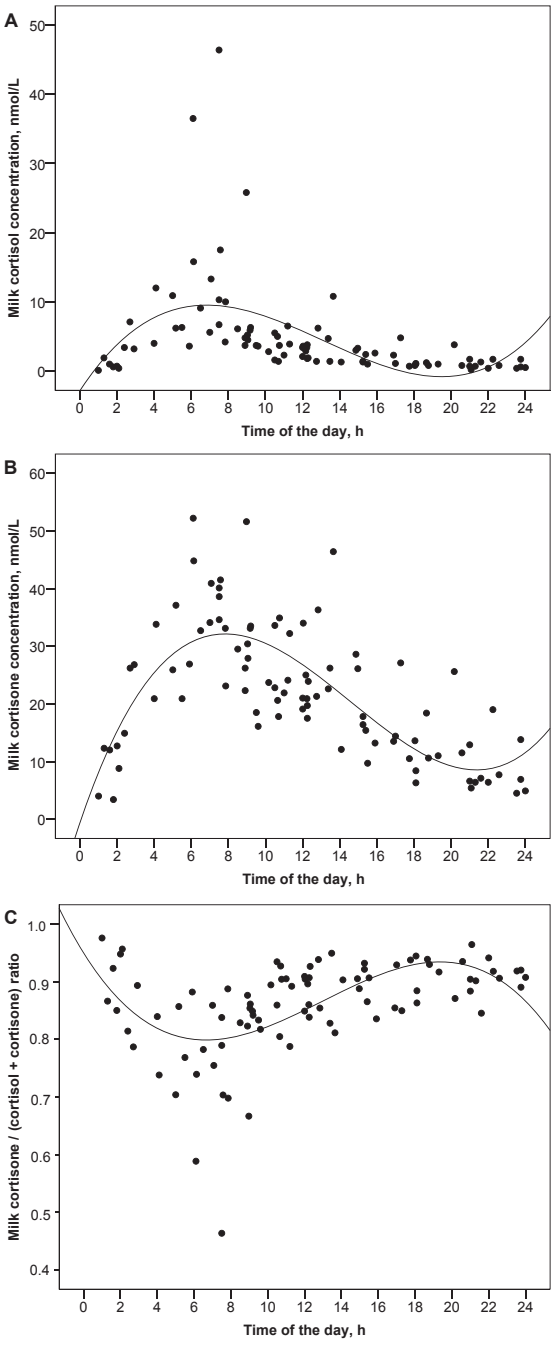
SUPPLEMENTAL TABLE 1. Cubic model of the association of collection time with milk cortisol, milk cortisone and the milk cortisone/(cortisol + cortisone) ratio during 24 hours in week 4 postpartum: Study 2

Dependent variable	Independent variables	ß (95% CI)	*P*-value
Cortisol, nmol/L	Time	2.62 (1.89 to 3.64)	< 0.01
	(Time * Time)	0.92 (0.89 to 0.94)	< 0.01
	(Time * Time * Time)	1.00 (1.00 to 1.00)	< 0.01
Cortisone, nmol/L	Time	1.65 (1.40 to 1.95)	< 0.01
	(Time * Time)	0.96 (0.95 to 0.97)	< 0.01
	(Time * Time * Time)	1.00 (1.00 to 1.00)	< 0.01
Ratio	Time	0.94 (0.91 to 0.97)	< 0.01
	(Time * Time)	1.01 (1.00 to 1.01)	< 0.01
	(Time * Time * Time)	1.00 (1.00 to 1.00)	< 0.01

Values are exponentiated betas (95% CI), n = 10 mothers; 92 milk samples.
Ratio is calculated as cortisone/(cortisol + cortisone).



SUPPLEMENTAL FIGURE 1. Scatter plot with the estimated cubic model in red, describing the diurnal variation of milk cortisol (A), milk cortisone (B) and the cortisone/(cortisol + cortisone) ratio (C) in week 4 postpartum: Study 2

CHAPTER 7

STABILITY OF CORTISOL AND CORTISONE IN HUMAN BREAST MILK DURING HOLDER PASTEURIZATION

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ABSTRACT

Human donor milk is the feeding of choice for preterm infants, when own mother's milk is not available. Holder pasteurization is necessary to secure the safety of donor milk, although it can affect milk quality by reduction of nutritional and bioactive components. Recently, research has focused on the potential role of breast-milk glucocorticoids for infant development. At this moment, it is unknown whether pasteurization affects milk glucocorticoid levels. Therefore, we assessed whether Holder pasteurization, the most frequently used method nowadays, reduces breast-milk cortisol and cortisone levels, using breast milk samples from 30 women who delivered at term. We found tight correlations between pre- and post-pasteurization levels of cortisol ($R^2 = 0.99$) and cortisone ($R^2 = 0.98$), as well as good agreement in Passing and Bablok regression analysis. In conclusion, cortisol and cortisone in human term breast milk are not significantly affected by Holder pasteurization.

INTRODUCTION

When own mother's milk is not available, human donor milk is advised as the second-best nutrition for preterm infants, because of its supportive effects on infant development [1]. Pasteurization of donor milk is imperative, minimizing transmission of pathogens. Currently, the most commonly used process by human milk banks for pasteurization of donor milk is Holder pasteurization, which includes 30 minutes of heating at 62.5°C followed by fast cooling [2]. Although this heating process can affect the milk quality by the reduction of nutritional and bioactive components, and inactivation of beneficial microbiota [1,3], it is thought to be the best available compromise between safety and quality [2].

A variety of non-nutritive bioactive components have been detected in human milk, including hypothalamic, pituitary, thyroid, parathyroid, adrenal, gastrointestinal and growth hormones [4]. We found that breast-milk glucocorticoids were highly correlated to maternal HPA axis activity [6]. Moreover, animal studies have shown that milk glucocorticoids are readily absorbed by the neonatal intestine, exerting a local, as well as a systemic anti-inflammatory effect on the gastrointestinal tract of the newborn [4,7]. In addition, observational studies suggested that milk cortisol influences infant neurodevelopment [8,9].

In infants born preterm the HPA axis is not yet fully maturated and is relatively insufficient during the first weeks of life, reflected by low cortisol levels for the degree of illness as well as increased risks of hypotension and hypoglycemia [10]. Glucocorticoids in breast milk could provide resilience against potentially life-threatening illnesses.

The bioavailability of some milk hormones has been shown to be altered by Holder pasteurization and reduced concentrations of insulin-like-growth-factors, growth hormone, leptin, adiponectin and insulin have been described [1]. There is little evidence with regard to the effects of Holder pasteurization on glucocorticoid levels in human milk [5]. The objective of this study was therefore to determine whether Holder pasteurization affects milk cortisol and cortisone concentrations, thereby influencing the bioavailability of milk glucocorticoids in human donor milk.

METHODS

The Dutch Human Milk Bank (located at VUMC) provided milk from 30 mothers who gave birth to a term infant and donated their breast milk. Written informed consent was obtained from all donors prior to study enrollment. All donors who are donating to the Dutch Human Milk Bank are women who produce more breast milk than their own infant needs. They donate their spare milk voluntarily. Donors are not actively recruited. They register themselves, after which they receive more information. Prior to the first donation, donors were screened according to international guidelines, and milk was collected by standardized procedures (described at https://www.moedermelkbank.nl). In short, donors collected breast milk at home in disposable bisphenol A free bottles (Sterifeed, Medicare Colgate Ltd, Devon, England), by use of a breast milk pump. They were asked to express all milk, including fore and hind milk, from one breast, completely. Immediately afterwards, the milk was stored at -20°C upon Holder pasteurization (30 minutes at 62.5°C) [1].

Samples that were used for this study were randomly selected, spare samples of pooled milk that was used to feed infants at the NICU (n = 30). A pooled sample contained milk from one mother collected at different moments during several days. As part of standard care, one pre- and one post-pasteurization sample from each pooled sample was stored. When safe administration of the donor milk to an infant was assured, these spare samples could be used for our study.

Pre- and post-pasteurisation samples were analysed with the use of an isotope dilution LC–MS/MS method as described previously [11], although with a slightly adjusted extraction method. In short, 0.5 mL of milk was used to assess cortisol and cortisone concentrations. Internal standards ($^{13}C_3$-labeled cortisol and $^{13}C_3$-labeled cortisone) were added to the samples. Lipids were removed by washing the milk 3 times with 2 mL hexane. Samples were extracted using Isolute plates (Biotage, Uppsala, Sweden) and analyzed by LC–MS/MS (Acquity with Quattro Premier XE, Waters Corporation). The lower limit of quantitation was 0.5 nmol/L for cortisol and 0.3 nmol/L for cortisone. The intra-assay CVs were 4% and 5% at cortisol concentrations of 7 and 23 nmol/L, and 5% at cortisone concentrations of 8 and 33 nmol/L, respectively. The interassay CVs were <9% for cortisol and cortisone.

The concentration found in the pre-pasteurization sample was set at 100% and plotted against the post-pasteurization concentration. Concentrations of cortisol and cortisone pre- and post-pasteurization were compared using Pearson correlation and

Passing and Bablok regression analysis. Assuming that pre- and post-pasteurization concentrations are equal, the intercept (95% CI) of the Passing and Bablok regression line should not differ from zero and the slope (95% CI) should not differ from one.

RESULTS

Thirty mothers provided milk samples at a median postpartum age of 18.1 weeks (range: 1.3 to 57.7). Their samples were collected at several moments during a time period ranging from 2 to 70 days (median 13.5). Their infants were born at a median gestational age of 40.2 weeks (range: 37.7 to 42.1), and 15 (50%) of them were male. **Table 1** shows mean cortisol and cortisone levels pre- and post-pasteurization. Pre- and post-pasteurization levels correlated highly: r = 0.997, R^2 =0.99, P < 0.01 for cortisol, and r = 0.990, R^2 = 0.98, P < 0.01 for cortisone.

TABLE 1. Mean breast-milk glucocorticoid levels pre- and post-pasteurization, n =30.

	Pre-pasteurization	Post-pasteurization
	Mean ± SD (range)	**Mean ± SD (range)**
Milk cortisol (nmol/L)	7.93 ± 4.88 (0.8 to 19.7)	7.96 ± 5.05 (0.8 to 19.8)
Milk cortisone (nmol/L)	20.98 ± 6.24 (3.7 to 29.6)	21.19 ± 6.36 (3.7 to 30.2)

Figure 1 shows the Passing and Bablok regression analysis, displaying good agreement between pre- and post-pasteurization concentrations. For cortisol the equation was cortisol post-pasteurization = 1.00 * cortisol pre-pasteurization, with the intercept ranging from -0.22 to 0.07 and the slope ranging from 0.98 to 1.04.

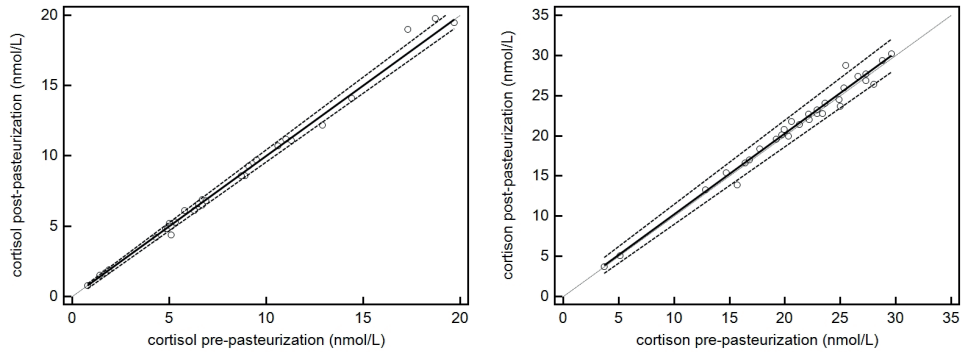

FIGURE 1. Passing and Bablok regression analysis of cortisol and cortisone in paired pre- and post-pasteurization milk samples. N = 30 paired samples.

For cortisone the equation was cortisone post-pasteurization = 0.14 + 1.01 * cortisone pre-pasteurization, with the intercept ranging from -0.66 to 0.99 and the slope ranging from 0.97 to 1.05.

DISCUSSION

Our study demonstrated that Holder pasteurization of human donor milk does not lead to a reduction in milk cortisol and cortisone levels. The levels found in the donated samples were within the range found in other studies [6,11]. Combined with results from our previous study in which we showed that human milk cortisol and cortisone levels are stable during 36h at room temperature and during multiple freeze-thaw cycles [11], we can now state that processing of human milk by donor milk banks does not affect milk cortisol and cortisone levels.

Steroid hormones are not thermolabile; cholesterol is the substrate for steroid biosynthesis and consists of carbon-based ring structures, making the molecule rather stabile below the melting point (148.5 ^{0}C) [12]. Therefore our results which showed that heating at 62.5°C for 30 minutes did not affect breast-milk cortisol and cortisone levels, were in line with our expectations. In contrast, protein hormones, such as insulin-like-growth-factors, growth hormone, leptin, adiponectin and insulin can denature upon heating and this explains decreased concentrations upon pasteurization [1]. Although, one study previously reported a possible heat-induced transformation of steroidal anti-inflammatory drugs in bovine milk [13], due to the thermostabile characteristics of steroid hormones, it is reasonable to assume that the unchanged milk glucocorticoidal levels retained their functionality.

The presence of milk glucocorticoids in donor human milk might exert direct and indirect beneficial effects on the gastro-intestinal tract of the vulnerable preterm infant. Evidence for direct effects were found in animal studies in which administration of glucocorticoids was found to result in maturation of neonatal intestinal enzymatic activity [14]. In addition in human studies on preterm infants, administration of antenatal glucocorticoids was found to result in a reduced incidence of necrotizing enterocolitis and a decrease in intestinal permeability [15,16]. Simultaneously, milk glucocorticoids might exert an indirect beneficial effect via the microbiome, and via human milk oligosaccharides (HMOs). Although Holder pasteurization is known to inactivate the bacteria of human milk [17], HMOs remain unaltered in donor milk [2]. These HMOs have the potential to support growth of Bifidobacterium in the gastrointestinal tract, which

have been positively associated with immunological protection, gastrointestinal health and disease [18].

In conclusion, Holder pasteurization does not affect cortisol and cortisone levels of term human breast milk. Whether these milk glucocorticoids maintain their functionality after the heating procedure and have the ability to exert effects in the vulnerable preterm, is yet unknown.

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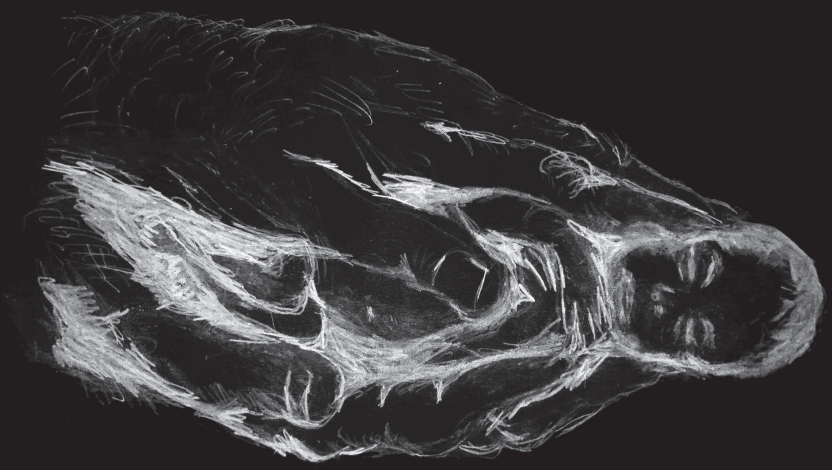
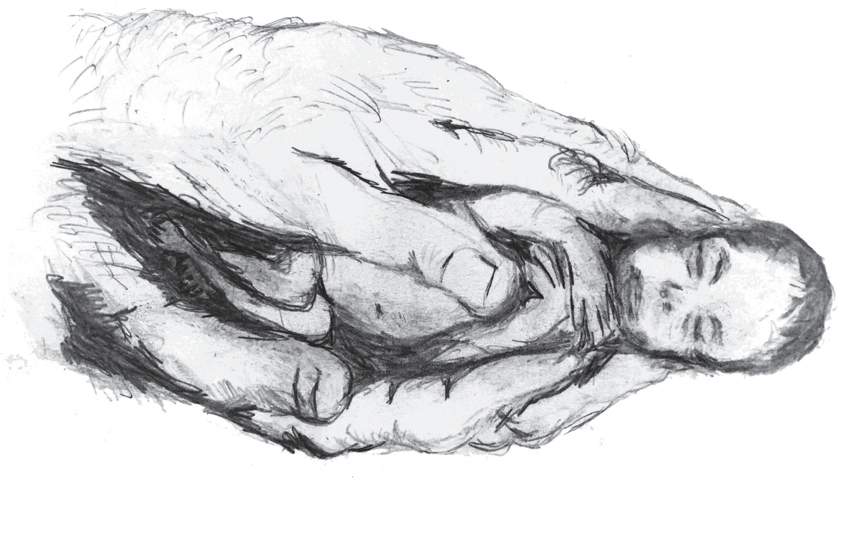
PART II
LONG -TERM CORRELATES





CHAPTER 8

ANTENATAL GLUCOCORTICOID TREATMENT AND POLYMORPHISMS OF THE GLUCOCORTICOID AND MINERALOCORTICOID RECEPTORS ARE ASSOCIATED WITH IQ AND BEHAVIOR IN YOUNG ADULTS BORN VERY PRETERM

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ABSTRACT

BACKGROUND

Preterm survivors exhibit neurodevelopmental impairments. Whether this association is influenced by antenatal glucocorticoid treatment and glucocorticoid sensitivity is unknown. This study aimed to study the effects of antenatal glucocorticoid treatment and glucocorticoid receptor (*GR*) and mineralocorticoid receptor (*MR*) polymorphisms on behavior and intelligence quotient (IQ).

METHODS

This multicenter study was part of the 19-year follow-up of the Project On Preterm and Small-for-gestational-age birth cohort. Three hundred forty-four 19-year-olds born very preterm (gestational age < 32 wk), of whom 71 had received a single antenatal treatment course of betamethasone. Main Outcome Measures: Behavior (Young Adult Self Report and Young Adult Behavior Checklist for parents) and IQ (digital Multicultural Capacity Test-intermediate level). Data were analyzed by linear regression and presented as regression coefficient (95% CI).

RESULTS

Sex ratio, *GR* (R23K; N363S) and *MR* (-2G/C; I180V) genotypes were equally distributed between treated and nontreated subjects. Independent of treatment, R23K carriers had improved IQ scores (β 9.3; 95% CI, 3.4 to 15.1) and a tendency toward more favorable total problem behavior scores (β -8.5;95%CI,-17.3 to 0.2) ; -2G/C CC carriers had poorer IQ scores (β -6.2;95%CI,-10.5 to -1.9); I180V carriers had more favorable internalizing behavior scores (β -2.0; 95% CI, -3.9 to -0.1). Antenatal glucocorticoid treatment was associated with more unfavorable behavior scores, especially internalizing behavior (β 2.4; 95% CI, 0.3 to 4.5). Interaction between GR and MR polymorphisms and antenatal glucocorticoid treatment was observed, with poorer IQ scores for exposed N363S carriers; poorer intellectual subdomain scores for exposed I180V-carriers; more favorable total problem behavior scores for exposed R23K carriers.

CONCLUSIONS

Genetic variations in glucocorticoid sensitivity and antenatal glucocorticoid treatment are associated with IQ and behavior in young adult preterm survivors.

INTRODUCTION

Children born prematurely have decreased brain volumes as well as higher frequencies of cognitive impairments and problems with internalizing behavior and attention [1,2]. During fetal maturation, development of the central nervous system follows a specific, coordinated chain of ontogenetic events [3]. Antenatal glucocorticoid treatment could disturb these processes [3,4].

Most women at risk of premature birth are treated with glucocorticoids to stimulate fetal lung maturation. This is highly effective in reducing short term mortality and morbidity [5]. However, despite these benefits, evidence from animal studies shows that exposure of the fetus to excess glucocorticoids can permanently alter neuroendocrine function, behavior and memory [3,4]. Accordingly, antenatal glucocorticoid treatment may affect the ontogenesis of the immature brain, causing life-long deleterious effects [3,4].

In 2006, a Cochrane meta-analysis, based on six studies, reported that antenatal glucocorticoid treatment is not associated with long term neurodevelopmental deficits [5]. However, 3 out of the 6 studies included in this meta-analysis were not limited to very preterm subjects (GA <32wks) [6-8]. Furthermore, 3 out of the 6 studies reported on the risk to develop a psychiatric disorder or become severely handicapped, and did not take subtle impairments into account [7,9,10].

The neural actions of glucocorticoids depend on the sensitivity of brain regions to these hormones, mediated by two corticosteroid receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) [3,11,12]. Polymorphisms of the *GR* and *MR* genes contribute to inter-individual variations in glucocorticoid sensitivity [11,13], cognitive performance and behavior [14,15]. Within the *GR* gene, the R23K (rs 6190) and N363S (rs 6195) SNPs have been associated with decreased and increased GR sensitivity, respectively [16]. Within the *MR* gene, the -2G/C (rs 2070951) and the I180V SNPs (rs5522) have been implicated in influencing MR sensitivity. The I180V SNP has been associated with decreased MR sensitivity and the CC-genotype of the -2G/C SNP is thought to increase MR sensitivity compared to the GG-genotype, although this was not a universal finding [17].

To date, no study has been performed in very preterm survivors, addressing the effects of antenatal synthetic glucocorticoid exposure and common variations in *GR* and *MR* genes on later cognitive and behavioral functioning. Therefore, we studied in young adults born very preterm the effects of antenatal glucocorticoid treatment, and GR and MR SNPs, as well as their interaction, on behavior and IQ.

METHODS

STUDY POPULATION

This study was part of the Project on Preterm and Small-for-Gestational-Age (POPS) study that recruited in 1983 at birth 94% (n=1,338) of all live born very preterm (GA <32 wks) and/or very low birth weight (<1,500 g) neonates in the Netherlands. The aim of this nationwide, multicenter, prospective follow-up study was to investigate causes of mortality and morbidity among this group [18]. The flowchart in **figure 1** represents the stepwise inclusion of subjects from the POPS birth cohort for the present study.

FIGURE 1. Flowchart of the inclusion procedure.

Responders and non-responders at age 19 did not differ in the perinatal risk factors small for gestational age (SGA) and mechanical ventilation to men, non-whites and subjects born to low-educated mothers were underrepresented [19]. Approval of the medical ethical committees of all participating centers was obtained, as well as written informed consent from all participants.

GLUCOCORTICOID TREATMENT

Seventy-one subjects had received antenatal glucocorticoid treatment. The antenatal glucocorticoid treatment protocol at that time consisted of two doses, with a 24-h interval, of 12 mg betamethasone. It is acknowledged that the side effects of a therapy can be studied with an observational study as reliably as with a randomized controlled trial, provided that these cannot be predicted from clinical data at the time of prescription [20]. In analogy, for the POPS study we have previously argued [21] that it is plausible that the limited knowledge in 1983 about long-term neurobiological side effects of antenatal glucocorticoid treatment precluded obstetricians from guiding their decisions by relevant prognostic data (e.g., level of parental education).
Postnatal glucocorticoid treatment had been given to 3 subjects who had also received glucocorticoids antenatally and to 27 subjects who had not received antenatal treatment.

SNPS OF THE GLUCOCORTICOID AND MINERALOCORTICOID RECEPTORS

Two SNPs in the *GR*, R23K (rs 6190) and N363S (rs 6195), and two in the *MR*, -2G/C (rs 2070951) and I180V (rs 5522), were analyzed. PCRs were performed using 2.5 ng of genomic DNA and standard reagents. SNPs were genotyped by mass spectrometry (homogeneous mass array system to Sequenom Inc., San Diego, CA), using standard conditions. Genotypes were analyzed, using Genotyper 3.0 software (Sequenom).

IQ

Intellectual functioning was assessed in 2002 by the digital Multicultural Capacity Test–Intermediate Level, developed and validated by Bleichrodt and Van den Berg in 2000 [22].

The test derives an IQ-score, indicating the level of general cognitive performance, together with scores on a wide spectrum of intellectual dimensions, giving a broader perspective on cognitive functioning. Overall, this test represents a person's capacities and skills: verbal and numerical intelligence, spatial visualization, speech fluency, memory, reasoning, and speed of perception. The test is validated for persons aged ≥ 16 years, from different backgrounds, whose level of education ranges from 5 years of secondary school to university. In the normal Dutch population this test reports an IQ-score of (mean ± SD) 100 ± 15 [22].

BEHAVIOR

Behavior was assessed by the young adult self-report (YASR) and the young adult behavior checklist (YABCL) for parents. These questionnaires are based on, respectively 130 and 109 items assessing behavior on a 3-point scale: 0 (not true), 1 (somewhat true) and 2 (often true). Both tests provide syndrome scores (anxious/depressed, withdrawn, somatic complaints, aggressive behavior, delinquent behavior, thought problems, attention problems, intrusive behavior and other problems), total sum-scores as well as internalizing and externalizing sum-scores. Internalizing behavior is calculated as the sum of anxious/depressed and withdrawn behavior. Externalizing behavior is calculated as the sum of aggressive, delinquent, and intrusive behavior [23]. A high score indicates that the person shows more of the related problem behavior.

STATISTICAL ANALYSES

IQ-scores were normally distributed and presented as mean ± SD. YASR and YABCL scores were skewed to the right, which did not change after logarithmic

transformation. Therefore, we decided not to correct for skewness and to present these as mean ± SD, in line with previous analyses in this cohort [23]. Given the sex specific differences in neurological outcome in previous analyses of this cohort [23,24], all analyses were corrected for sex.
Statistical analysis was done by stepwise linear regression, with IQ or behavior as dependent factor. First, the influence of antenatal glucocorticoid treatment on IQ and behavior was investigated using multivariate regression, with antenatal glucocorticoid treatment and sex as independent factors. Second, the influence of the GR and MR polymorphisms on IQ and behavior were independently analyzed using multivariate regression, with the SNP concerned and sex as independent factors. Third, the interaction term, antenatal glucocorticoid treatment and the SNP concerned were added to the model. Fourth, we repeated all analyses, corrected for perinatal risk factors and socioeconomic status (**table 1**).

RESULTS

The allele frequencies of these polymorphisms in our sample, of 4.0%, 2.6%, 48.5% and 9.9% respectively, were well in range with those observed in the normal population [17, 25, 26]. The genotype distributions were in agreement with the distribution predicted by the Hardy-Weinberg equilibrium, except for the -2G/C SNP. This has been described earlier in a normal Caucasian population sample [17]. Compound SNP carriage was not present for the GR polymorphisms, in contrast to the MR SNPs. In addition, intercourse compound variation (the combination of both a GR and a MR polymorphism) was observed in a small number of subjects (**table 2**).
Table 1 shows that perinatal variables, sex, low socioeconomic status and GR and MR SNPs were equally divided between treated and non-treated subjects.

Table 3 presents the relation between antenatal glucocorticoid treatment versus IQ and behavioral problem scores. Antenatal glucocorticoid treatment was not associated with IQ-score. Antenatal glucocorticoid treatment was associated with unfavorable scores on self-reported and parent-reported internalizing behavior and with unfavorable scores on parent-reported externalizing behavior and total problem scores.

Table 4 shows the relation between the GR polymorphisms versus IQ, YASR and YABCL scores. Carriers of the R23K SNP had a significantly higher IQ-score compared to non-carriers, as well as higher Z-scores of the intellectual subdomains logical

reasoning and spatial visualization and a trend (p=0.06) towards a higher Z-score for mathematical capacity. Carriage of the R23K SNP was not related to self-reported behavior scores. Carriage of the R23K SNP was associated with a decrease in parent-reported attention problems and a trend (p= 0.06) towards a decrease in parent-reported total sum score for problem behavior. Interaction between the R23K SNP and antenatal glucocorticoid treatment (n=8) was observed, with a decrease of -4.1 points (95% CI -7.7 to -0.4 [p=0.03]) in parent-reported other problems and a trend towards a decrease in parent-reported total sum score for problem behavior (-16.3 [95% CI -34.5 to 1.9] p=0.08).

TABLE 1. Baseline characteristics

		Antenatal glucocorticoid treatment (n=71)	No treatment (n=273)	P-value
Gestational age (wks)		29.5 ± 1.5	29.9 ± 1.5	0.07
Birth weight (g)		1311 ± 322	1332 ± 340	0.62
Birth weight (sds)		-0.1 ± 0.8	-0.2 ± 1.0	0. 40
SGA < -1 SD		8 (11%)	50 (18%)	0.16
Male		42 (59%)	132 (48%)	0.11
Postnatal glucocorticoid treatment		3 (4%)	27 (10%)	0.13
Necrotizing enterocolitis		2 (3%)	19 (7%)	0.19
Apgar score <7 5 minutes postpartum		5 (7%)	35 (13%)	0.14
Neonatal convulsions		1 (1%)	10 (4%)	0.34
Low socioeconomic status		25 (35%)	98 (36%)	0.91
GR genotype	R23K carrier	8 (11%)	19 (7%)	0.24
	N363S carrier	4 (6%)	13 (5%)	0.75
MR genotype	-2G/C CC genotype	16 (22%)	52 (20%)	0.59
	-2G/C GG genotype	17 (24%)	61 (23%)	0.87
	I180V carrier	11 (16%)	51 (19%)	0.40

Continuous variables are presented as mean ± SD and compared with an independent t-test.
Dichotomous variables are presented as N (percentages) and compared with a Chi-Square test.
Data are shown for subjects who received antenatal glucocorticoid treatment and subjects who did not.

TABLE 2. Genetic characteristics

		GR genotype		MR genotype		
		R23K Carrier (N=27)	N363S Carrier (N=17)	-2G/C CC genotype (N=68)	-2G/C GG genotype (N=78)	I180V Carrier (N=62)
GR genotype	R23K carrier	-	0	6	8	4
	N363S carrier	0	-	2	7	2
MR genotype	-2G/C CC genotype	6	2	-	-	24
	-2G/C GG genotype	8	7	-	-	0
	I180V carrier	4	2	24	0	-

Dichotomous variables are presented as N

TABLE 3. Relation between antenatal glucocorticoid treatment versus IQ, YASR and YABCL scores.

IQ	**IQ-score**	-1.2 (-5.3 to 2.8)
	Linguistic capacity Z-score	-0.1 (-0.3 to 0.1)
	Mathematical capacity Z-score	-0.1 (-0.4 to 0.1)
	Logical reasoning Z-score	-0.1 (-0.4 to 0.1)
	Spatial visualization Z-score	-0.1 (-0.4 to 0.1)
YASR	**Internalizing sum score**	2.4 (0.3 to 4.5) *
	Externalizing sum score	0.7 (-0.8 to 2.1)
	Total sum score	5.2 (-0.8 to 11.2)
	Anxious/depressed	1.8 (0.2 to 3.4) *
	Withdrawn	0.6 (-0.04 to 1.3)
	Somatic complaints	0.6 (-0.3 to 1.5)
	Aggressive behavior	0.5 (-0.2 to 1.3)
	Delinquent behavior	0.1 (-0.4 to 0.5)
	Thought problems	0.2 (0.0 to 0.5) *
	Attention problems	0.2 (-0.4 to 0.8)
	Intrusive behavior	0.1 (-0.5 to 0.6)
	Other problems	1.0 (-1.0 to 3.0)
YABCL	**Internalizing sum score**	3.9 (2.2 to 5.7) *
	Externalizing sum score	3.2 (1.3 to 5.1) *
	Total sum score	13.3 (7.5 to 19.2) *
	Anxious/depressed	2.9 (1.5 to 4.2) *
	Withdrawn	1.1 (0.6 to 1.6) *
	Aggressive behavior	2.0 (0.8 to 3.3) *
	Delinquent behavior	0.8 (0.4 to 1.2) *
	Somatic complaints	0.8 (0.1 to 1.4) *
	Thought problems	0.8 (0.4 to 1.2) *
	Attention problems	1.9 (0.7 to 3.0) *
	Intrusive behavior	0.4 (-0.2 to 0.9)
	Other problems	2.8 (1.6 to 4.0) *

IQ, intellectual subdomain, YASR and YABCL scores are presented as regression coefficient (95% CI) and are corrected for sex. * p<0.05

Carriage of the N363S SNP was not related to IQ score or the intellectual subdomains, except for linguistic capacity, which tended to be higher (p=0.06). Carriage of the N363S SNP was not related to self-reported or parent-reported behavior. Interaction between the N363S SNP and antenatal glucocorticoid treatment (n=4) was observed, with a decrease in the intellectual subdomains linguistic capacity of -1.2 SD (95% CI -2.3 to -0.1 [p=0.04]) and logical reasoning of -1.4 SD (95% CI -2.5 to -0.4 [p=0.01]), as well as a trend towards a decrease in IQ-score (-16.4 [95% CI -34.0 to 1.2] p=0.07).

Table 5 shows the relation between the MR polymorphisms versus IQ, YASR and YABCL scores. The -2G/C GG-genotype was not related to IQ, YASR or YABCL scores, except for parent-reported intrusive behavior and other problems. The -2G/C CC-genotype was associated with a decrease in IQ-score, as well as with decreases in all intellectual subdomains, except for spatial visualization. The -2G/C CC-genotype was not related to YASR or YABCL scores. Interaction between -2G/C variation and antenatal glucocorticoid treatment (n=33) was observed and was associated with a

decrease of -0.7 SD (95% CI -1.2 to -0.1 [p=0.02]) in spatial visualization in exposed carriers of the GG-genotype, as well as a decrease of -0.7 points (95% CI -1.3 to -0.1 [p=0.02]) for self-reported thought problems and an increase of 4.2 points (95% CI 1.0 to 7.4 [p=0.01]) for parent-reported aggressive behavior in exposed carriers of the CC-genotype.

TABLE 4. Relation between GR polymorphisms versus IQ, YASR and YABCL scores.

		R23K SNP	N363S SNP
IQ	**IQ-score**	9.3 (3.4 to 15.1) *	5.8 (-1.9 to 13.4)
	Linguistic capacity Z-score	0.2 (-0.1 to 0.5)	0.4 (-0.01 to 0.8)
	Mathematical capacity Z-score	0.4 (-0.01 to 0.8)	0.1 (-0.4 to 0.6)
	Logical reasoning Z-score	0.4 (0.1 to 0.7) *	0.2 (-0.2 to 0.7)
	Spatial visualization Z-score	0.5 (0.2 to 0.8) *	0.3 (-0.2 to 0.7)
YASR	**Internalizing sum score**	-1.5 (-4.6 to 1.7)	0.7 (-3.2 to 4.5)
	Externalizing sum score	-1.8 (-4.0 to 0.3)	-0.6 (-3.2 to 2.0)
	Total sum score	-5.2 (-13.9 to 3.5)	0.4 (-10.1 to 10.9)
	Anxious/depressed	-1.4 (-3.8 to 1.1)	-0.1 (-3.2 to 2.6)
	Withdrawn	-0.1 (-1.1 to 0.9)	1.0 (-0.2 to 2.2)
	Aggressive behavior	-0.8 (-1.9 to 0.4)	-0.3 (-1.7 to 1.2)
	Delinquent behavior	-0.5 (-1.1 to 0.2)	-0.3 (-1.1 to 0.4)
	Somatic complaints	-0.1 (-1.5 to 1.2)	0.4 (-1.2 to 2.0)
	Thought problems	-0.1 (-0.5 to 0.2)	-0.1 (-0.5 to 0.3)
	Attention problems	-0.7 (-1.6 to 0.2)	0.6 (-0.5 to 1.6)
	Intrusive behavior	-0.6 (-1.4 to 0.3)	-0.02 (-1.0 to 1.0)
	Other problems	-1.4 (-4.3 to 1.6)	-0.8 (-4.3 to 2.8)
YABCL	**Internalizing sum score**	-1.7 (-4.3 to 0.9)	-0.04 (-3.2 to 3.2)
	Externalizing sum score	-2.3 (-5.1 to 0.5)	-0.4 (-3.8 to 3.1)
	Total sum score	-8.5 (-17.3 to 0.2)	-0.7 (-11.5 to 10.1)
	Anxious/depressed	-1.4 (-3.5 to 0.6)	-0.5 (-3.0 to 2.0)
	Withdrawn	-0.3 (-1.0 to 0.5)	0.5 (-0.4 to 1.4)
	Aggressive behavior	-1.5 (-3.3 to 0.3)	0.5 (-1.8 to 2.7)
	Delinquent behavior	-0.3 (-0.9 to 0.4)	-0.3 (-1.1 to 0.4)
	Somatic complaints	-0.2 (-1.1 to 0.8)	0.3 (-0.9 to 1.4)
	Thought problems	-0.4 (-1.0 to 0.2)	-0.1 (-0.9 to 0.6)
	Attention problems	-2.5 (-4.2 to -0.8) *	0.1 (-2.0 to 2.2)
	Intrusive behavior	-0.5 (-1.4 to 0.3)	-0.5 (-1.5 to 0.5)
	Other problems	-1.6 (-3.4 to 0.2)	-0.6 (-2.8 to 1.6)

IQ, intellectual subdomain, YASR and YABCL scores are presented as regression coefficient (95% CI) and are corrected for sex. SNP carriers are compared with non-carriers. * p<0.05

Carriage of the MR I180V SNP was not related with IQ-score or the intellectual subdomains, but it was associated with a decrease in self-reported and parent-reported anxious/depressed behavior, a decrease in parent-reported internalizing behavior and a trend (p=0.06) towards a decrease in self-reported internalizing behavior. Interaction between the I180V SNP and antenatal glucocorticoid treatment (n=11) was observed, with a decrease in logical reasoning of -0.6 SD (95%CI -1.2 to -0.02 [p=0.04]) and a trend towards a decrease in linguistic capacity of -0.5 SD

(95%CI -1.0 to 0.03 [p=0.07]).
Intercourse compound variation did not have an additional effect on the outcomes (data not shown).
The results shown in tables 3, 4 and 5 remained unchanged after correction for perinatal risk factors and socioeconomic status (data not shown).

TABLE 5. Relation between MR polymorphisms versus IQ, YASR and YABCL scores.

		-2G/C GG-genotype	-2G/C CC-genotype	I180V SNP
IQ	**IQ-score**	-1.9 (-6.0 to 2.1)	-6.2 (-10.5 to -1.9) *	-1.5 (-5.9 to 2.8)
	Linguistic capacity Z-score	-0.1 (-0.3 to 0.2)	-0.3 (-0.5 to -0.1) *	-0.2 (-0.4 to 0.02)
	Mathematical capacity Z-score	-0.2 (-0.5 to 0.03)	-0.5 (-0.8 to -0.2) *	-0.02 (-0.3 to 0.3)
	Logical reasoning Z-score	-0.2 (-0.4 to 0.1)	-0.3 (-0.6 to -0.1) *	-0.04 (-0.3 to 0.2)
	Spatial visualization Z-score	-0.1 (-0.3 to 0.2)	-0.2 (-0.4 to 0.1)	-0.03 (-0.3 to 0.2)
YASR	**Internalizing sum score**	-0.7 (-2.9 to 1.4)	1.5 (-0.8 to 3.8)	-2,2 (-4.4 to 0.1)
	Externalizing sum score	-0.9 (-2.4 to 0.5)	0.9 (-0.7 to 2.4)	0.3 (-1.3 to 1.8)
	Total sum score	-3.4 (-9.5 to 2.7)	2.2 (-4.2 to 8.6)	-1.7 (-8.0 to 4.6)
	Anxious/depressed	-0.4 (-2.1 to 1.2)	0.9 (-0.9 to 2.6)	-1.7 (-3.4 to -0.02) *
	Withdrawn	-0.3 (-0.9 to 0.4)	0.6 (-0.1 to 1.3)	-0.4 (-1.2 to 0.3)
	Aggressive behavior	-0.7 (-1.5 to 0.1)	0.3 (-0.6 to 1.1)	-0.1 (-0.9 to 0.7)
	Delinquent behavior	-0.2 (-0.7 to 0.2)	0.1 (-0.4 to 0.6)	0.1 (-0.3 to 0.5)
	Somatic complaints	-0.5 (-1.4 to 0.4)	0.7 (-0.3 to 1.6)	0.3 (-0.6 to 1.3)
	Thought problems	0.1 (-0.1 to 0.3)	0.1 (-0.2 to 0.4)	-0.2 (-0.4 to 0.1)
	Attention problems	0.02 (-0.6 to 0.6)	0.3 (-0.4 to 0.9)	-0.2 (-0.8 to 0.5)
	Intrusive behavior	-0.1 (-0.6 to 0.5)	0.5 (-0.1 to 1.1)	0.3 (-0.3 to 0.9)
	Other problems	-0.8 (-2.8 to 1.2)	0.7 (-1.5 to 2.8)	-0.6 (-2.6 to 1.6)
YABCL	**Internalizing sum score**	0.2 (-1.7 to 2.1)	0.3 (-1.7 to 2.5)	-2.0 (-3.9 to -0.1) *
	Externalizing sum score	-1.4 (-3.4 to 0.6)	1.2 (-0.9 to 3.3)	1.4 (-0.7 to 3.5)
	Total sum score	-3.7 (-10.0 to 2.6)	2.2 (-4.5 to 8.8)	-1.3 (-7.9 to 5.2)
	Anxious/depressed	-0.3 (-1.7 to 1.2)	0.3 (-1.3 to 1.8)	-1.8 (-3.3 to -0.3) *
	Withdrawn	0.5 (-0.1 to 1.0)	0.02 (-0.6 to 0.6)	-0.2 (-0.8 to 0.4)
	Aggressive	-0.5 (-1.8 to 0.8)	0.8 (-0.5 to 2.2)	0.6 (-0.7 to 2.0)
	Delinquent	-0.2 (-0.6 to 0.2)	0.2 (-0.3 to 0.6)	0.3 (-0.1 to 0.7)
	Somatic complaints	-0.5 (-1.2 to 0.2)	-0.3 (-1.0 to 0.5)	-0.3 (-1.1 to 0.4)
	Thought problems	-0.1 (-0.5 to 0.3)	0.2 (-0.3 to 0.6)	-0.3 (-0.8 to 0.1)
	Attention problems	-0.6 (-1.8 to 0.6)	0.3 (-1.0 to 1.5)	-0.2 (-1.5 to 1.1)
	Intrusive behavior	-0.6 (-1.2 to -0.04) *	0.3 (-0.4 to 0.9)	0.5 (-0.2 to 1.1)
	Other problems	-1.4 (-2.6 to -0.1) *	0.5 (-0.9 to 1.8)	0.1 (-1.3 to 1.4)

IQ, intellectual subdomain, YASR and YABCL scores are presented as regression coefficient (95% CI) and are corrected for sex. SNP carriers are compared with non-carriers. * p<0.05

DISCUSSION

Consistent with our hypothesis, we found that both common genetic variants of the GR and MR and antenatal glucocorticoid treatment were associated with cognitive and behavioral outcomes in very preterm subjects. Independent of treatment, the R23K SNP was associated with an improved IQ-score and the -2G/C CC-genotype with

a poorer IQ-score. In addition, the R23K SNP was associated with a more favorable parent-reported total problem behavior score and the I180V SNP with a more favorable internalizing behavior score. Interaction between the *GR* and *MR* polymorphisms and antenatal exposure to synthetic glucocorticoids was observed, with poorer IQ-scores for exposed N363S carriers and I180V carriers and more favorable total problem behavior score for exposed R23K carriers. Antenatal glucocorticoid treatment was associated with more unfavorable behavior, especially internalizing behavior, but not with IQ.

Previous follow-up studies in this cohort [23,24] showed, similar to other studies in preterm survivors [2,27,28], poorer cognitive functioning and more unfavorable internalizing behavior, with male subjects being disadvantaged. In animals, antenatal glucocorticoid treatment was found to affect the limbic system, primarily the hippocampus [3,4,12]. Antenatal exposure to synthetic glucocorticoids was associated with a decreased hippocampal volume, degeneration and depletion of hippocampal neurons, reduced levels of *GR* and *MR* mRNA, cognitive deficits and increased anxiety-like and hypoactive behavior [12,29-31]. In humans, longitudinal studies assessing neurodevelopment after antenatal glucocorticoid treatment for fetal lung maturation are scarce [5]. The present study is the first to show that variation in *GR* and *MR* signaling contributes to cognitive and behavioral outcomes after preterm birth and might be augmented by the effects of antenatal glucocorticoid exposure.

In human fetuses, both GRs and MRs are expressed in the limbic system as early as 24 weeks gestational age [30]. The *MR* mRNA expression appears to be high at the end of the second trimester and decreases through the third trimester. In contrast, the *GR* mRNA expression is low at the end of the second trimester and increases during the third trimester. These dissimilarities in the spatiotemporal distribution of GRs and MRs, as well as person-specific affinity for synthetic glucocorticoids, have the potential to affect neurodevelopment [12,13,32,33].
In general, *GR* and *MR* SNPs have been associated with depression, dementia, metabolism and body composition [14,15,34-38]. At 19 years of age, we found already associations between GR and MR SNPs and neurobiological outcomes in a population with a high risk of cognitive impairments and psychopathology. Furthermore, we found that a single antenatal course of glucocorticoids had an additional effect on these outcomes. Yet it is unknown whether these could be modulated by subsequent functioning of the HPA axis.
The major strength of this study lies in the relatively large size of the cohort, given its unique nature. Another strength is that we assessed both parent- as well as self-

reported behavior, since preterm survivors appear to underreport deviating behavior [23,28,39] and parents provide data with a high validity [39].

A limitation of our study is that there was an underrepresentation of subjects who followed special education and subjects with a severe handicap [19]. However, if these individuals with unfavorable outcomes had participated, this would probably have enhanced the effects found. Another weakness is that genotypes were not known for the entire birth cohort. Therefore, we were unable to rule out a survival advantage associated with *GR* or *MR* carriage [15,40]. However, the allele frequencies in our sample were similar to the normal Caucasian population. Furthermore, most results were consistent with expectations based on in vitro receptor signaling studies [16,17]. In the interpretation of genetic association studies several other factors which affect the outcome must be acknowledged, including challenges during life and health status. Last, in our cohort numbers of exposed GR SNP carriers were small, as only 8 R23K carriers and 4 N363S carriers were exposed to antenatal glucocorticoid treatment. Even though, statistically significant differences were found. Furthermore, also the numbers with intercourse compound variation were low.

In conclusion, our data provide evidence for a possible mechanism explaining the association between prematurity and impairments in cognitive functioning and behavior. We showed that individual variations in glucocorticoid sensitivity and antenatal exposure to synthetic glucocorticoids during a critical period of neural ontogenesis were associated with IQ and behavior in young adults with a history of very preterm birth. Replication of these findings in independent samples is warranted.

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CHAPTER 9

BIRTH WEIGHT AND POSTNATAL GROWTH IN PRETERM BORN CHILDREN ARE ASSOCIATED WITH CORTISOL IN EARLY INFANCY, BUT NOT AT AGE 8 YEARS

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ABSTRACT

BACKGROUND

Preterm birth has been associated with altered HPA axis activity as well as cardiometabolic diseases and neurodevelopmental impairments later in life. We assessed cortisol from term age to age 8 y in children born preterm, to explore the development of HPA axis activity in association with intrauterine and early-postnatal growth until 6 mo. corrected age.

METHODS

In 152 children born at a gestational age ≤32 wks and/or with a birth weight ≤1,500g, random serum cortisol was assessed at term age (n=150), 3 mo. (n=145) and 6 mo. corrected age (n= 144), and age 8 y (n=59). Salivary cortisol was assessed at age 8 y (n=75): prior to bedtime, at awakening, 15 min after awakening, and before lunch. Cortisol was analyzed in association with birth weight SDS, being born small for gestational age (SGA), and combinations of intrauterine and postnatal growth: appropriate for gestational age [1] with or without growth restriction (AGA GR+ or AGA GR-) at 6 mo. corrected age, and SGA with or without catch-up growth (SGA CUG+ or SGA CUG-) at 6 mo. corrected age. Cross-sectional associations at all time points were analyzed using linear regression, and longitudinal associations were analyzed using generalized estimating equations.

RESULTS

Longitudinally, birth weight-SDS was associated with cortisol (β [95%CI]): lower cortisol over time was seen in infants with a birth weight ≤–2 SDS (–50.69 [–94.27 to –7.11] P=0.02), infants born SGA (–29.70 [–60.58 to 1.19] P=0.06), AGA GR+ infants (–55.10 [–106.02 to –4.17] P=0.03), SGA CUG– infants (–61.91 [–104.73 to –19.10] P=0.01). In cross-sectional analyses at age 8 y, no associations were found between either serum or salivary cortisol and birth weight-SDS, SGA-status, or growth from birth to 6 mo. corrected age.

CONCLUSION

In children born preterm, poor intrauterine and postnatal growth were associated with lower cortisol in early infancy, but not at age 8 y. Even though HPA axis activity no longer differed between groups at age 8 y, or differences could not be confirmed due to attrition, it is unknown whether the differences found in early infancy could attribute to increased health risks later in life.

INTRODUCTION

In infants born very preterm ,i.e., born at a gestational age ≤32 wks, the HPA axis is not yet fully maturated. Relative adrenal insufficiency is common in this group during the first weeks of life, and is characterized by relatively low basal and stress-induced cortisol levels, and increased risks of hypotension, hypoglycemia and bronchopulmonary dysplasia [2-5]. This is partly attributable to the sudden disruption of the maturation of the fetal HPA axis, which in full-term pregnancies is stimulated in the third trimester by an increase in the secretion of placental CRH and alterations in the expression of 11βHSD type 2 by the placenta [2]. Conversely, there is some evidence suggesting that HPA axis activity is upregulated years after preterm birth [3], which might contribute to the association between prematurity and long-term sequelae like cardiometabolic diseases and neurodevelopmental impairments.

Little is known about the impact of intrauterine and early-postnatal growth patterns on these associations in preterm infants. Intrauterine growth restriction is accompanied by a reduced expression and activity of the placental barrier enzyme 11Bhsd type 2, which converts cortisol to inert cortisone [6]. The subsequent fetal overexposure to maternal cortisol has been suggested to permanently alter HPA axis settings [7], initially by suppressing the axis, followed by increased activity later in life. This is strengthened by animal studies suggesting that the presence of abundant glucocorticoids in-utero, could result in a reduced expression of glucocorticoid receptors in tissues, and thereby, a compensatory upregulation of HPA axis activity [8-10].

Moreover, animal studies suggest that fetal growth restriction as well as early-postnatal growth restriction may predispose to cardiometabolic diseases later in life. Also, rapid postnatal growth after being born with a low birth weight has been associated with cardiometabolic disease risk, and alterations in HPA axis functioning have been suggested to explain these associations [10,11].

In term born subjects, there are few studies that have explored whether the HPA axis could explain part of the association between low birth weight and cardiometabolic disease [1,12]. In preterm born infants, rapid early-postnatal and childhood growth have been associated with risks of cardiometabolic diseases [13,14], but their relation with HPA axis activity throughout the lifespan has never been described.

To study the development of HPA axis activity after preterm birth in association with intrauterine and early-postnatal growth, we assessed cortisol levels of infants who were born very preterm and/or with a very low birth weight (≤1,500g), from term age until the age of 8 y.

METHODS

All subjects were originally included in a nutritional RCT ('Study Towards the Effect of Postdischarge nutrition' [STEP-1]) that compared the effects of postdischarge formula, term formula, and human milk on growth and body composition of very preterm (gestational age ≤32 wks) and/or very low birth weight (≤1,500 g) infants, as described previously [15]. Exclusion criteria were congenital malformations or other conditions known to affect growth or body composition. At term age, infants fed formula were randomized to receive either a protein- and mineral-enriched postdischarge formula or term formula between term age and 6 mo. corrected age. Corrected age is the age of the preterm born child calculated from the term date (i.e., 40 weeks gestation), and not from birth. This correction is usually maintained until the corrected age of 24 months.
At the age of 8 y, parents from the STEP-study participants were asked to participate in the follow-up study, STEP-2. Exclusion criteria were incomplete follow-up, severe physical impairment or other conditions known to affect growth or body composition.

DATA COLLECTION

For STEP-1, the following data were extracted from medical records: birth weight, birth length, gestational age and gender. The neonatal therapeutic intervention scoring system (NTISS), an indicator for neonatal illness severity and mortality risk [16], was calculated, and parents were asked to report their ethnicity, which was categorized as Caucasian or non-Caucasian. At term age, 3 mo. and 6 mo. corrected age, weight was measured with a digital scale to the nearest 1.0 g, and length with a length board to the nearest 0.1 cm. SD-scores (SDS's), which quantify the deviation from a reference population, were calculated for all auxological parameters. At birth and at term age, this was done by the use of neonatal anthropometric charts, adjusting for sex and gestational age [17]. At 3 and 6 mo. corrected age, this was done by the use of postnatal growth curves, adjusting for sex and (corrected) age [18]. At term age, 3 mo. and 6 mo. corrected age, fasting venous blood samples were collected. Mean fasting duration was recorded as the interval between blood sampling and the last feed before blood sampling. Mean fasting duration was 3.4 ± 0.7h at term age, 3.6 ± 0.7h at 3 mo. corrected age, and 3.5 ± 0.7h at 6 mo. corrected age.

We used the following definitions [19]:

1. Appropriate for gestational age (AGA) [1]: birth weight and birth length > –2SDS
2. Growth restriction (AGA GR+): weight and/or length ≤–2SDS at 6 mo. corrected age, after being born AGA
3. Small for gestational age (SGA): birth weight and/or birth length ≤–2SDS
4. Catch-up growth (SGA CUG+): weight and length >–2SDS at 6 mo. corrected age, after being born SGA

For STEP-2, children aged 8 y visited the outpatient clinic in the morning. Venous blood samples were obtained after an overnight fast, and anthropometric measurements were performed. Weight was measured to the nearest 0.05 kg with an electronic scale (Seca) and standing height was measured to the nearest 0.1 cm using a digital stadiometer (DGI 250D, De Grood Metaaltechniek, Nijmegen, the Netherlands), and expressed as SDS based on Dutch reference data adjusted for gender and age [18]. Salivary samples for cortisol measurement were collected at 4 moments: prior to bedtime at the evening before the study visit, immediately after awakening at the morning of the study visit, 15min after awakening, and before lunch during the study visit. Participants were instructed to refrain from eating and drinking at least 30min before sampling.
Parents were asked to report their education level, which was categorized as neither, one, or both parent(s) having finished higher education.
The study protocols were approved by the ethics committee of VUMC, Amsterdam. All parents of subjects gave written informed consent.

LABORATORY PARAMETERS

Serum was stored at −80°C and thawed only once just before the analyses.

In STEP-1, total serum cortisol (nmol/L) was measured using 2 different methods as the assay changed during the course of the study. In 90 infants, total serum cortisol at term age, 3 mo. and 6 mo. corrected age was measured using a competitive immunoassay (Advantage, Nichols Institute Diagnostics, San Juan Capistrano, USA) with an intra-assay coefficient of variation (CV) of 3%, 3% and 4% (at levels of 100, 500 and >600 nmol/L, respectively), an inter-assay CV of 8%, 7% and 6% (at levels of 140, 400 and 850 nmol/L, respectively), and a lower limit of quantitation (LLOQ) of 30 nmol/L. In 62 infants, total serum cortisol at term age, 3 mo. and 6 mo. corrected age was measured using a competitive immunoassay (DPC, Los Angeles, USA) with an inter-assay CV of 8%, 7% and 6% (at levels of 150, 400 and 900 nmol/L, respectively).

In STEP-2, total serum cortisol was measured using a competitive immunoassay (Luminescence Advia Centaur, Siemens Medical Solutions Diagnostics, USA) with an intra-assay CV of 3% (at a level of 700 nmol/L), an inter-assay CV of 7%, 6% and 6% (at levels of 80, 300 and 1,000 nmol/L, respectively), and a LLOQ of 30 nmol/L.
Salivary samples were stored at −80°C and thawed just before analyses. Free cortisol in saliva was measured using a competitive immunoassay (Luminescence Architect, Abbott Laboratories, Diagnostics Division Abbott Park, Illinois, USA) with an intra-assay CV of 9% (at a level of 5 nmol/L), an inter-assay CV of 11% (at a level of 7 nmol/L), and a LLOQ of 1 nmol/l.

All laboratory tests were performed by the Endocrine Laboratory of VUMC, Amsterdam, The Netherlands.

DATA ANALYSIS

To assess the effect of attrition at follow-up, baseline characteristics of participants, non-participants and excluded subjects were compared using one-way ANOVA, Chi-square or Kruskal-Wallis tests, with STEP-2 participants as reference group (**Table 1**). Since cortisol is associated with BMI, we compared BMI at age 8 y between the groups. No significant differences were found between SGA and AGA groups (p=0.560), and we therefore decided not to adjust our cross-sectional analyses at age 8 y for BMI.

SERUM CORTISOL

To test whether groups differed at any of the time points, cross-sectional, univariable, linear regression analyses were performed with either cortisol at term age, 3 mo. or 6 mo. corrected age, or 8 y as dependent factor.
Subsequently, generalized estimating equations (GEEs) were used for longitudinal analysis of cortisol, i.e., the assessment of differences between groups, adjusted for intra-individual variation over time. We assumed that attrition at age 8 y resulted in 'missings completely at random' and therefore used all available data of participants of the original RCT (n=152) without exclusion of dropouts, while accounting for 'missings completely at random' by use of GEE. GEE is designed for the handling of missing data, provided that missingness is completely at random [20,21]. In addition, GEE adjusts for grouped samples, collected from the same subject at different times, by using a correlation structure. For our data analyses, we chose an exchangeable correlation structure, in which one average within-subject correlation between samples over time is assumed. Stepwise GEEs were performed with cortisol over time (at term age, 3 mo. corrected age, 6 mo. corrected age, and 8 y) as dependent, continuous factor. First, the association between birth weight SDS and cortisol was

investigated using univariable regression, with birth weight SDS as independent, continuous factor. Second, the association between birth weight ≤–2SDS and cortisol was investigated, with birth weight SDS as independent, dichotomous factor. Third, the association between SGA-status and cortisol was investigated, with SGA-status as independent, dichotomous factor. Fourth, infants were classified into 4 groups according to birth weight SDS and growth from birth to 6 mo. corrected age (see also **section 2.1**): AGA GR– (1), AGA GR+ (2), SGA CUG– (3) and SGA CUG+ (4). Subsequently, the influence of growth pattern on cortisol was investigated, with the groups as independent, categorical factor and AGA GR– as reference category. Fifth, in order to test the stability of our data, all analyses were repeated, adjusted for perinatal or other characteristics that were significantly different between AGA and SGA subjects, one by one (**Table 2**).

TABLE 1. Baseline characteristics of all subjects, and STEP-2 participants, STEP-2 non-participants and excluded subjects.

		All subjects		STEP-2 Participants	STEP-2 Non-participants	STEP-2 Excluded subjects	P-value[a]
n		152		79	52	21	
Perinatal characteristics							
Male		78 (51)		40 (51)	26 (50)	12 (57)	NS
Gestational age	wks	30 [30 to 31]	range 25 to 33	31 [29 to 32]	30 [30 to 31]	31 [30 to 32]	NS
Birth weight	g	1339 ± 29	range 710 to 2065	1314 ± 304	1350 ± 290	1404 ± 265	NS
	SDS	–0.3 ± 1.0	range –2.8 to 1.9	–0.4 ± 1.0	–0.3 ± 0.9	–0.2 ± 1.2	NS
Birth length	cm	38 ± 3.0		38 ± 3.1	38 ± 3.0	38 ± 2.9	NS
	SDS	–0.9 ± 1.2	range –3.9 to 2.0	–0.8 ± 1.3	–1.1 ± 1.1	1.0 ± 1.3	NS
SGA		35 (23)		17 (22)	13 (28)	5 (24)	NS
Birth head circumference	cm	27.7 ± 1.9		27.6 ± 1.9	27.7 ± 1.9	28.0 ± 1.8	NS
	SDS	–0.2 ± 1.1		–0.2 ± 1.1	–0.3 ± 1.0	–0.21 ± 1.3	NS
NTISS		21.4 ± 7.6		21.9 ± 7.8	21.4 ± 7.1	19.8 ± 8.0	NS
Weight 6 mo. corrected age	kg	7.3 ± 1.1		7.2 ± 1.0	7.4 ± 1.0	7.6 ± 1.4	NS
	SDS	–0.4 ± 1.2		–0.5 ± 1.2	–0.3 ± 1.2	–0.3 ± 1.7	NS
Length 6 mo. corrected age	cm	66.4 ± 2.9		66.4 ± 2.8	66.5 ± 2.9	66.4 ± 3.4	NS
	SDS	–0.3 ± 1.1		–0.4 ± 1.1	–0.3 ± 1.1	–0.5 ± 1.4	NS
Demographics							
Parents that finished higher education	neither	77 (52)		41 (53)	23 (45)	13 (62)	NS
	one	32 (22)		15 (19)	13 (26)	4 (21)	
	both	39 (26)		22 (28)	15 (29)	2 (11)	
≥1 parent non-Caucasian		43 (28)		17 (22)	13 (25)	13 (62)	<0.01

Data are expressed as mean ± SD, median [IQR], or *n* (%). Abbreviations: NS, non-significant; NTISS, neonatal therapeutic intervention scoring system; SDS, SD-score; SGA, small for gestational age (birth weight and/or birth length ≤–2SDS) [a] STEP-2 participants, STEP-2 non-participants and excluded subjects were compared using one-way ANOVA, Chi-square or Kruskal-Wallis tests, as appropriate.

SALIVARY CORTISOL AT AGE 8 YEARS

Data were examined for outliers. Levels at awakening that were 10 times higher than 15 min. after awakening, and levels in the evening that were 10 times higher than the cohort mean, were excluded.

The cortisol awakening response (CAR) was calculated as the difference between levels at awakening and 15 min after awakening. We calculated the AUC increase (AUC_i; with reference to the lowest individual cortisol level) of all samples according to the formula by Pruessner [22], and a time-weighted AUC_i (divided by the total time of collection, AUC_i/h). Analyses of salivary cortisol parameters in association with birth weight SDS, SGA-status and growth from birth to 6 mo. corrected age were performed with univariable linear regression analysis.
Statistical significance was defined as a P-value of <0.05. Statistical analyses were performed with IBM SPSS Statistics version 22.

RESULTS

One hundred and fifty two subjects were included in STEP-1. Over time, 13 children were lost to follow-up, 21 children were excluded, and 39 children refused to participate in STEP-2, resulting in 79 subjects that could be included at age 8 y in STEP-2 (**Figure 1**).

No differences were found in baseline characteristics of STEP-2 participants vs. non-participants and excluded subjects (P > 0.3), except for parental ethnicity which was more often "non-Caucasian" in the excluded group compared to participants and non-participants (**Table 1**). AGA and SGA subjects differed in gestational age, gender distribution, preeclampsia/HELLP syndrome and exposure to antenatal glucocorticoid treatment (**Table 2**). We considered these variables as possible confounders and therefore tested their influence on the longitudinal analyses.

TABLE 2. Perinatal and study characteristics that could potentially confound the research question

	AGA	SGA	P-value [a]
n	109	35	
PIH/Preeclampsia/HELLP	36 (33)	21 (60)	<0.01
Antenatal glucocorticoid treatment	57 (52)	24 (69)	<0.01
Male	49 (45)	25 (71)	<0.01
Gestational age (wks)	30 [29 to 31]	31 [30 to 32]	<0.01
STEP-1 human milk group	37 (34)	11 (31)	NS
NTISS score	21.4 ± 7.8	21.6 ± 6.9	NS
Caucasian ethnicity	78 (72)	24 (69)	NS
Time of collection serum samples (24h) [b]	13:27 ± 2:05	13:29 ± 2:09	NS

Data are expressed as mean ± SD, median [IQR], or *n* (%). Abbreviations: AGA, appropriate for gestational age (birth weight and birth length >–2SDS); NS, non-significant; NTISS, neonatal therapeutic intervention scoring system; PIH, pregnancy induced hypertension; SGA, small for gestational age (birth weight and/or birth length ≤–2SDS) [a] Groups were compared using independent t-test, Fisher's exact test, or Mann-Whitney U test as appropriate [b] Mean time of collection of all sampling moments: term age, 3mo. or 6 mo. corrected age and age 8 y.

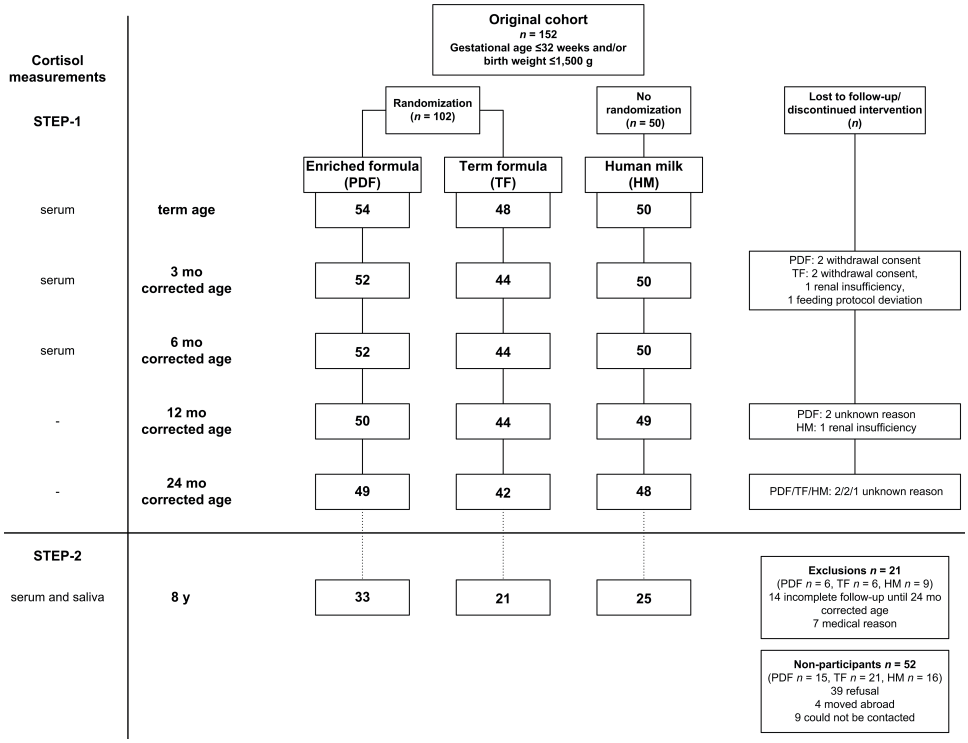

FIGURE 1. Flowchart of STEP-1 and -2
Abbreviations: HM, human milk; PDF, postdischarge formula; TF, term formula

CROSS SECTIONAL ANALYSES

Birth weight SDS was positively associated with cortisol at 3 mo. and 6 mo. corrected age, β [95%CI]: 32.78 [8.74 to 56.81], P=0.01 and 20.90 [–0.35 to 42.14], P=0.05 respectively, and birth weight ≤–2 SDS was associated with lower cortisol compared to birth weight >–2 SDS at 6 mo. corrected age (–91.60 [–172.86 to –10.35], P=0.03). At 3 mo. corrected age, a non-significant association between cortisol and SGA-status was found (–49.83 [–105.85 to 6.19], P=0.08) (**Figure 2A** and **Table 3**).

At term age, cortisol was lower in SGA CUG– compared to AGA GR– infants (–115.69 [–212.06 to –19.31], P= 0.02). At 3 mo. corrected age, a non-significant association between cortisol and SGA CUG+ compared to AGA GR– was found (–62.40 [–133.03 to 8.23], P=0.08), and at 6 mo. corrected age, a non-significant association between cortisol and SGA CUG– compared to AGA GR– was found (–64.08 [–134.65 to 6.49], P=0.08) (**Figure 2B** and **Table 3**).

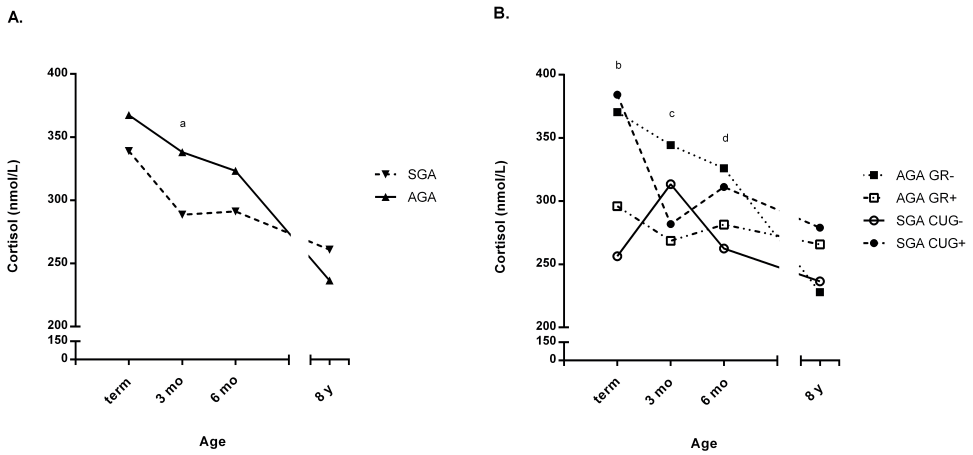

FIGURE 2. Serum cortisol (means): SGA vs. AGA infants (A), growth birth-6 mo. corrected age (AGA GR–, AGA GR+, SGA CUG–, and SGA CUG+) (B).

Abbreviations: AGA GR+/GR–, appropriate for gestational age with or without growth restriction at 6 mo. corrected age; SGA CUG+/CUG–, small for gestational age with or without catch-up growth at 6 mo. corrected age a SGA vs. AGA p=0.08, b SGA CUG– vs. AGA GR– p=0.02, c SGA CUG+ vs. AGA GR– p=0.08, d SGA CUG– vs. AGA GR– p=0.08. All P-values represent unadjusted cross-sectional analyses.

TABLE 3. Serum cortisol (nmol/L), mean ± SD at every sampling moment

		n	Term age	n	3 mo. corrected age	n	6 mo. corrected age	n	8 y
Birth weight SDS, continuous		150	367.3 ± 175.4	145	328.1 ± 148.9	144	319.8 ± 127.1	59	241.2 ± 113.5
SGA	AGA	108	367.3 ± 175.6	103	337.7 ± 143.1 [a]	103	323.0 ± 126.1	43	233.7 ± 114.8
	SGA	35	339.0 ± 166.2	34	287.9 ± 143.6	34	291.1 ± 120.5	14	265.2 ± 117.5
Early postnatal growth	AGA GR–	95	372.0 ± 179.1	95	343.6 ± 146.2	95	326.5 ± 129.5	38	229.2 ± 106.8
	AGA GR+	8	296.0 ± 117.6	8	268.5 ± 73.8	8	268.7 ± 64.3	5	267.6 ± 177.1
	SGA CUG–	14	256.3 ± 157.3 [b]	14	313.4 ± 110.5	14	262.4 ± 119.8 [d]	6	236.7 ± 84.8
	SGA CUG+	20	384.2 ± 148.2	19	281.2 ± 159.8 [c]	20	311.2 ± 119.8	8	286.6 ± 138.8

Abbreviations: AGA, appropriate for gestational age (birth weight and birth length >–2SDS); CUG, catch-up growth; GR, growth restriction; SGA, small for gestational age (birth weight and/or birth length ≤–2SDS). [a] SGA vs. AGA *p*=0.08, [b] SGA CUG– vs. AGA GR– *p*=0.02, [c] SGA CUG+ vs. AGA GR– *p*=0.08, [d] SGA CUG– vs. AGA GR– *p*=0.08. All P-values represent unadjusted cross-sectional analyses.

LONGITUDINAL ANALYSES GEE

The results of the longitudinal analyses are presented in **Table 4**.

A birth weight <0 SDS was associated with lower cortisol as compared to a birth weight ≥0 SDS. (–17.33 [–30.96 to –3.69], P=0.01). A birth weight ≤–2 SDS was associated with a lower cortisol over time, compared to a birth weight >–2 SDS (–50.69 [–94.27 to –7.11], P=0.02). Being born SGA was associated with lower cortisol over time, as compared to being born AGA (–29.70 [–60.58 to 1.19], P=0.05).

At 6 mo. corrected age, AGA GR+ and SGA CUG – infants had lower cortisol over time compared to AGA GR– infants (–55.10 [–106.02 to –4.17], P = 0.03, and –61.91 [–104.73 to –19.10], P = 0.01, respectively). No differences in cortisol over time were found between SGA CUG+ and AGA GR– infants.

After adjustment for confounders, similar results were found, with the exception of pregnancy induced hypertensive diseases, which resulted in a loss of significance (data not shown). After exclusion of visually impaired STEP-1 subjects (n=2) , similar results were found (data not shown).

TABLE 4. Longitudinal associations between birth weight SDS, SGA-status, growth from birth to 6 mo. corrected age, and serum cortisol

				Crude		
			n	β	(95% CI)	*p* value
Birth weight SDS, continuous			152	17.33	(3.69 to 30.96)	0.01
Birth weight SDS , dichotomous	**≤–2SDS**	vs. **>–2SDS**	11 vs. 141	–50.69	(–94.27 to –7.11)	0.02
SGA	**SGA**	vs. **AGA**	35 vs. 109	–29.70	(–60.58 to 1.19)	0.06
Early postnatal growth	**AGA GR+**	vs. **AGA GR–**	8 vs. 96	–55.10	(–106.02 to –4.17)	0.03
	SGA CUG–	vs. **AGA GR–**	14 vs. 96	–61.91	(–104.73 to –19.10)	0.01
	SGA CUG+	vs. **AGA GR–**	20 vs. 96	–13.26	(–49.62 to 23.10)	0.48

Abbreviations: AGA, appropriate for gestational age (birth weight and birth length >–2SDS); CI, confidence interval; CUG, catch-up growth; GR, growth restriction; SDS, standard deviation score; SGA, small for gestational age (birth weight and/or birth length ≤–2SDS)

SALIVARY CORTISOL

Table 5 shows salivary cortisol data at age 8 y; mean per sampling moment, CAR, AUC_i, AUC_i/h and maximum level. Two outliers with levels at awakening that were 10 times higher than 15 min. after awakening, or levels in the evening that were 10 times higher than the cohort mean, were excluded from the analysis. Linear regression analyses showed no association between birth weight SDS, SGA-status or growth from birth to 6 mo. corrected age, and either of the salivary cortisol parameters (data not shown).

TABLE 5. Salivary cortisol (nmol/L), mean at every sampling moment and CAR, AUC_i and maximal level

		n	Bedtime	Awakening	+ 15 min	Lunch	CAR	AUC_i	AUC_i/h	Max.
Birth weight SDS , continuous		75	1.0 ± 0.1	4.9 ± 2.9	6.5 ± 3.2	2.3 ± 1.8	1.5 ± 2.8	38.2 ± 22.6	2.5 ± 1.5	6.9 ± 3.1
SGA	AGA	57	1.0 ± 0.2	4.9 ± 3.0	6.6 ± 3.4	2.3 ± 1.9	1.7 ± 2.9	38.8 ± 24.2	2.5 ± 1.5	7.1 ± 3.3
	SGA	15	1.0 ± 0.0	4.6 ± 2.2	6.0 ± 2.6	2.1 ± 1.1	1.3 ± 2.4	34.7 ± 16.1	2.2 ± 1.1	6.3 ± 2.4
Early postnatal growth	AGA GR–	51	1.0 ± 0.2	5.0 ± 3.1	6.4 ± 3.5	2.3 ± 2.0	1.4 ± 2.7	38.5 ± 25.4	3.4 ± 1.6	7.0 ± 3.4
	AGA GR+	6	1.0 ± 0.0	4.1 ± 1.9	7.9 ± 2.7	2.3 ±1.5	3.8 ± 3.7	41.6 ± 6.5	2.6 ± 0.3	8.1 ± 2.5
	SGA CUG–	5	1.0 ± 0.0	3.9 ± 1.5	6.0 ± 1.7	2.1 ± 1.0	2.0 ± 2.0	30.2 ± 11.4	1.1 ± 0.8	6.0 ± 1.7
	SGA CUG+	10	1.0 ± 0.0	4.9 ± 2.5	6.0 ± 3.0	2.1 ± 1.2	0.9 ± 2.7	37.5 ± 18.7	2.4 ± 1.2	6.6 ± 2.9

Data are expressed as mean ± SD. Abbreviations: AGA, appropriate for gestational age (birth weight and birth length >-2SDS); AUC_i (/h), area under the curve with respect to the increase (per hour); CAR, cortisol awakening response; CUG, catch-up growth; GR, growth restriction; max, maximal salivary cortisol level of the 4 sampling moments; SGA, small for gestational age (birth weight and/or birth length ≤–2SDS).

DISCUSSION

We showed that differences in growth between birth and 6 mo. corrected age are related to the pattern of serum cortisol decline during infancy. In our longitudinal analyses, the growth-restricted groups (i.e., low BW, SGA, AGA with GR and SGA without CUG) all had a lower cortisol over time, compared to non-growth-restricted infants (AGA infants without GR). These differences were not explained by gestational age, antenatal glucocorticoid treatment or gender. Not surprisingly, statistical correction for pregnancy induced hypertensive disorders reduced the strength of our associations (Aufdenblatten et al., 2009; McCalla et al., 1998). In cross-sectional analyses at age 8 y, the differences between the growth-restricted and non-growth-restricted groups were no longer present. In addition, salivary cortisol at age 8 y was not different between these groups at any sampling moment throughout the day.

In the first weeks of life, the HPA axis of preterm newborns is still immature. Among the impairments are insensitivity of the pituitary gland to synthetic CRH, decreased 11β-hydroxylase activity in the adrenal cortex, and a cortisol-cortisone shuttle favoring cortisone [3]. In animal studies, adverse events occurring in early life have been associated with permanent alterations in HPA axis activity [8]. In line with our results, in previous studies among infants of whom the majority were born at term, a blunted cortisol response to painful procedures was found in those born SGA [23,24]. In contrast, from childhood onwards, lower birth weight has been associated with increases in glucocorticoid metabolite excretion (Clark 1996), basal cortisol [25] and the cortisol response to psychosocial stress [26]. Preterm infants were found to exhibit altered responses to different kinds of stressors, as compared to their term counterparts [27,28]. Furthermore, prematurity has been associated with a lower cortisol response, in spite of a higher pretest cortisol level, during a psychosocial stress test [27,29,30]. These findings suggest that the HPA axis is hypoactive after being born SGA and/or preterm, and becomes hyperactive with age, although not indisputable. Similar shifts were observed in extremely preterm infants (gestational age <29 wks) compared to very preterm (gestational age 29-32 wks) and term infants [31]. Our study suggests that these longitudinal patterns in HPA axis activity of preterm infants are augmented by poor intrauterine and early-postnatal growth, although our follow-up might have been too short to demonstrate a subsequent increase in HPA axis activity.

We found no differences in salivary cortisol parameters at age 8 y, which included diurnal rhythmicity and CAR, between growth-restricted and non-growth-restricted subjects. At school age, a lower CAR has been described in preterm born children compared to term born control subjects [32]. Studies regarding the diurnal rhythm linked

preterm birth with cortisol levels that were either higher at bedtime [33] or throughout the day [34]. Since we did not include a term control group, the results of these studies cannot be compared to ours. Moreover, we collected only 2 salivary samples post-awakening, and may therefore have missed the peak cortisol concentration. In addition, a single collection day may not be sufficient to demonstrate differences at age 8 year [35].

It is increasingly recognized that preterm birth constitutes a major risk factor for cardiometabolic disease [36]. However, in studies providing long-term follow-up of preterm populations, no differences in insulin resistance or blood pressure were demonstrated between subjects with and without intrauterine growth restriction [37,38]. Possibly, the subjects within these cohorts were too young to demonstrate such differences. This may also partly explain the lack of association in our sample at age 8 y. Life-long follow-up of preterm populations is warranted, since it is conceivable that alterations in HPA axis activity in preterm infants during early life are involved in pathways leading to cardiometabolic disease and neurodevelopmental impairments.

The main strength of this study is the use of a well-described birth cohort, in which extensive information on early growth was available. In addition, serial measurement of serum cortisol in early infancy as well as in childhood, and measurement of salivary cortisol at age 8 y were performed.
There are several limitations. First, this study was a post-hoc analysis of a nutritional RCT, in which a term born control group was not included. Second, attrition at age 8 y limited our sample size. We therefore carefully assessed the possibility of attrition bias by comparing participants with non-participants and excluded subjects. Since there were no differences, analyzing with GEE gave us the opportunity to use all available data in longitudinal analyses, while accounting for missing data [20]. With this approach, we followed the suggested reporting requirements for addressing attrition as described by Fewtrell [21]. Third, subgroup analyses were performed in relatively few subjects. Fourth, the exact timing of cortisol sampling was not the same for all subjects and the early morning would have had our preference. Since this was not possible, we assessed fasting blood levels at term age, 3 and 6 mo. corrected age, as a second best option [39]. However, since there was no difference in mean sampling time between AGA and SGA subjects we considered this to have no influence on our results. In addition, despite our effort to standardize our salivary cortisol collection protocol by giving clear instructions and reporting time of sampling, it was not optimal, and the LLOQ limited the sensitivity of values in the lower ranges (i.e., ≤1 nmol/L). Considering the recently published guidelines of Stalder et al., the small

number of samples to determine the CAR, as well as a single day of collection, may have influenced our results [35].

CONCLUSION

In children born preterm, poor intrauterine and postnatal growth were associated with lower cortisol during early infancy, irrespective of gestational age. However, at age 8 y these differences were no longer present or could not be confirmed due to attrition. It is unknown whether alterations in HPA axis activity in early infancy could attribute to increased health risks later in life.

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CHAPTER 10

GENDER-SPECIFIC DIFFERENCES IN HYPOTHALAMUS–PITUITARY–ADRENAL AXIS ACTIVITY DURING CHILDHOOD: A SYSTEMATIC REVIEW AND META-ANALYSIS

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ABSTRACT

BACKGROUND

Gender-specific differences in HPA axis activity have been postulated to emerge during puberty. We conducted a systematic review and meta-analysis to test the hypothesis that gender-specific differences in HPA axis activity are already present in childhood.

METHODS

From inception to January 2016, PubMed and Embase.com were searched for studies that assessed non-stimulated cortisol in serum or saliva, or cortisol in 24h-urine in healthy males and females aged ≤18yr. Studies were reported conform the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement. Standardized mean differences (95%CIs) were calculated and analyzed using fixed-effect meta-analysis stratified for age: <8yr (prepubertal) and 8-18yr (peri-/postpubertal). For comparison, we ran the same analyses using random-effects models.

RESULTS

Two independent assessors selected 413 out of 6,158 records (7%) for full-text screening, of which 79 articles were included. Of these, 58 (with data on 16,551 subjects) were included in the meta-analysis. Gender differences in cortisol metabolism differed per age group. Boys aged <8yr had 0.18 (0.06 to 0.30) nmol/L higher serum and 0.21 (0.05 to 0.37) nmol/L higher salivary cortisol levels, while between 8-18yr, boys had 0.34 (0.28 to 0.40) nmol/L lower serum and 0.42 (0.38; 0.47) nmol/L lower salivary cortisol levels. In 24h-urine, cortisol was consistently higher in boys, being 0.34 (0.05 to 0.64) and 0.32 (0.17 to 0.47) μg/24h higher in the <8yr and 8-18yr groups, respectively. However, gender-differences in serum cortisol <8 yr and between 8-18 yr were absent when using random-effects models.

CONCLUSIONS

Gender differences in cortisol metabolism are already present in childhood, with higher salivary cortisol in boys aged <8yr compared to girls. This pattern was reversed after age 8 yr. In contrast, the gender-specific difference in cortisol production as assessed through 24h-urine did not change with age. Although differences were small, and analyses of gender differences in serum cortisol were inconclusive, they might contribute to gender-specific origins of health and disease.

BACKGROUND

The hypothalamus-pituitary-adrenal (HPA) and hypothalamus-pituitary-gonadal (HPG) axes are closely connected. Animal studies demonstrated that CRH inhibits the HPG axis at all levels, while testosterone inhibits the HPA axis at the hypothalamic level. Additionally, estrogens stimulate the HPA axis at both the hypothalamic and adrenal levels. Moreover, CRH levels were dependent on the phase of the menstrual cycle, with the highest concentrations occurring during the follicular phase [1,2].

Human studies suggested that estrogens decrease the hepatic A-ring reduction of cortisol, albeit not in the short term [3], and increase the production of CBG, thereby affecting the bioavailability of cortisol [1,4,5]. The latter being enhanced by the use of oral contraceptives. Furthermore, HPA axis responses to acute psychological stress were different depending on the phase of the menstrual cycle [2,4].

Due to an increase in sex steroid concentrations, gender differences in HPA axis activity have been postulated to emerge during puberty [6,7]. However, more recent evidence suggests that gender differences in HPA axis activity are already present early in life [1,8,9]. Putative mediators of these prepubertal gender differences are the postnatal reproductive hormone surge, also known as mini-puberty [10], and sex-specific effects of styles in parental care, such as psychosocial stress reactivity to maternal over-controlling behavior [11]. However, physiological gender differences in cortisol concentrations during childhood have not been studied yet.

Therefore, the question was raised whether gender differences in unstimulated HPA axis activity emerge during puberty or whether they are already present earlier in life. Accordingly, we conducted a systematic review and meta-analysis with the hypothesis that gender-specific differences in unstimulated HPA axis activity are present in early life and are subsequently influenced by puberty.

METHODS

SEARCH STRATEGY

From inception up to 14 January 2016, PubMed and Embase.com were searched (by BvdV and JCFK) for studies that reported non-stimulated cortisol in serum or saliva, or cortisol in 24h-urine for healthy boys and girls aged ≤18 yr separately.

Appendix 1 presents the full search strategy, which was based on the following index terms or free-text words: 'cortisol' or 'glucocorticoid', and 'sex difference' or 'sexual characteristics', and 'child' or 'adolescent'. Studies in children with (psycho)pathology, on synthetic glucocorticoids, or with risk for abnormal HPA axis activity (e.g., a history of maltreatment) were excluded. An English language restriction was applied for abstracts of published articles. No restrictions for year of publication or study design, apart from reviews and case reports, were applied. The review protocol was based on the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement.

DATA COLLECTION

Two independent assessors (BvdV and JJH) screened 6,158 titles and abstracts without consideration of outcomes. Studies were not assessed blindly. Disagreement between assessors was discussed until consensus was reached. When gender differences were analyzed without reporting on cortisol levels for boys and girls separately or when data were only presented in graphs, authors were requested for additional quantitative data. Data were stratified into two age groups: <8 yr (prepubertal) and between 8–18 yr (peri-/postpubertal). Ideally, stratification would have been based on pubertal staging according to Tanner. Unfortunately, only a minority of the included studies reported on the subjects' Tanner stages. Because pubertal onset before age 8 years is considered to be pathologic [12], we chose 8 yr as cut off for stratification. When articles reported on serial cortisol measurements, we included only data on the youngest assessment age. When cortisol levels were reported prepubertally as well as peri-/postpubertally within the same individual, we included one sampling moment for each stratified group. When articles reported on the same study population, we included the article with the lowest bias risk. When articles reported on dynamic tests of HPA axis activity, we only included baseline cortisol. We only included the control subjects of case-control studies. If known, we excluded female subjects on oral contraceptives. When gender differences were described but not quantified, the articles were included in the descriptive analysis rather than the meta-analysis.

META-ANALYSIS

When necessary, we converted serum and salivary cortisol levels into nmol/L, and 24h-urine cortisol levels into μg/24h. When means ± SDs were not reported, the SD was calculated based on the following assumptions: the 95% CI is 3.92 SDs wide (2 × 1.96); the inter-quartile range is 1.35 SDs wide; the range is 4 SDs wide; the SD is the SE multiplied by the square root of the sample size [13]. To assess parametricity, we

assumed that a normal distribution extends no more than 2 SDs from the mean [14], i.e., when normally distributed, the mean minus 2 SDs should be >0 nmol/L. Data analyses were performed using Review Manager (RevMan) version 5.3.5, 2014. For each study, the standardized mean gender difference (95% CI) in cortisol concentration was calculated by combining the SD with the sample size. Subsequently, fixed-effect meta-analyses were performed first, which assumes that the effect estimate of the group differences was fixed across studies. Second, the results of these analyses were compared with random-effects meta-analysis, which weigh studies of variable sample sizes more equally. We reported any source of bias from each included article conform the PRISMA statement and assessed selection, performance, detection and other biases. Bias was assessed as low, unclear or high (**Figure 1, Appendix 2**). A sensitivity analysis was done by excluding studies that had ≥ 1 high bias risks. Heterogeneity of the data was assessed by the I^2 statistic, with significance defined as I^2 >50%. Publication bias was assessed through funnel plots.

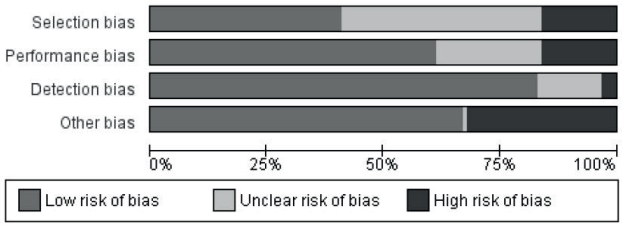


FIGURE 1. Risk of bias graph presenting a summary of the judgements of the accessors concerning risk of bias across all studies included in the meta-analysis. Bias risk is presented as percentage of total studies (n = 58).

RESULTS

Figure 2 shows the flowchart of the descriptive analysis and meta-analysis. Of the 6,158 titles and abstracts, 414 (7%) were eligible for full-text screening, from which 79 articles (19%) were included. Thirty-one authors of articles with insufficient quantitative data were contacted, of whom 12 responded: six provided the necessary quantitative data, five did not have access to the raw data anymore and one was not willing to participate. Two articles reported the cortisol production rate assessed through 24h-serum sampling, which hampered inclusion in the meta-analysis. The authors of 27 articles that only provided gender-specific data in figures were contacted, but could not be reached. Subsequently, these articles were excluded. Finally, 21 articles were included only in the descriptive analysis, and 58 articles (with data on 16,551 subjects) had sufficient data for inclusion in the meta-analysis.

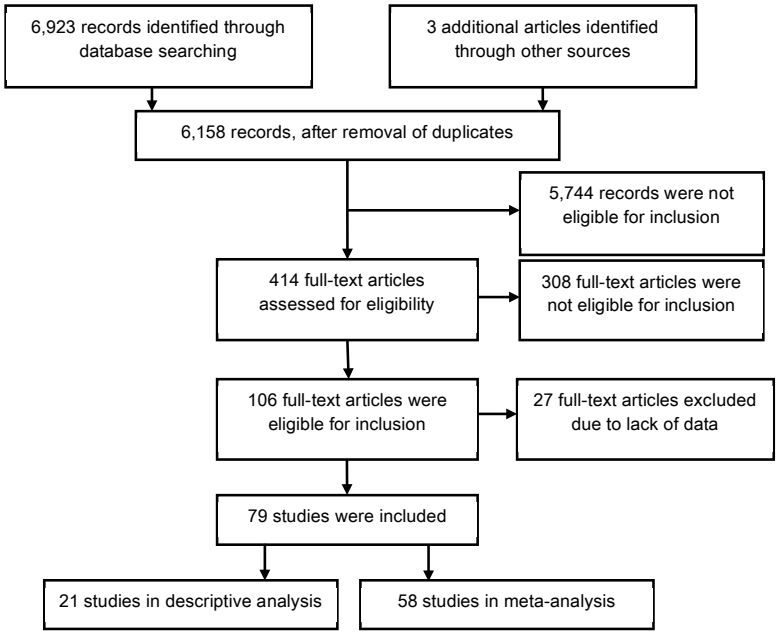


FIGURE 2. This flow chart presents the different phases of the systematic review and meta-analysis, conform the PRISMA-statement. (www.prisma-statement.org)

DESCRIPTION OF INCLUDED STUDIES

Studies were conducted in Europe (n = 36), North-America (n = 37), Asia (n = 3), South-America (n = 2) or Africa (n = 1), and were published between 1973 and 2016. Sample sizes ranged from 11 to 2,824 subjects, with seven studies having a sample size >500 subjects. Study designs were as follows: randomized placebo-controlled (n = 2), prospective observational (n = 29), non-randomized intervention, i.e., stress tests (n = 15), cross-sectional (n = 16), longitudinal (n = 11) and case-control (n = 6). All studies that assessed serum or salivary cortisol used immunoassays, except for one that used high-performance liquid chromatography (HPLC). Studies that assessed 24h-urine cortisol used immunoassays (n = 4), gas chromatography–mass spectrometry (n = 3), HPLC (n = 1), and liquid chromatography-UV detection (n = 1). Twenty-two studies (28%) did not collect morning samples, of which 11 did not report the time of collection and 11 described specifically that samples were collected in the afternoon. **Online supplementary File 1** presents the data extracted from the articles included in the meta-analysis. Three out of 21 studies (14%) included in the descriptive analysis had no high bias risk (**Table 1**), while 16 out of 58 studies (28%) included in the meta-analysis had no high bias risk (**Figure 1**).

TABLE 1. Summary of studies included in the descriptive analysis.

Group	First author (year)	N (%girls)	Age (yr)	Sample protocol	Assay	Result	Bias*
Saliva <8 yr	Klug (2000) [35]	119 (46%)	0	3 point day curve	Immunoassay	No gender differences	2
	Eiden (2015) [36]	257 (?)	0.75	Laboratory Temperament Assessment	Immunoassay	No gender differences	3
	Plusquellec (2011) [37]	466 (?)	1.6 ± 0. 1	Morning sample	Immunoassay	No gender differences	2
	Spinrad (2009) [38]	84 (49%)	4.5	Preschool Laboratory Assessment	Immunoassay	No gender differences	2
	Hatzinger (2007) [15]	102 (42%)	4.9 ± 0. 4	CAR	Immunoassay	Cortisol levels were lower in boys at awakening (p<0.1)	1
Saliva 8–18 yr	Safarzadeh (2005) [39]	100 (58%)	6 – 14	Morning sample	Immunoassay	No gender differences	1
	Isaksson (2015) [40]	68 (50%)	9	Morning sample	Immunoassay	No gender differences	2
	Kjölhede (2014) [41]	231 (50%)	9.5 ± 1.5	Morning sample	Immunoassay	No gender differences	1
	Vaillancourt (2008) [8]	154 (52%)	12.3 ± 0.8	Six samples standardized across time and day	Immunoassay	On Saturday morning boys had significantly lower morning levels. On Monday and Thursday no gender differences were found.	1
	Gunnar (2009) [42]	82 (49%)	9 – 15	TSST	Immunoassay	No gender differences	1
Serum <8 yr	Fadalti (1999) [43]	72 (49%)	0 – 2	Morning sample	Immunoassay	No gender differences	0
	Ballerini (2010) [44]	319 (45%)	0 – 5	Surplus serum	Immunoassay	No gender differences	2
	Parker (1978) [45]	106 (43%)	2 – 12	Morning sample	Immunoassay	No gender differences	2
Serum 8–18 yr	Kulasingam (2010) [46]	419 (?)	0 – 15	Surplus serum	Immunoassay	No gender differences	3
	Soldin (2005) [47]	376 (?)	0 – 18	Surplus serum	Immunoassay	No gender differences	1
	Karbasy (2015) [48]	711 (?)	0 – 19	?	Immunoassay	No gender differences	1
	Fadalti (1999) [43]	82 (49%)	6 – 18	Morning sample	Immunoassay	No gender differences	0
	Barra (2015) [49]	120 (45%)	12.4 ± 3	Morning sample	Immunoassay	No gender differences	1
	Chalew (1997) [50]	15 (73%)	12.7 ± 2.2	24h-blood withdrawal	Immunoassay	No gender differences	1
	Linder (1990) [51]	82 (58%)	8 – 17	24h-blood withdrawal	HPL	No gender differences .	0
Urine <8 yr	-						
Urine 8–18 yr	Dorn (1996) [52]	20 (55%)	15.2 ± 1.1	24h-urine sample	Immunoassay	No gender differences	1

* number of high risks of bias out of 4 bias categories (selection, performance, detection and other biases)

GENDER-SPECIFIC DIFFERENCES

Descriptive analysis

Table 1 summarizes the data on the 21 studies included in the descriptive analysis. The majority (90%) of these studies reported no significant gender differences in cortisol levels. Before age 8 yr, one study [15] found significantly lower salivary cortisol levels for boys at awakening. Between ages 8-18 yr, one study [8] found significantly lower morning salivary cortisol levels in boys.

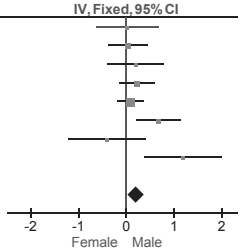
Meta-analysis

Nine articles (16%) did not report mean and SD values, which were therefore calculated. **Figure 3** shows the results of the fixed-effect meta-analysis.

A

Study or Subgroup	Male Mean	Male SD	Male Total	Female Mean	Female SD	Female Total	Weight	Std. Mean Difference IV, Fixed, 95% CI
0 yr (Davis 1995)	30.1	36.4	18	29.5	32.8	18	6.1%	0.02 [-0.64, 0.67]
3 yr (Gunnar 2010)	4.03	4.58	37	3.89	3.01	54	14.9%	0.04 [-0.38, 0.46]
3.3 yrs (De Bruijn 2009)	2.82	1.86	19	2.45	1.82	27	7.5%	0.20 [-0.39, 0.79]
4 yr (Pérez-Edgar 2008)	11.9	4.7	51	10.8	5	60	18.6%	0.22 [-0.15, 0.60]
4.1yr (Mills 2008)	3.03	2.76	115	2.76	2.76	85	33.1%	0.10 [-0.18, 0.38]
4.3 yr (Tout 1998)	5.79	2.48	38	4.41	1.38	37	12.0%	0.68 [0.21, 1.14]
4yr (Gunnar 2010)	2.92	1.35	11	4.47	4.88	13	3.9%	-0.40 [-1.22, 0.41]
7.3yr (Törnhage 2002)	10.5	5.2	19	5.2	1.88	11	4.0%	1.19 [0.38, 2.00]
Total (95% CI)			**308**			**305**	**100.0%**	**0.21 [0.05, 0.37]**

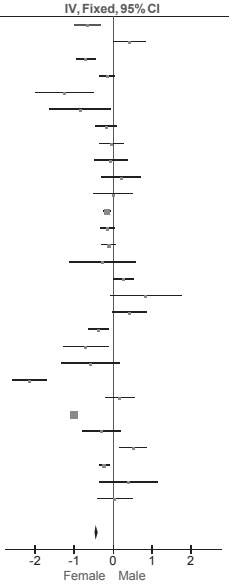
Heterogeneity: Chi² = 13.34, df = 7 (P = 0.06); I² = 48%
Test for overall effect: Z = 2.53 (P = 0.01)



B

Study or Subgroup	Male Mean	Male SD	Male Total	Female Mean	Female SD	Female Total	Weight	Std. Mean Difference IV, Fixed, 95% CI
8yr (Jones 2006)	9.58	1.51	68	10.59	1.49	72	1.6%	-0.67 [-1.01, -0.33]
8 yr (Azurmendi 2016)	8	4.14	49	6.35	3.59	41	1.0%	0.42 [-0.00, 0.84]
8yr (Martikainen 2013)	6.8	1.7	126	8	1.7	132	2.9%	-0.70 [-0.96, -0.45]
8.4 yr (Michels 2012)	12.66	6.12	183	13.84	8.96	202	4.6%	-0.15 [-0.35, 0.05]
9 yr (Alghadir 2009) active	0.13	0.026	25	0.16	0.017	12	0.3%	-1.25 [-2.00, -0.50]
9yr (Alghadir 2009) sedentary	0.18	0.014	12	0.2	0.028	16	0.3%	-0.84 [-1.62, -0.05]
9.2 yr (Cicchetti 2001)	8.64	5.16	124	9.52	5.21	80	2.3%	-0.17 [-0.45, 0.11]
9.4 yr (Turan 2015)	5.2	3.2	79	5.6	10.8	74	1.8%	-0.05 [-0.37, 0.27]
9.9 yr (Osika 2007)	5.34	2.1	34	5.5	3.33	50	1.0%	-0.05 [-0.49, 0.38]
10yr (Törnhage 2002)	11	4.08	28	8.9	13.03	31	0.7%	0.21 [-0.30, 0.72]
10.4±0.4yr (Cieslak 2003)	3	0.5	29	3	0.3	32	0.7%	0.00 [-0.50, 0.50]
11.1±0.55yr (Dietrich 2013)	11.2	4.7	818	11.9	4.73	786	19.0%	-0.15 [-0.25, -0.05]
11.9±0.6yr (Chen 2014)	4.69	5.52	213	5.52	5.24	212	5.0%	-0.15 [-0.34, 0.04]
11.9 yr (Portnoy 2015)	4.06	3.72	224	5.79	22.18	214	5.2%	-0.11 [-0.30, 0.08]
12 yr (Tzortzi 2009)	9.05	3.63	11	10.24	4.86	10	0.2%	-0.27 [-1.13, 0.59]
12.7 ± 2.9yr (Allen 2009)	0.17	0.18	119	0.13	0.09	116	2.8%	0.28 [0.02, 0.54]
12.7 yr (Yu 2009)	6.22	0.92	10	4.83	2.04	10	0.2%	0.84 [-0.08, 1.76]
13.4 yr (Mrug 2016)	3.43	2.83	40	2.31	2.5	39	0.9%	0.42 [-0.03, 0.86]
14.0±0.5yr (Belva 2013)	24.6	10.6	115	29.2	13.2	108	2.6%	-0.38 [-0.65, -0.12]
14.9yr (Alghadir 2009) active	0.22	0.015	20	0.24	0.034	30	0.5%	-0.70 [-1.29, -0.12]
14.9 yr (Alghadir 2009) sedentary	0.16	0.018	25	0.17	0.014	10	0.3%	-0.57 [-1.32, 0.17]
14 -16yr (Fransson 2014)	6.6	2	43	10.9	2	89	0.9%	-2.14 [-2.59, -1.69]
15.3 ± 1.1yr (Covelli 2012)	27	11	49	25	11.2	57	1.2%	0.18 [-0.20, 0.56]
15.5 yr (West 2010)	10.64	1.02	1418	11.67	1.02	1406	29.8%	-1.01 [-1.09, -0.93]
16 ±1yr (Georgopoulos 2011)	9.1	8.3	35	11.6	8.3	29	0.7%	-0.30 [-0.79, 0.20]
16.1 ±1.0yr (Daughters 2013)	3.22	2.13	59	2.3	1.38	73	1.5%	0.52 [0.17, 0.87]
16.6 ± 0,5yr (Reynolds 2013)	25.93	14.1	573	29.5	19	362	10.5%	-0.22 [-0.35, -0.09]
17.9 yr (Minckley 2012)	10.47	6.29	14	8.42	2.84	14	0.3%	0.41 [-0.34, 1.16]
18 yr (Colomina 1997)	13.7	7.7	37	13.4	7.6	36	0.9%	0.04 [-0.42, 0.50]
Total (95% CI)			**4580**			**4343**	**100.0%**	**-0.42 [-0.47, -0.38]**

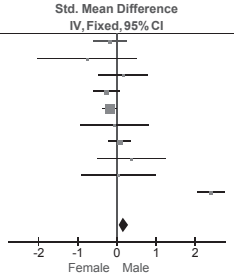
Heterogeneity: Chi² = 481.05, df = 28 (P < 0.00001); I² = 94%
Test for overall effect: Z = 19.37 (P < 0.00001)



C

Study or Subgroup	Male Mean	Male SD	Male Total	Female Mean	Female SD	Female Total	Weight	Std. Mean Difference IV, Fixed, 95% CI
0 yr (Elmlinger 2002)	253	95.7	42	271	97.4	44	7.7%	-0.18 [-0.61, 0.24]
0 yr (Forest 1978)	312	168	15	448	187	3	0.9%	-0.76 [-2.03, 0.51]
0 yr (Tennes 1973)	435.9	313.6	23	389.8	227.6	17	3.5%	0.16 [-0.47, 0.79]
0 yrs (Garagorri 2008)	249.41	99.32	80	280.31	124.16	58	12.0%	-0.28 [-0.62, 0.06]
0-2 yr (Bailey 2013)	159.8	135.3	261	190.2	186.2	208	41.6%	-0.19 [-0.37, -0.01]
0.4 yr (Lashansky 1991)	342	146	13	353	196	8	1.8%	-0.06 [-0.94, 0.82]
2-8 yr (Bailey 2013)	134.4	75.6	110	129.9	71.5	86	17.4%	0.06 [-0.22, 0.34]
3 yr (Lashansky 1991)	337	160	14	281	113	8	1.8%	0.37 [-0.51, 1.25]
4-10 yr (Tsvetkova 1977)	518.69	193.1	9	510.42	157.3	8	1.5%	0.04 [-0.91, 1.00]
6.8 yr (Soriano-Rodriguez 2010)	441.4	13.8	106	408.3	13.8	117	11.6%	2.39 [2.05, 2.74]
Total (95% CI)			**673**			**557**	**100.0%**	**0.17 [0.05, 0.29]**

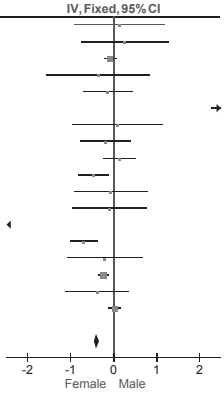
Heterogeneity: Chi² = 186.39, df = 9 (P < 0.00001); I² = 95%
Test for overall effect: Z = 2.79 (P = 0.005)



D

Study or Subgroup	Male Mean	Male SD	Male Total	Female Mean	Female SD	Female Total	Weight	Std. Mean Difference IV, Fixed, 95% CI
8 yr (Apter 1979)	240	80.3	7	226.2	109.5	7	0.4%	0.13 [-0.91, 1.18]
8yr (Lashansky 1991)	254	86	8	229	94	7	0.4%	0.26 [-0.76, 1.28]
8yr (Ong 2004)	210	58	423	215	69	347	22.4%	-0.08 [-0.22, 0.06]
9 yr (Ilias 2009)	112.97	49.14	5	153.26	131.05	6	0.3%	-0.36 [-1.56, 0.84]
6-15 yr (Ross 1986)	74.5	35.9	23	80	44.1	24	1.4%	-0.13 [-0.71, 0.44]
10yr (Stroud 2011)	164.4	5.9	39	146.5	5.99	30	0.9%	2.98 [2.28, 3.68]
11-14 (Tsvetkova 1977)	424.89	171.1	7	408.3	160	7	0.4%	0.09 [-0.95, 1.14]
11y (Elmlinger 2002)	341	113.5	22	364	117.3	23	1.3%	-0.20 [-0.78, 0.39]
12.5 yr (Susman 1991)	364.7	149.5	56	344.3	148.7	52	3.2%	0.14 [-0.24, 0.51]
13 yr (Hackney 2003)	397.3	126.9	62	466.3	162.8	63	3.6%	-0.47 [-0.82, -0.11]
13yr (Lashansky 1991)	223	74	10	229	94	11	0.6%	-0.07 [-0.92, 0.79]
14.6 yr (Ghaziuddin 2003)	1,757.5	1,191.9	12	1,914.7	1,658.2	9	0.6%	-0.11 [-0.97, 0.76]
14.7 yr (Huybrechts 2014)	299.5	1.6	297	305.6	1.7	426	7.8%	-3.67 [-3.91, -3.43]
15 yr (Syme 2008)	546.3	140.7	77	714.6	306.24	82	4.4%	-0.70 [-1.02, -0.38]
15.5yr (Lashansky 1991)	262	80	13	279	77	8	0.6%	-0.21 [-1.09, 0.68]
16.6 ± 0,5yr (Reynolds 2013)	551	154	653	590.98	202.79	401	29.1%	-0.23 [-0.35, -0.10]
17 yr (Stupnicki 1995)	347	130	14	397	125	15	0.8%	-0.38 [-1.12, 0.35]
8-18yr (Bailey 2013)	218.3	98.9	370	215.7	110.7	369	21.7%	0.02 [-0.12, 0.17]
Total (95% CI)			**2098**			**1887**	**100.0%**	**-0.39 [-0.46, -0.32]**

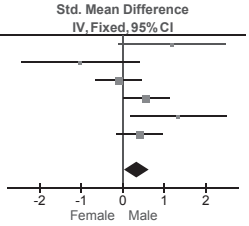
Heterogeneity: Chi² = 876.34, df = 17 (P < 0.00001); I² = 98%
Test for overall effect: Z = 11.36 (P < 0.00001)



E

Study or Subgroup	Male Mean	Male SD	Male Total	Female Mean	Female SD	Female Total	Weight	Std. Mean Difference IV, Fixed, 95% CI
3yr (Lundberg 1981)	3.35	1.12	4	2.46	0.44	9	5.2%	1.19 [-0.11, 2.49]
4.5yr (Lundberg 1983)	8.5	1.8	8	11.9	5.5	3	4.3%	-1.02 [-2.45, 0.41]
3-4 yr (Wudy 2007)	20.5	6.63	25	21.2	6.49	25	28.6%	-0.11 [-0.66, 0.45]
5-6 yr (Wudy 2007)	30.5	10.2	25	25.4	7.52	25	27.4%	0.56 [-0.01, 1.13]
5-10 yr (Nakamura 1984)	14.8	3.6	7	9.98	3.2	8	6.6%	1.34 [0.18, 2.50]
7-8 yr (Wudy 2007)	36.9	12.9	25	31.9	10.9	25	28.0%	0.41 [-0.15, 0.97]
Total (95% CI)			**94**			**95**	**100.0%**	**0.34 [0.05, 0.64]**

Heterogeneity: Chi² = 11.11, df = 5 (P = 0.05); I² = 55%
Test for overall effect: Z = 2.28 (P = 0.02)



F

Study or Subgroup	Male Mean	Male SD	Male Total	Female Mean	Female SD	Female Total	Weight	Std. Mean Difference IV, Fixed, 95% CI
8yr (Honour 2007)	2,826.86	774.81	244	2,557.71	655.56	188	57.7%	0.37 [0.18, 0.56]
9-10 yr (Wudy 2007)	35.5	9.26	25	34.9	12.5	25	6.9%	0.05 [-0.50, 0.61]
4-15yr (Canalis 1982)	15.2	6.3	14	13	5.6	15	3.9%	0.36 [-0.38, 1.09]
11-12 yr (Wudy 2007)	54.1	13.8	25	43.6	12.2	25	6.4%	0.79 [0.22, 1.37]
13 ±3yr (Vaindirlis 2000)	94	32	12	69	33	10	2.8%	0.74 [-0.13, 1.61]
13-14 yr (Wudy 2007)	53.2	21.4	25	49.5	15.1	25	6.9%	0.20 [-0.36, 0.75]
14-19yr (Nakamura 1984)	40.9	23	6	33.3	3.1	7	1.7%	0.45 [-0.66, 1.56]
15-16 yr (Wudy 2007)	68.5	21	25	65.1	22	25	6.9%	0.16 [-0.40, 0.71]
17-18 yr (Wudy 2007)	75	25.3	25	81.1	32.3	25	6.9%	-0.21 [-0.76, 0.35]
Total (95% CI)			**401**			**345**	**100.0%**	**0.32 [0.17, 0.47]**

Heterogeneity: Chi² = 8.67, df = 8 (P = 0.37); I² = 8%
Test for overall effect: Z = 4.31 (P < 0.0001)

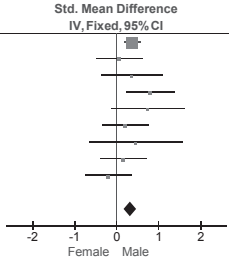


FIGURE 3. Forest plots of gender differences per subgroup (Fixed effect analyses)

A Salivary cortisol (nmol/L) <8 yr of age
B Salivary cortisol (nmol/L) 8–18 yr of age
C Serum cortisol (nmol/L) <8 yr of age
D Serum cortisol (nmol/L) 8–18 yr of age
E 24h-urine cortisol (µg/24h) <8 yr of age
F 24h-urine cortisol (µg/24h) 8–18 yr of age

Compared to girls, boys <8 yr had 0.21 (0.05 to 0.37) nmol/L (P = 0.01, I^2 = 48%) higher salivary and 0.18 (0.06 to 0.30) nmol/L (P < 0.01, I^2 = 94%) higher serum cortisol levels. Between ages 8-18 yr, boys had 0.42 (0.38 to 0.47) nmol/L (P < 0.01, I^2 = 94%) lower salivary and 0.34 (0.28 to 0.40) nmol/L (P < 0.01, I^2 = 97%) lower serum cortisol levels. In contrast, free cortisol in 24h-urine was 0.34 (0.05 to 0.64) µg/24h (P = 0.02, I^2 = 55%) higher in boys aged <8 yr and 0.32 (0.17 to 0.47) µg/24h (P <

0.01, I^2 = 8%) higher in boys between ages 8-18 yr. The sensitivity analyses did not significantly change the results, although it decreased the heterogeneity: boys <8 yr had 0.40 (0.11 to 0.69) nmol/L (P < 0.01, I^2 = 55%) higher salivary, 0.45 (0.30 to 0.61) nmol/L (P < 0.01, I^2 = 94%) higher serum and 0.28 (-0.04 to 0.61) µg/24h (P = 0.08, I^2 = 33%) higher 24h-urine cortisol; boys 8–18 yr had 0.20 (0.13 to 0.26) nmol/L (P < 0.01, I^2 = 47%) lower salivary, 0.10 (0.02 to 0.18) nmol/L (P = 0.01, I^2 = 33%) lower serum and 0.24 (0.02 to 0.47) µg/24h (P = 0.04, I^2 = 24%) higher 24h-urine cortisol.

Appendix 3 shows the results of the random-effects meta-analyses. When analyzed by the random-effects method, the effect estimates of serum cortisol <8 yr and between 8-18 yr became non-significant (P = 0.46 and P = 0.62, respectively). This also applied to salivary cortisol <8 yr (P = 0.06) and urinary cortisol <8yr (P = 0.12), although trends in the same direction were observed.

Funnel plots showed no evidence of publication bias. (**Appendix 4**)

DISCUSSION

The results from this meta-analysis suggest that gender-specific differences in HPA axis activity are already present early in life. They also support previous observations which show that cortisol metabolism diverges between genders at pubertal age. Before age 8 yr, cortisol in both serum and saliva was higher in boys compared to girls, at least in fixed-effect meta-analysis. These patterns were reversed after age 8 yr. In contrast, gender differences in 24h-urine cortisol remained consistent with age, with higher cortisol levels in urine for boys before and after age 8 yr.

Total serum cortisol and free salivary cortisol reflect the balance between cortisol production and degradation, i.e., the bioavailability. Our meta-analysis suggests that puberty induces gender-specific changes in the bioavailability of cortisol, as reflected by similar changes in both total serum and free salivary cortisol levels, at least in fixed-effect models. Even though associations were absent for total serum cortisol in random-effects models, the change in free salivary cortisol could not be explained by an estrogen-induced increase in the production of CBG [4]. Moreover, the gender difference in cortisol in 24hr-urine (i.e., non-metabolized, free cortisol, representing cortisol production rate) remained consistent with age. Consequently, sex-hormone dependent effects on the hepatic metabolism of cortisol are more likely to explain our observations. Cortisol is metabolized reversibly by 11βHSD type 2,

and irreversibly by α- and β-ring reductases, and CYP3A. Animal studies showed a lower bioavailability of glucocorticoids in females due to decreased 11βHSD type 1 [16–18] and relatively increased 11βHSD type 2 activity [18], as compared to males. In addition, previous observations in humans suggest that estrogens could alter hepatic cortisol metabolism through increased CYP3A activity [19, 20], and decreased A-ring reduction [3, 21]. In contrast, sex-specificity in the activities of 11βHSD isozymes is debated in humans [3,21,22]. Since analyses of gender-specific differences in total serum cortisol were inconclusive in random-effects models (**Appendix 3**) and only one of the included studied had assessed CBG levels next to cortisol, we cannot exclude a gender-specific influence of CBG [4] on the serum cortisol level.

The HPA axis set point can be modified through an altered balance between mineralocorticoid and glucocorticoid receptor expression [23]. Animal studies have suggested that patterns in receptor expression develop in a gender-specific manner from birth onwards [24]. In humans, behavioral patterns that impact a child's stress vulnerability have been associated with gender-specific changes in cortisol levels from age 1.5 yr onwards [11,25]. Therefore, even in our sample of normal children, gender-specific effects of stress exposure could be an explanation for our results [9].

Even subtle disturbances in HPA axis activity have been associated with cardiovascular disease and its risk factors [26–28]. Cardiovascular disease susceptibility is gender-specific [7, 29], which has been suggested to be due to gender differences in HPA axis activity, stress vulnerability and responsivity [4,30–32]. Early in life, developmental plasticity offers the child the capacity to change his HPA axis set point based on stress experiences [9,33]. This ability offers opportunities to withstand early-life challenges, but it has also been suggested to affect disease risk later in life. Accordingly, although the gender differences found in our study were small, these patterns might contribute to gender-specific origins of health and disease [9].

The major strength of this study is our systematic approach and the effort to contact all authors of eligible publications, enabling us to include the data on 16,551 healthy children. Moreover, articles with a lack of quantitative data were included in our descriptive analysis with the aim to be as complete as possible. The large sample size enabled us to perform a sensitivity analysis, which decreased the heterogeneity between studies. Furthermore, we accounted for this heterogeneity by calculating standardized mean differences, based on the intervention effects relative to the variability observed [13]. Additionally, we chose fixed-effect meta-analysis, because the studies with a large sample size were most likely conducted with greater

methodological accuracy [13]. Fixed-effect meta-analysis has the advantage of increasing the impact of large studies on the effect estimate. For comparison, results of random-effects meta-analyses, which put more weight on studies with small sample sizes, were also included. (**Appendix 3**).

A limitation of this study is that only a subset of studies (16%) considered gender differences as the primary outcome. In addition, in 22 studies (28%) samples were not collected specifically during mornings. Both could have led to a selection or performance bias, which we accounted for in our sensitivity analysis. Furthermore, 21 articles with data on 3,985 subjects could not be included in the meta-analysis due to lack of gender-stratified quantitative data, while most of these articles reported no significant gender differences. However, funnel plots of the articles included in the meta-analysis were not suggestive of publication bias. Instead, the plots seem to indicate that most articles reported on the nonexistence of gender differences, which might be a result of the common idea that gender differences are nonexistent at this early age. Nonetheless, our meta-analysis shows that significant gender differences are already present early in life. Another limitation is that almost all studies that reported on salivary or serum cortisol used immunoassays. Due to its superior specificity, liquid chromatography-tandem mass spectrometry is the method of choice for steroid hormone analysis [34]. Furthermore, we stratified studies based on the mean age or age range of the study group. Since study samples differed in age range, we have probably included some subjects < 8 yr of age in the 8-18 yr groups, and vice versa. An overview of the age ranges of studies included in the meta-analysis is presented in **Appendix 5**. Moreover, only a minority of the included studies assessed Tanner pubertal staging. Therefore, we were unable to address the question at which maturational stage the direction of the gender-specific dimorphism in cortisol changes.

CONCLUSIONS

In conclusion, gender differences in HPA axis activity are present early in life, with higher salivary cortisol concentrations in boys. A gender-specific evolution of cortisol metabolism is suggested to be induced by puberty, resulting in lower bioavailability of cortisol in boys. Although results from random-effects analyses were inconclusive for serum cortisol, the gender difference in cortisol production seems to be consistent between genders with age. Future research should take gender differences in HPA axis activity into account, regardless of age. Whether gender differences in stress-induced cortisol levels also exist is unknown and remains to be explored.

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APPENDIX 1

Search strategy for PubMed (14 January 2016)

Search	Query	Records (n)
#1	"Hydrocortisone"[Mesh] OR "Glucocorticoids"[Mesh] OR "11-beta-Hydroxysteroid Dehydrogenases"[Mesh] OR "Tetrahydrocortisone"[Mesh] OR "Tetrahydrocortisol"[Mesh] OR cortisol*[tiab] OR hydrocortison*[tiab] OR epicortisol*[tiab] OR cortifair*[tiab] OR cortril*[tiab] OR glucocorticoid*[tiab] OR beta hydroxysteroid dehydrogenase*[tiab] OR 11 oxoreductase*[tiab] OR 11 oxidoreductase*[tiab] OR 11 hydroxysteroid dehydrogenase*[tiab] OR 11b hydroxysteroid dehydrogenase*[tiab] OR 11 reductase*[tiab] OR 11beta hydroxysteroid dehydrogenase*[tiab] OR tetrahydrocortiso*[tiab] OR "hsd11b2"[tiab] OR "11bhsd2"[tiab] OR "11betahsd2"[tiab] OR 11beta hsd*[tiab] OR hydroxycortisol*[tiab] OR "Circadian Rhythm"[Mesh] OR "twenty four hour"[tiab] OR circadian*[tiab] OR diurnal*[tiab] OR nyctohemeral*[tiab]	250,920
#2	child*[tw] OR schoolchild*[tw] OR infan*[tw] OR adolescen*[tw] OR pediatri*[tw] OR paediatr*[tw] OR neonat*[tw] OR boy[tw] OR boys[tw] OR boyhood[tw] OR girl[tw] OR girls[tw] OR girlhood[tw] OR youth[tw] OR youths[tw] OR baby[tw] OR babies[tw] OR toddler*[tw] OR "Mental Disorders Diagnosed in Childhood"[MeSH] OR teen[tw] OR teens[tw] OR teenager*[tw] OR newborn*[tw] OR postneonat*[tw] OR postnat*[tw] OR perinat*[tw] OR puberty[tw] OR preschool*[tw] OR suckling*[tw] OR picu[tw] OR nicu[tw] OR "Arthritis, Juvenile"[Mesh] OR "Myoclonic Epilepsy, Juvenile"[Mesh] OR "Leukemia, Myelomonocytic, Juvenile"[Mesh] OR "Xanthogranuloma, Juvenile"[Mesh] OR "Juvenile Delinquency"[Mesh] OR "Corneal Dystrophy, Juvenile Epithelial of Meesmann"[Mesh]	3,664,351
#3	"Sex Characteristics"[Mesh] OR "Sex Factors"[Mesh] OR sex characteristic*[tiab] OR sex difference*[tiab] OR sex dimorphism*[tiab] OR sexual dimorphism*[tiab] OR sexual difference*[tiab] OR sexual characteristic*[tiab] OR sex factor*[tiab] OR sexual factor*[tiab] OR sexual dimorphi*[tiab] OR sex influenc*[tiab] OR sexual influenc*[tiab] OR gender*[tiab]	451,894
#4	(#1 AND #2 AND #3)	2,643

Abbreviations: Mesh = Medical subject headings; tiab = words in title OR abstract; tw = words in title, abstract, MeSH and other content related fields

Search strategy for Embase.com (14 January 2016)

Search	Query	Records (n)
#1	'hydrocortisone'/exp OR 'glucocorticoid'/de OR '11beta hydroxysteroid dehydrogenase'/exp OR 'tetrahydrocortisone'/exp OR 'tetrahydrocortisol'/exp OR cortisol*:ab,ti OR hydrocortison*:ab,ti OR epicortisol*:ab,ti OR cortifair*:ab,ti OR cortril*:ab,ti OR glucocorticoid*:ab,ti OR ('beta hydroxysteroid' NEAR/3 dehydrogenase*):ab,ti OR (11 NEXT/1 oxoreductase*):ab,ti OR (11 NEXT/1 oxidoreductase*):ab,ti OR ('11 hydroxysteroid' NEXT/1 dehydrogenase*):ab,ti OR ('11b hydroxysteroid' NEXT/1 dehydrogenase*):ab,ti OR (11 NEXT/1 reductase*):ab,ti OR ('11beta hydroxysteroid' NEXT/1 dehydrogenase*):ab,ti OR tetrahydrocortiso*:ab,ti OR 'hsd11b2':ab,ti OR '11bhsd2':ab,ti OR '11betahsd2':ab,ti OR (11beta NEXT/1 hsd*):ab,ti OR hydroxycortisol*:ab,ti OR ('trier social stress' NEXT/1 test*):ab,ti OR tsst:ab,ti OR (stress NEAR/3 hormone*):ab,ti OR (stress NEAR/3 marker*):ab,ti	235,221
#2	adolescen*:ab,ti OR 'adolescence'/exp OR 'adolescent coping orientation for problem experiences'/exp OR 'adolescent development'/exp OR 'adolescent disease'/exp OR 'adolescent health'/exp OR 'adolescent parent'/exp OR 'adolescent pregnancy'/exp OR 'adolescent smoking'/exp OR 'adolescent'/exp OR 'adolescent-family inventory of life events and changes'/exp OR babies:ab,ti OR baby:ab,ti OR 'birth weight'/exp OR boy:ab,ti OR boyhood:ab,ti OR boys:ab,ti OR 'brazelton neonatal behavioral assessment scale'/exp OR 'child abuse'/exp OR 'child advocacy'/exp OR 'child behavior checklist'/exp OR 'child behavior'/exp OR 'child care'/exp OR 'child death'/exp OR 'child health care'/exp OR 'child health'/exp OR 'child nutrition'/exp OR 'child parent relation'/exp OR 'child psychology'/exp OR 'child restraint system'/exp OR 'child safety'/exp OR 'child welfare'/exp OR child*:ab,ti OR 'child'/exp OR 'childhood disease'/exp OR 'childhood mortality'/exp OR 'childhood'/exp OR girl:ab,ti OR girlhood:ab,ti OR girls:ab,ti OR 'high risk infant'/exp OR infan*:ab,ti OR 'infant disease'/exp OR 'infant mortality'/exp OR 'infant nutrition'/exp OR 'infant welfare'/exp OR 'infanticide'/exp OR 'infantile diarrhea'/exp OR 'infantile hypotonia'/exp OR 'juvenile delinquency'/exp OR neonat*:ab,ti OR 'neonatal weight loss'/exp OR 'newborn disease'/exp OR 'newborn morbidity'/exp OR 'newborn period'/exp OR newborn*:ab,ti OR 'newborn'/exp OR nicu:ab,ti OR 'only child'/exp OR paediatr*:ab,ti OR pediatri*:de,ab,ti OR 'pediatric advanced life support'/exp OR 'pediatric anesthesia'/exp OR 'pediatric cardiology'/exp OR 'pediatric hospital'/exp OR 'pediatric intensive care nursing'/exp OR 'pediatric nurse practitioner'/exp OR 'pediatric nursing'/exp OR 'pediatric rehabilitation'/exp OR 'pediatric surgery'/exp OR 'newborn hypoxia'/exp OR 'pediatric ward'/exp OR 'pediatrics'/exp OR perinat*:ab,ti OR 'perinatal development'/exp OR 'perinatal period'/exp OR 'persistent hyperinsulinemic hypoglycemia of infancy'/exp OR picu:ab,ti OR postnat*:ab,ti OR 'postnatal care'/exp OR 'postnatal development'/exp OR 'postnatal growth'/exp OR postneonat*:ab,ti OR preschool*:ab,ti OR puberty:ab,ti OR 'runaway behavior'/exp OR 'school child':ab,ti OR schoolchild*:ab,ti OR 'severe myoclonic epilepsy in infancy'/exp OR suckling*:ab,ti OR teen:ab,ti OR teenager*:ab,ti OR teens:ab,ti OR toddler*:ab,ti OR 'transient hypogammaglobulinemia of infancy'/exp OR youth:ab,ti OR youths:ab,ti	4,477,134
#3	'sex difference'/exp OR 'sex ratio'/exp OR ('boy'/exp AND 'girl'/exp) OR (sex NEAR/3 characteristic*):ab,ti OR (sex NEAR/3 difference*):ab,ti OR (sex NEAR/3 dimorphism*):ab,ti OR (sexual NEAR/3 dimorphism*):ab,ti OR (sexual NEAR/3 difference*):ab,ti OR (sexual NEAR/3 characteristic*):ab,ti OR (sex NEAR/3 factor*):ab,ti OR (sexual NEAR/3 factor*):ab,ti OR (sexual NEAR/3 dimorphi*):ab,ti OR (sex NEAR/3 influenc*):ab,ti OR (sexual NEAR/3 influenc*):ab,ti OR gender*:ab,ti OR (boy*:ab,ti AND girl*:ab,ti) OR sex:ab,ti	1,014,014
#4	(#1 AND #2 AND #3)	4,280

/exp = EMtree keyword with explosion; /de = EMtree keyword without explosion; :ab,ti = words in title or abstract; NEXT/x = words in that order next to each other, x places apart; NEAR/x = words near to each other, x places apart

APPENDIX 2

RISK OF BIAS OF STUDIES INCLUDED IN THE META-ANALYSIS

(See for argumentation Online Supplementary File 2)

Risk of selection bias included: participants' age range, and sex-specific differences in participation or baseline characteristics. Risk of performance bias included: time of sample collection, protocol transparency, and sex-specific differences in protocol compliance. Risk of detection bias included: sex-specific differences in assay methods. Non-parametric distribution of the data was recorded as a risk of other biases. Bias could be assessed as low (i.e., unlikely to alter the results), unclear (i.e., raises doubt about results) or high (i.e., weakens confidence in results). Colored squares indicate: ■ = Low risk ■ = Unclear risk, ■ = High risk of bias.

A. Serum <8 yr

	Selection bias	Performance bias	Detection bias	Other bias
Bailey 2013	+	-	+	+
Elmlinger 2002	?	?	+	+
Forest 1978	?	?	+	+
Garagorri 2008	+	+	+	+
Lashansky 1991	?	+	?	+
Soriano-Rodriguez 2010	+	?	+	+
Tennes 1973	?	+	-	-
Tsvetkova 1977	?	+	+	+

B. Serum 8–18 yr

	Selection bias	Performance bias	Detection bias	Other bias
Apter 1979	?	?	+	-
Bailey 2013	+	-	+	+
Elmlinger 2002	?	?	+	+
Ghaziuddin 2003	+	+	+	-
Hackney 200	-	+	+	+
Huybrechts 2014	-	+	+	+
Ilias 2009	-	+	+	-
Lashansky 1991	?	+	?	+
Ong 2004	+	+	+	+
Reynolds 2013	?	+	+	+
Ross 1986	-	-	+	-
Stroud 2011	+	-	+	+
Stupnicki 1995	-	-	+	+
Susman 1991	?	+	+	+
Syme 2008	-	?	?	+
Tsvetkova 1977	?	+	+	+

C. Saliva

	Selection bias	Performance bias	Detection bias	Other bias
Davis 1995	?	-	+	+
De Bruijn 2009	+	-	+	-
Gunnar 2010	+	+	?	-
Mills 2008	?	-	+	-
Pérez-Edgar 2008	+	+	+	+
Törnhage 2002	?	+	+	-
Tout 1998	+	+	+	+

D. Saliva 8–18 yr

	Selection bias	Performance bias	Detection bias	Other bias
Alghadir 2009	+	+	+	-
Allen 2009	+	-	+	+
Azurmendi 2016	?	+	-	-
Belva 2013	+	+	+	+
Chen 2014	?	+	+	+
Cicchetti 2001	-	+	+	
Cieslak 2003	+	-	+	+
Colomina 1997	-	+	+	
Covelli 2012	-	+	+	
Daughters 2013	+	-	+	+
Dietrich 2013	?	?	+	+
Fransson 2014	+	+	+	-
Georgopoulos 2011	-	?	-	-
Jones 2006	?	+	+	+
Martikainen 2013	-	+	+	+
Michels 2012	+	+	+	+
Minckley 2012	?	+	+	?
Mrug 2016	-	+	+	-
Osika 2007	+	+	+	-
Portnoy	+	-	+	-
Reynolds 2013	?	+	+	+
Törnhage 2002	?	+	+	-
Turan 2015	+	-	?	-
Tzortzi 2009	+	+	+	+
West 2010	-	+	+	
Yu 2009	?	-	+	+

E. Urine <8 yr

	Selection bias	Performance bias	Detection bias	Other bias
Lundberg 1981	?	+	+	-
Lundberg 1983	?	+	?	-
Nakamura 1984	-	?	+	+
Wudy 2007	+	+	+	+

F. Urine 8–18 yr

	Selection bias	Performance bias	Detection bias	Other bias
Canalis 1982	?	?	?	+
Honour 2007	+	+	+	-
Nakamura 1984	-	?	+	+
Vaindirlis 2000	?	+	?	+
Wudy 2007	+	+	+	+

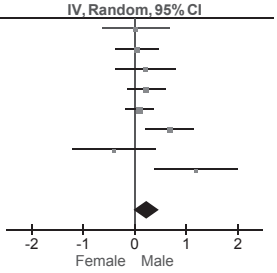
APPENDIX 3

FOREST PLOTS OF GENDER DIFFERENCES PER SUBGROUP (RANDOM EFFECT ANALYSES)

A. Saliva <8 yr

	Male			Female				Std. Mean Difference
Study or Subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, Random, 95% CI
0 yr (Davis 1995)	30.1	36.4	18	29.5	32.8	18	9.2%	0.02 [-0.64, 0.67]
3 yr (Gunnar 2010)	4.03	4.58	37	3.89	3.01	54	15.4%	0.04 [-0.38, 0.46]
3.3 yrs (De Bruijn 2009)	2.82	1.86	19	2.45	1.82	27	10.6%	0.20 [-0.39, 0.79]
4 yr (Pérez-Edgar 2008)	11.9	4.7	51	10.8	5	60	16.9%	0.22 [-0.15, 0.60]
4.1yr (Mills 2008)	3.03	2.76	115	2.76	2.76	85	20.6%	0.10 [-0.18, 0.38]
4.3 yr (Tout 1998)	5.79	2.48	38	4.41	1.38	37	13.8%	0.68 [0.21, 1.14]
4yr (Gunnar 2010)	2.92	1.35	11	4.47	4.88	13	6.7%	-0.40 [-1.22, 0.41]
7.3yr (Törnhage 2002)	10.5	5.2	19	5.2	1.88	11	6.8%	1.19 [0.38, 2.00]
Total (95% CI)			**308**			**305**	**100.0%**	**0.23 [-0.01, 0.48]**

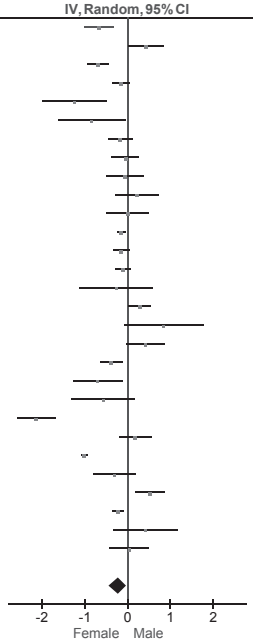
Heterogeneity: Tau² = 0.05; Chi² = 13.34, df = 7 (P = 0.06); I² = 48%
Test for overall effect: Z = 1.90 (P = 0.06)



B. Saliva 8-18 y

	Male			Female				Std. Mean Difference
Study or Subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, Random, 95% CI
8yr (Jones 2006)	9.58	1.51	68	10.59	1.49	72	3.7%	-0.67 [-1.01, -0.33]
8 yr (Azurmendi 2016)	8	4.14	49	6.35	3.59	41	3.5%	0.42 [-0.00, 0.84]
8yr (Martikainen 2013)	6.8	1.7	126	8	1.7	132	3.9%	-0.70 [-0.96, -0.45]
8.4 yr (Michels 2012)	12.66	6.12	183	13.84	8.96	202	4.0%	-0.15 [-0.35, 0.05]
9 yr (Alghadir 2009) active	0.13	0.026	25	0.16	0.017	12	2.6%	-1.25 [-2.00, -0.50]
9yr (Alghadir 2009) sedentary	0.18	0.014	12	0.2	0.028	16	2.5%	-0.84 [-1.62, -0.05]
9.2 yr (Cicchetti 2001)	8.64	5.16	124	9.52	5.21	80	3.8%	-0.17 [-0.45, 0.11]
9.4 yr (Turan 2015)	5.2	3.2	79	5.6	10.8	74	3.8%	-0.05 [-0.37, 0.27]
9.9 yr (Osika 2007)	5.34	2.1	34	5.5	3.33	50	3.5%	-0.05 [-0.49, 0.38]
10yr (Törnhage 2002)	11	4.08	28	8.9	13.03	31	3.3%	0.21 [-0.30, 0.72]
10.4±0.4yr (Cieslak 2003)	3	0.5	29	3	0.3	32	3.3%	0.00 [-0.50, 0.50]
11.1±0.55yr (Dietrich 2013)	11.2	4.7	818	11.9	4.73	786	4.1%	-0.15 [-0.25, -0.05]
11.9±0.6yr (Chen 2014)	4.69	5.52	213	5.52	5.24	212	4.0%	-0.15 [-0.34, 0.04]
11.9 yr (Portnoy 2015)	4.06	3.72	224	5.79	22.18	214	4.0%	-0.11 [-0.30, 0.08]
12 yr (Tzortzi 2009)	9.05	3.63	11	10.24	4.86	10	2.4%	-0.27 [-1.13, 0.59]
12.7 ± 2.9yr (Allen 2009)	0.17	0.18	119	0.13	0.09	116	3.9%	0.28 [0.02, 0.54]
12.7 yr (Yu 2009)	6.22	0.92	10	4.83	2.04	10	2.2%	0.84 [-0.08, 1.76]
13.4 yr (Mrug 2016)	3.43	2.83	40	2.31	2.5	39	3.5%	0.42 [-0.03, 0.86]
14.0±0.5yr (Belva 2013)	24.6	10.6	115	29.2	13.2	108	3.9%	-0.38 [-0.65, -0.12]
14.9yr (Alghadir 2009) active	0.22	0.015	20	0.24	0.034	30	3.1%	-0.70 [-1.29, -0.12]
14.9 yr (Alghadir 2009) sedentary	0.16	0.018	25	0.17	0.014	10	2.6%	-0.57 [-1.32, 0.17]
14 -16yr (Fransson 2014)	6.6	2	43	10.9	2	89	3.4%	-2.14 [-2.59, -1.69]
15.3 ± 1.1yr (Covelli 2012)	27	11	49	25	11.2	57	3.6%	0.18 [-0.20, 0.56]
15.5 yr (West 2010)	10.64	1.02	1418	11.67	1.02	1406	4.1%	-1.01 [-1.09, -0.93]
16 ±1yr (Georgopoulos 2011)	9.1	8.3	35	11.6	8.3	29	3.3%	-0.30 [-0.79, 0.20]
16.1 ±1.0yr (Daughters 2013)	3.22	2.13	59	2.3	1.38	73	3.7%	0.52 [0.17, 0.87]
16.6 ± 0,5yr (Reynolds 2013)	25.93	14.1	573	29.5	19	362	4.1%	-0.22 [-0.35, -0.09]
17.9 yr (Minckley 2012)	10.47	6.29	14	8.42	2.84	14	2.6%	0.41 [-0.34, 1.16]
18 yr (Colomina 1997)	13.7	7.7	37	13.4	7.6	36	3.4%	0.04 [-0.42, 0.50]
Total (95% CI)			**4580**			**4343**	**100.0%**	**-0.23 [-0.43, -0.03]**

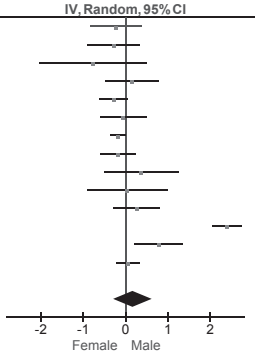
Heterogeneity: Tau² = 0.25; Chi² = 481.05, df = 28 (P < 0.00001); I² = 94%
Test for overall effect: Z = 2.23 (P = 0.03)



C. Serum <8 yr

	Male			Female				Std. Mean Difference	Std. Mean Difference
Study or Subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, Random, 95% CI	IV, Random, 95% CI
1-7d (Elmlinger 2002)	524	190.82	28	569	184.44	17	7.2%	-0.23 [-0.84, 0.37]	
8-15d (Elmlinger 2002)	311	99.75	20	342	119.39	20	7.1%	-0.28 [-0.90, 0.35]	
0 yr (Forest 1978)	312	168	15	448	187	3	5.0%	-0.76 [-2.03, 0.51]	
0 yr (Tennes 1973)	435.9	313.6	23	389.8	227.6	17	7.1%	0.16 [-0.47, 0.79]	
0 yrs (Garagorri 2008)	249.41	99.32	80	280.31	124.16	58	7.8%	-0.28 [-0.62, 0.06]	
0.4 yr (Lashansky 1991)	342	146	13	353	196	420	7.3%	-0.06 [-0.61, 0.50]	
0-2 yr (Bailey 2013)	159.8	135.3	261	190.2	186.2	208	8.1%	-0.19 [-0.37, -0.01]	
16d-3y (Elmlinger 2002)	253	95.66	42	271	97.44	44	7.7%	-0.18 [-0.61, 0.24]	
3 yr (Lashansky 1991)	337	160	14	281	113	8	6.3%	0.37 [-0.51, 1.25]	
4-10 yr (Tsvetkova 1977)	518.69	193.1	9	510.42	157.3	8	6.1%	0.04 [-0.91, 1.00]	
4-6y (Elmlinger 2002)	253	143.88	28	218	117.86	23	7.3%	0.26 [-0.29, 0.81]	
6.8 yr (Soriano-Rodriguez 2010)	441.4	13.8	106	408.3	13.8	117	7.8%	2.39 [2.05, 2.74]	
7-8y (Elmlinger 2002)	400	141.58	26	294	123.47	24	7.2%	0.78 [0.21, 1.36]	
2-8 yr (Bailey 2013)	134.41	75.57	110	129.91	71.48	86	7.9%	0.06 [-0.22, 0.34]	
Total (95% CI)			**775**			**1053**	**100.0%**	**0.17 [-0.28, 0.63]**	

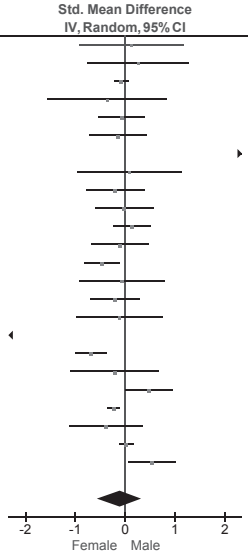
Heterogeneity: Tau² = 0.67; Chi² = 194.87, df = 13 (P < 0.00001); I² = 93%
Test for overall effect: Z = 0.75 (P = 0.46)



D. Serum 8-18 yr

	Male			Female				Std. Mean Difference	Std. Mean Difference
Study or Subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, Random, 95% CI	IV, Random, 95% CI
8 yr (Apter 1979)	240	80.3	7	226.2	109.5	7	3.6%	0.13 [-0.91, 1.18]	
8yr (Lashansky 1991)	254	86	8	229	94	7	3.7%	0.26 [-0.76, 1.28]	
8yr (Ong 2004)	210	58	423	215	69	347	4.5%	-0.08 [-0.22, 0.06]	
9 yr (Ilias 2009)	112.97	49.14	5	153.26	131.05	6	3.4%	-0.36 [-1.56, 0.84]	
9-10y (Elmlinger 2002)	317	165.82	31	328	153.32	40	4.3%	-0.07 [-0.54, 0.40]	
6-15 yr (Ross 1986)	74.5	35.9	23	80	44.1	24	4.2%	-0.13 [-0.71, 0.44]	
10yr (Stroud 2011)	164.4	5.9	39	146.5	5.99	30	4.1%	2.98 [2.28, 3.68]	
11-14 (Tsvetkova 1977)	424.89	171.1	7	408.3	160	7	3.6%	0.09 [-0.95, 1.14]	
11y (Elmlinger 2002)	341	113.5	22	364	117.3	23	4.2%	-0.20 [-0.78, 0.39]	
12y (Elmlinger 2002)	386	114.29	22	388	117.35	23	4.2%	-0.02 [-0.60, 0.57]	
12.5 yr (Susman 1991)	364.7	149.5	56	344.3	148.7	52	4.4%	0.14 [-0.24, 0.51]	
13y (Elmlinger 2002)	430	131.12	21	442	103.32	25	4.2%	-0.10 [-0.68, 0.48]	
13 yr (Hackney 2003)	397.3	126.9	62	466.3	162.8	63	4.4%	-0.47 [-0.82, -0.11]	
13yr (Lashansky 1991)	223	74	10	229	94	11	3.9%	-0.07 [-0.92, 0.79]	
14y (Elmlinger 2002)	440	109.18	32	466	131.63	30	4.3%	-0.21 [-0.71, 0.29]	
14.6 yr (Ghaziuddin 2003)	1,757.5	1,191.9	12	1,914.7	1,658.2	9	3.9%	-0.11 [-0.97, 0.76]	
14.7 yr (Huybrechts 2014)	299.5	1.6	297	305.6	1.7	426	4.5%	-3.67 [-3.91, -3.43]	
15 yr (Syme 2008)	546.3	140.7	77	714.6	306.24	82	4.5%	-0.70 [-1.02, -0.38]	
15.5yr (Lashansky 1991)	262	80	13	279	77	8	3.9%	-0.21 [-1.09, 0.68]	
16y (Elmlinger 2002)	444	111.99	31	388	116.58	40	4.3%	0.48 [0.01, 0.96]	
16.6 ± 0,5yr (Reynolds 2013)	551	154	653	590.98	202.79	401	4.6%	-0.23 [-0.35, -0.10]	
17 yr (Stupnicki 1995)	347	130	14	397	125	15	4.1%	-0.38 [-1.12, 0.35]	
8-18yr (Bailey 2013)	218.3	98.9	370	215.7	110.7	369	4.5%	0.02 [-0.12, 0.17]	
17-19y (Elmlinger 2002)	465	124.74	30	403	103.57	42	4.3%	0.54 [0.07, 1.02]	
Total (95% CI)			**2265**			**2087**	**100.0%**	**-0.11 [-0.56, 0.33]**	

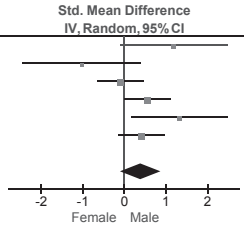
Heterogeneity: Tau² = 1.12; Chi² = 906.47, df = 23 (P < 0.00001); I² = 97%
Test for overall effect: Z = 0.50 (P = 0.62)



E. Urine <8 yr

Study or Subgroup	Male Mean	Male SD	Male Total	Female Mean	Female SD	Female Total	Weight	Std. Mean Difference IV, Random, 95% CI
3yr (Lundberg 1981)	3.35	1.12	4	2.46	0.44	9	9.9%	1.19 [-0.11, 2.49]
4.5yr (Lundberg 1983)	8.5	1.8	8	11.9	5.5	3	8.6%	-1.02 [-2.45, 0.41]
3-4 yr (Wudy 2007)	20.5	6.63	25	21.2	6.49	25	23.4%	-0.11 [-0.66, 0.45]
5-6 yr (Wudy 2007)	30.5	10.2	25	25.4	7.52	25	23.1%	0.56 [-0.01, 1.13]
5-10 yr (Nakamura 1984)	14.8	3.6	7	9.98	3.2	8	11.6%	1.34 [0.18, 2.50]
7-8 yr (Wudy 2007)	36.9	12.9	25	31.9	10.9	25	23.3%	0.41 [-0.15, 0.97]
Total (95% CI)			**94**			**95**	**100.0%**	**0.39 [-0.10, 0.87]**

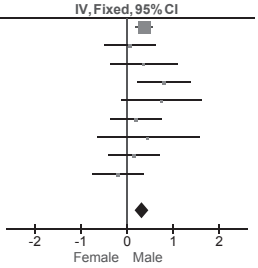
Heterogeneity: Tau² = 0.18; Chi² = 11.11, df = 5 (P = 0.05); I² = 55%
Test for overall effect: Z = 1.55 (P = 0.12)



F. Urine 8-18 yr

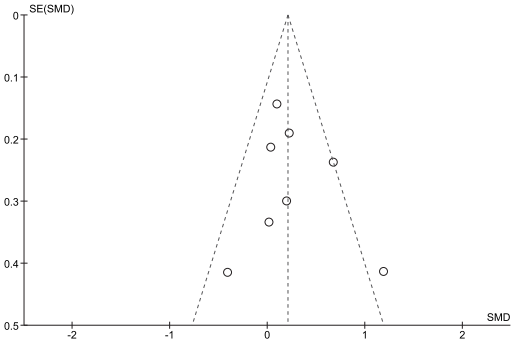
Study or Subgroup	Male Mean	Male SD	Male Total	Female Mean	Female SD	Female Total	Weight	Std. Mean Difference IV, Fixed, 95% CI
8yr (Honour 2007)	2,826.86	774.81	244	2,557.71	655.56	188	57.7%	0.37 [0.18, 0.56]
9-10 yr (Wudy 2007)	35.5	9.26	25	34.9	12.5	25	6.9%	0.05 [-0.50, 0.61]
4-15yr (Canalis 1982)	15.2	6.3	14	13	5.6	15	3.9%	0.36 [-0.38, 1.09]
11-12 yr (Wudy 2007)	54.1	13.8	25	43.6	12.2	25	6.4%	0.79 [0.22, 1.37]
13 ±3yr (Vaindirlis 2000)	94	32	12	69	33	10	2.8%	0.74 [-0.13, 1.61]
13-14 yr (Wudy 2007)	53.2	21.4	25	49.5	15.1	25	6.9%	0.20 [-0.36, 0.75]
14-19yr (Nakamura 1984)	40.9	23	6	33.3	3.1	7	1.7%	0.45 [-0.66, 1.56]
15-16 yr (Wudy 2007)	68.5	21	25	65.1	22	25	6.9%	0.16 [-0.40, 0.71]
17-18 yr (Wudy 2007)	75	25.3	25	81.1	32.3	25	6.9%	-0.21 [-0.76, 0.35]
Total (95% CI)			**401**			**345**	**100.0%**	**0.32 [0.17, 0.47]**

Heterogeneity: Chi² = 8.67, df = 8 (P = 0.37); I² = 8%
Test for overall effect: Z = 4.31 (P < 0.0001)

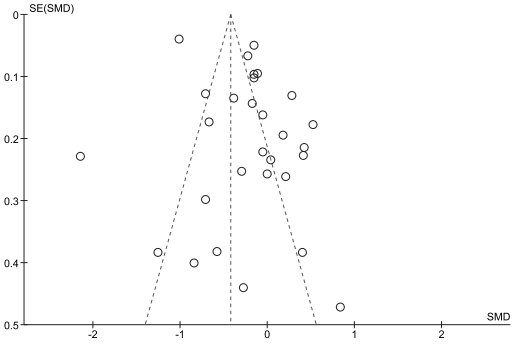


APPENDIX 4

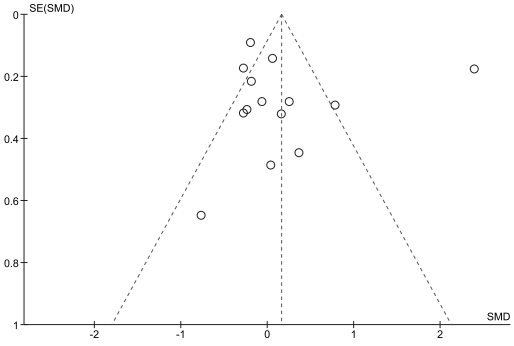
A. Saliva <8 yr



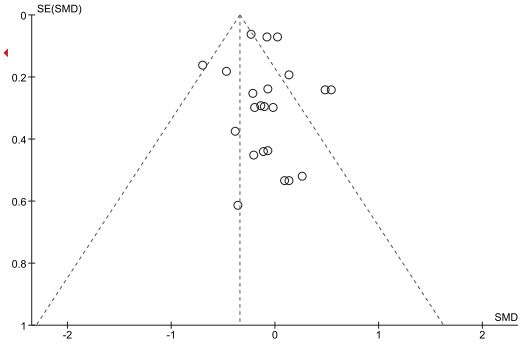
B. Saliva 8-18 yr



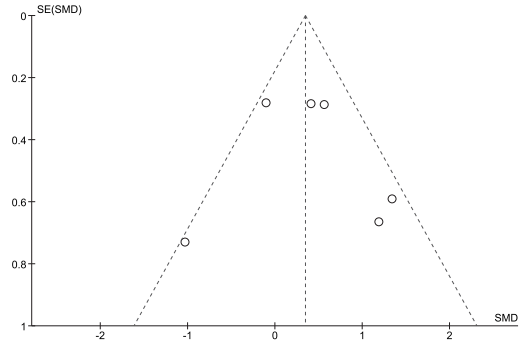
C. Serum <8 yr



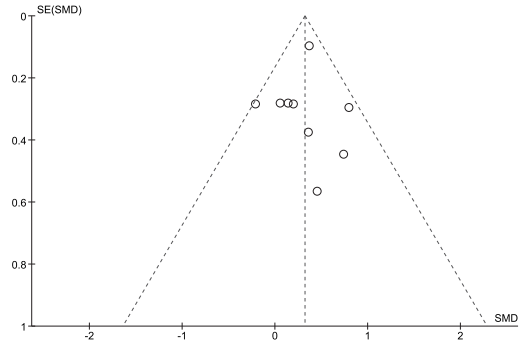
D. Serum 8-18 yr



E. Urine <8 yr



F. Urine 8-18 yr



APPENDIX 5

Overview of age ranges of studies included in meta-analysis

Study	Mean age ± SD*	Study	Mean age ± SD*
Davis 1995	2 days	Mrug 2016	13.36 ± 0.95 yr
Forest 1978	115.3 ± 120.1 days	Vaindirlis 2000	13 yr ± 3.5 yr
Tennes 1973	3 days	Hackney 2003	13.4 ± 0.9 yr
Garagorri 2008	3 days	Belva 2013	girls 14.0 ± 0.5 yr, boys 14.0 ± 0.4 yr
De Bruijn 2009	38.61 ± 9.4 months	Ghaziuddin 2003	14.6 ± 1.5 yr
Lundberg 1981	3 yr	Huybrechts 2014	14.7 ± 1.2 yr
Gunnar 2010	3.81 ± 0.23 yr	Fransson 2014	ranges 14 - 16 yr
Mills 2008	4.14 ± 0.24 yr	Nakamura 1984	5 – 10 yr and 14 - 19 yr
Tout 1998	mean 4.3 yr	Covelli 2012	15.3 ± 1.1 yr
Lundberg 1983	boys mean 52.3 months, girls 54.9 months	West 2010	15.4 ± 0.4 yr
Soriano-Rodriguez 2010	6.8 ± 0.19 yr	Syme 2008	boys 14.4 ± 1.7 yr, girls 14.4 ± 1.9 yr
Michels 2012	boys 8.44 ± 1.18 yr, girls 8.39 ± 1.20 yr	Daughters 2013	16.1 yr ± 1.0 yr
Apter 1979	range 7.5 – 8.5 yr	Reynolds 2013	16.6 yr ± 0.5 yr
Azurmendi 2016	8 yr	Georgopoulos 2011	boys 15.3 ± 2.0 yr, girls 16.0 ± 1.4 yr
Honour 2007	range 8.2 – 8.4 yr	Minckley 2012	17.86 ± (S.E.M.) 0.096 yr
Jones 2006	range 7- 9 yr	Stupnicki 1995	boys 17.3 ± 0.8 yr, girls 16.4 ± 0.6 yr
Martikainen 2013	boys 8.2 ± 0.3 yr, girls 8.1 ± 0.3 yr	Colomina 1997	range 17.5 – 18.5 yr
Ong 2004	8.2 ± 0.1 yr	Elmlinger 2002	ranges 16 days – 3 yr, 11 yr
Cicchetti 2001	9.24 ± 2.33 yr	Tsvetkova 1977	ranges 4-10 yr and 11-14 yr
Turan 2015 Osika 2007 Ilias 2009 Cieslak 2003	9.38 ± 0.62 yr 9.9 ± 0.6 yr boys 9.5 ± 1.9 yr, girls 9.1 ± 1.3 yr 10.4 ± 0.4 yr	Lashansky 1991	Boys 0.42 ± 0.24, 3.2 ± 1.6, 7.4 ± 1.8, 13.1 ± 1.2, 15.2 ± 1.4 yr Girls 0.42 ± 0.2,2.5 ± 1.5, 9.3 ± 2.2, 12.5 ± 0.9, 15.9 ± 0.7 yr
Stroud 2011	10.5 ± 1.7 yr	Bailey 2013	0.41 ± 0.37, 5.29 ± 1.74, 13.48 ± 3.03 yr
Dietrich 2013 Chen 2014	11.1 yr ± 0.55 yr 11.87 ± 0.60 yr	Wudy 2007	ranges 3-4, 5-6, 7-8, 9-10, 11-12, 13-14, 15-16, 17-18 yr
Portnoy 2015 Susman 1991	11.92 ± 0.59 yr mean boys 12.72 yr, girls 11.99 yr	Alghadir 2009	boys 9.3 ± 1.5 and 14.9 ± 3.7 yr girls 8.96 ± 1.8 and 14.82 ± 4.6 yr
Allen 2009 Yu 2009	12.7 yr ± 2.9 yr 12.6 ± 1.8 yr	Törnhage 2002	median girls 7.4 and 10.3 yr, boys 7.1 and 10.2 yr
Ross 1986 Canalis 1982	range 6-15 yr range 4-15 yr	Tzortzi 2009	ranges boys 10 yr and 3 months – 13 yr and 7 months, girls 10 yr and 3 months – 13 yr and 3 months

* unless otherwise indicated

ONLINE SUPPLEMENTARY FILES

1. Extracted data of studies included in the meta-analysis
   https://goo.gl/RBPvoT

2. Risk of bias of studies included in the meta-analysis
   https://goo.gl/iZX9vk

CHAPTER 11

IS HPA AXIS REACTIVITY IN CHILDHOOD GENDER-SPECIFIC? A SYSTEMATIC REVIEW

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Joost Rotteveel
Martijn J.J. Finken

[1] BvdV and JJH contributed equally to this manuscript.

Biology of Sex Differences (2017) 8:23

ABSTRACT

BACKGROUND

In adults, hypothalamic-pituitary-adrenal (HPA) axis activity shows sexual dimorphism, and this is thought to be a mechanism underlying sex-specific disease incidence. Evidence is scarce on whether these sex differences are also present in childhood. In a meta-analysis, we recently found that basal (non-stimulated) cortisol in saliva and free cortisol in 24-h urine follow sex-specific patterns. We explored whether these findings could be extended with sex differences in HPA axis reactivity.

METHODS

From inception to January 2016, PubMed and EMBASE.com were searched for studies that assessed HPA axis reactivity in healthy girls and boys aged ≤18 years. Articles were systematically assessed, and reported in the categories: (1) diurnal rhythm, (2) cortisol awakening response (CAR), (3) protocolled social stress tests similar or equal to the Trier Social Stress Test for children (TSST-C), (4) pharmacological (ACTH and CRH) stress tests, (5) miscellaneous stress tests.

RESULTS

Two independent assessors selected 109 out of 6,158 records for full-text screening, of which 81 studies (with a total of 14,591 subjects) were included. Studies showed that girls had a tendency towards a more variable diurnal rhythm (12 out of 29 studies), a higher CAR (8 out of 18 studies), and a stronger cortisol response to social stress tests (9 out of 21 studies). We found no evidence for sex differences in cortisol response after a pharmacological challenge or to miscellaneous stress tests.

DISCUSSION

Sex differences in HPA axis reactivity appear to be present in childhood, although evidence is not unequivocal. For a better evaluation of sex differences in HPA axis reactivity, standardization of protocols and reports of stress tests is warranted.

BACKGROUND

Marked gender differences exist in the incidence of several diseases. While men are more prone to obesity, cardiovascular disease and infectious diseases, women are more susceptible to anxiety, depression and autoimmune diseases. Sex-specific risks for chronic, non-communicable diseases are thought to result from a combination of genotype, phenotype and environmental influences during life. Whereas adjustment to environmental challenges is healthy in the short term, developmental plasticity can cause sex-specific adverse effects in the long term.[1]

One of the possible explanations for this sexual dimorphism in disease is a sex-specific reactivity of the hypothalamic-pituitary-adrenal (HPA) axis. HPA axis functioning can be distinguished by on the one hand the maintenance of homeostasis by controlling basal activity as well as the sensitivity to stressors, and on the other hand coping with, adapting to and recovery from reactions to stressors. These processes are controlled by mineralocorticoid and glucocorticoid receptors (MRs and GRs). MRs are mainly involved with basal HPA axis activity, whereas GRs predominantly regulate HPA axis reactivity.[2] In animals, receptor expression patterns appear to develop in a sex-specific manner, with sex differences already present at birth.[3] In humans, sexually dimorphic HPA axis reactivity has also been reported in adulthood: men showed a greater cortisol response to acute real-life or controlled laboratory psychological stress compared to women.[4] Additionally, cortisol responses increased with age in both men and women, but the effect was three-fold stronger in women compared to men, which could possibly be attributed to menopause.[5] These patterns closely resemble those of cardiovascular disease mortality and morbidity.[6] While the setting of HPA axis functioning results from the balance between MR and GR expression,[2] interactions with the hypothalamic–pituitary–gonadal (HPG) axis are thought to mediate sex-specific stress reactions as well as pathophysiology.[7]

It has previously been hypothesized that disease susceptibility can originate in childhood, possibly through permanent alterations in HPA axis activity to environmental challenges.[1] We recently showed that basal HPA axis activity, represented by non-stimulated cortisol concentrations in saliva and free cortisol in 24h-urine, show sexual dimorphism, with a sex-specific change induced by puberty.[8] In addition, gender differences in the reactivity of the HPA axis have also been described in children,[4,9,10] although evidence is scarce and not systematically reviewed. Therefore, we aimed to examine whether sex-specific differences in HPA axis reactivity are present in childhood.

To study this sex-specific reactivity of the HPA axis, we performed a systematic review of the literature. Reactivity of the HPA axis was defined as the response to either

exogenous (e.g., pharmacological, physical or social) or endogenous (e.g., cortisol awakening response (CAR)) stimuli. In addition, we included diurnal rhythm as a marker of the responsiveness of the HPA axis, although it functions differently from reactions of the HPA axis to stressors. We hypothesized that sex-specific HPA axis reactivity is already present early in life.

METHODS

SEARCH STRATEGY

PubMed and Embase.com were searched from inception up to January 14th, 2016 for studies addressing HPA axis reactivity in serum or saliva in boys and girls aged ≤18 years by reports of either absolute cortisol values, slopes, AUC's and/or through visualization of the data in figures. The full search strategy is detailed in **Appendix 1, Chapter 9** and was based on the index terms or free-text words 'cortisol' or 'glucocorticoid', and 'sex difference' or 'sexual characteristics', and 'child' or 'adolescent'. We excluded studies on children with (psycho)pathology, on synthetic glucocorticoids or with a risk of abnormal HPA axis reactivity (e.g., maltreatment). We did not impose restrictions on the year of publication or study design, apart from reviews and case reports, but we did apply an English language restriction. The review protocol was based on the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement.

DATA COLLECTION

Two independent assessors (BvdV and JJH) screened 6,158 titles and abstracts for assessment of sex-specific HPA axis reactivity. Studies were not assessed blindly. Disagreement between assessors was discussed until consensus was reached. One-hundred and nine were eligible for full-text screening, of which 81 studies were included in the systematic review.

Figure 1 shows the flowchart of the search. When reports of results were unclear, the authors were contacted (n=4); two authors responded. One author did not reply and one replied but could not provide sufficient data, resulting in exclusion of these studies. Additionally, articles were excluded when (1) no statistical analysis of reactivity was performed (n=9), (2) pharmacological stress tests did not use CRH and/or ACTH (n=2), (3) HPA axis reactivity was presented stratified by gender, without analyzing gender differences (n=6), (4) gender was analyzed only as a confounder or

effect modifier (n=3), 5) analyses of sex differences were performed with cases and controls combined (n=2) or (5) cortisol reactivity was defined as the variability of cortisol concentrations over several days to months (n=3). Several articles reported on the same cohort. Provided that extra information was presented, all articles were included in the review. Two articles were excluded as no new information was provided compared to other articles describing the same cohort. With respect to case-control studies, we included only the control group.

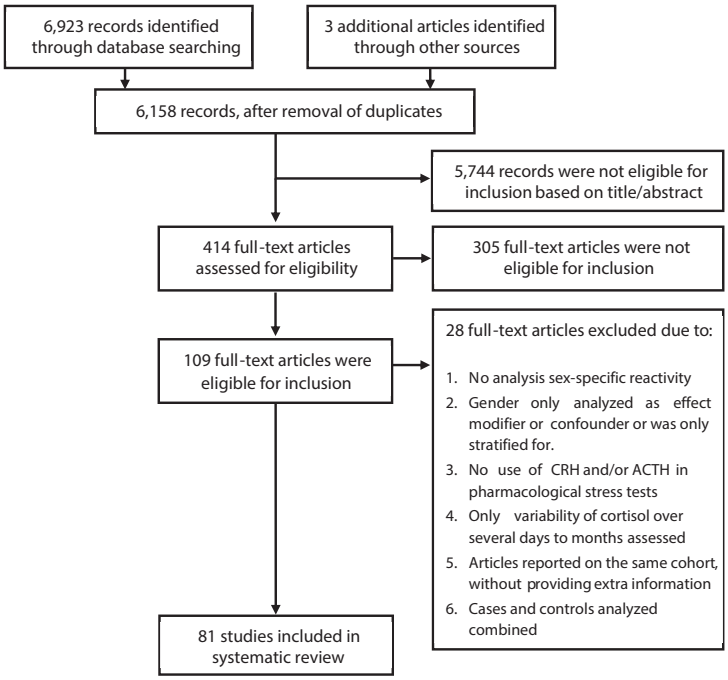

FIGURE 1. This flow chart presents the different phases of the systematic review, conform the PRISMA-statement. (www.prisma-statement.org)

DATA ANALYSIS

HPA axis reactivity was classified as follows: (1) diurnal rhythm, (2) cortisol awakening response (CAR), (3) protocolled social stress tests similar or equal to the Trier Social Stress Test for children (TSST-C), (4) pharmacological (ACTH and/or CRH) tests, or (5) miscellaneous stress tests. One assessor (JJH) assessed all the articles and sorted them according to the categories above. Data were extracted from the articles and systematically summarized. If more than one type of reactivity was assessed within one article, the data were included in all applicable categories.

RESULTS

A short overview of all articles is presented in **Tables 1 through 5**. For a more in-depth summary of the articles, see **Online Supplementary File 3**. Data on 14,591 subjects were included in this review, with an age range of 31 hours to 18 years.

DIURNAL RHYTHM

Twenty-nine studies (with the data of 8,971 subjects) described diurnal rhythmicity and/or decline of cortisol throughout the day in children, of which fifteen studies reported no significant sex differences.[11-25] Fourteen studies reported significant sex differences, of which twelve reported higher cortisol levels and/or a steeper decline over the day in girls. Both Adam et al. (2010) [26] (n=230, age: 17.04±0.36 years) and Williams et al. (2013) [27] (n=27, age: 9.13±1.41 years) reported a steeper diurnal cortisol curve in girls. Morin-Major et al. (2016) [28] (n=88, age: 14.5±1.8 years) found a higher area under the curve as measured from the ground (AUCg) in girls. Martikainen et al. (2013) [29] (n=252, age: 8.1±0.3 years) reported a higher cortisol level at awakening in girls, while there was no difference between sexes at nadir, suggesting a steeper cortisol decline over the day in girls compared to boys. This was also found by Rosmalen et al. (2005) [30] (n=1768, age: 11.08±0.55 years), who found this to be already present pre-pubertally, while age and pubertal status were not associated with diurnal rhythm. Fransson et al. (2014) [31] (n=157, age: 14-16 years) found a higher cortisol level at awakening and a steeper diurnal decline in girls. Kelly et al. (2008) [32] (n=2,995, age: 15.4±0.3 years) found a greater decrease in cortisol concentration in girls as compared to boys between +/- 9am and 9:30am. Ruttle et al. (2013) [33] (n=346, age: 11, 13 and 15 years) and Shirtcliff et al. (2012) [34] (n=357, age: 9, 11, 13 and 15 years) examined the same cohort. Ruttle et al. (2013) found a significantly steeper diurnal decline in girls aged 11 and 13 years. At age 15, gender differences in cortisol slope had disappeared, although girls had higher cortisol levels throughout the day. Shirtcliff et al. (2012) found similar differences, with higher cortisol and steeper slopes, as well as more curvature, in girls. Moreover, the circadian rhythm became flatter with advancing puberty, particularly among girls. Vaillancourt et al. 2008) [35] (n=154, age: 147±9.1 months) examined morning and evening cortisol levels on Monday, Thursday and Saturday. They only found a higher cortisol concentration in girls on Saturday morning. Moreover, after modeling the circadian pattern, they found that girls consistently had higher cortisol levels than boys throughout the day. Bae et al. (2015) [36] (n=138, age: 10.7±1.7 years) found higher cortisol levels in girls at awakening and 30 minutes after awakening, as well as a higher total daily output.

TABLE 1. Summary of articles describing sex differences in diurnal rhythmicity

Author (year)	Sample size	Age	Sampling points	Medium	Results
Adam (2010) [26]	230	17.04±0.36 yrs	6x/day on 3 days	Saliva	Lower diurnal cortisol curves in boys
Bae (2015) [36]	138 (70 controls)	10.7±1.7 yrs	3x/day on 3 days	Saliva	Higher levels at awakening, 30 minutes after awakening and higher total daily output in girls; levels in the evening and diurnal slope: no sex differences
Barbosa (2012) [22]	145	8-10yr group: 9.0±0.8 yrs; 11-14yr group: 11.9±1.0 yrs	2x	Saliva	No sex differences, higher diurnal decline in children aged 11-14 yrs old
Bartels (2003) [23]	360	12 yrs	4x/day on 2 days	Saliva	No sex differences; pubertal status not assessed
Carrion (2002) [24]	31	Mean: 10.9 yrs	4x/day on 3 days	Saliva	No sex differences; pubertal status not associated with reactivity
Doom (2013) [25]	110	9.42±0.88 yrs	3x/day on 5 days	Saliva	No sex differences; pubertal status not assessed
Fransson (2014) [31]	157	14-16 yrs	4x (including CAR)	Saliva	Steeper decline in girls
Garcia (1990) [12]	76 (21 controls)	11.2±0.37 yrs	3-hourly during 24 hours	Blood	No sex differences; pubertal status not assessed
Haen (1984) [11]	64	1 mo. to 15 yrs	6 hourly (4x)	Blood	No sex differences; pubertal status not assessed
Jones (2006) [14]	140	7-9 yrs	5x	Saliva	No sex differences; pubertal status not assessed
Kelly (2008) [32]	2995	15.4±0.32 yrs	2x, 30min apart in the morning	Saliva	Steeper decline in girls
Kjolhede (2014) [19]	342	9.5±1.9 yrs	3x/day on 4 days	Saliva	No sex differences; pubertal status not assessed
Knutsson (1997) [13]	235	2.2-18.5 yrs	7x	Blood	No sex differences, except for higher values in girls at pubertal stage 2
Kuhlman (2015) [38]	121	12.8±2.3 yrs	4x/day on 2 days	Saliva	No impact of sex on cortisol at awakening or linear decline, but boys showed less deceleration of the diurnal decline between dinner and bed-time.
Lumeng (2014) [20]	331	3-4 yrs	3x/day on 3 days	Saliva	No sex differences; pubertal status not assessed
Martikainen (2013) [29]	252	8.1±0.3 yrs	7x	Saliva	Higher morning cortisol in girls, no sex difference in nadir
Matchock (2007) [39]	120	Boys: 9, 11 or 13 yrs; girls: 8, 10 or 12 yrs	6x (including CAR)	Saliva	Cortisol peak occurred later in boys than girls during later puberty. Higher morning cortisol in boys at pubertal stage 2. AUCg: no effect of sex, but significant pubertal stage effect
Michels (2012) [18]	385	5-10 yrs	4x (including CAR)	Saliva	No sex differences except somewhat steeper decline in girls (p=0.30)
Morin-Major (2016) [28]	88	14.5±1.8 yrs	4x/day on 2 days	Saliva	Higher AUC in girls
Netherton (2004) [37]	129	12.8±0.19 yrs	2x/day on 4 days	Saliva	Mid-post pubertal girls higher morning cortisol than boys. No sex differences in variance across the four days
Osika (2007) [15]	84	9.9±0.55 yrs	5x (including CAR)	Saliva	No sex differences; pubertal status not assessed
Rosmalen (2005) [30]	1768	11.08±0.55 yrs	3x (including CAR)	Saliva	Higher morning cortisol levels in girls, no sex differences in evening cortisol, already present in pre-pubertal children. Age or pubertal status not associated with cortisol levels.
Ruttle (2013) [33]	346	11, 13 and 15 yrs	3x/day on 3 days	Saliva	Steeper slope in girls at ages 11 and 13, and in longitudinal analyses; higher cortisol levels in girls throughout the day at age 15
Shirtcliff (2012) [34]	357	9, 11, 13 and 15 yrs	3x/day on 3 days	Saliva	Steeper slopes, more curvature in girls. Advancement through puberty: rhythm becomes flatter, especially in girls
Susman (2007) [16]	111	Boys: 9, 11 or 13 yrs; girls: 8, 10 or 12 yrs	6x (including CAR)	Saliva	No sex differences; pubertal status not associated with reactivity
Tzortzi (2009) [17]	21	10-14 yrs	20x (including CAR)	Saliva	No sex differences; pubertal status not assessed
Vaillancourt (2008) [35]	154	147±9.07 mo.	2x/day on 3 days	Saliva	Higher morning levels in girls on Saturday, multilevel regression: consistently higher production in girls
Vanaelst (2013) [21]	355	5-10 yrs	4x/day on 2 days (including CAR)	Saliva	No sex differences; pubertal status not assessed
Williams (2013) [27]	27	9.13±1.41 yrs	3x/day on 2 days (including CAR)	Saliva	Boys exhibited flatter slopes than girls

TABLE 2. Summary of articles describing sex differences in cortisol awakening response (CAR)

Author (year)	Sample size	Age	Sampling points	Medium	Results
Adam (2010) [26]	230	17.04 ± 0.36 yrs	0 and 40 min after awakening	Saliva	No sex differences; pubertal status not assessed
Bae (2015) [36]	138 (70 controls)	10.7±1.7 yrs	0 and 30 min after awakening	Saliva	Higher levels in girls at awakening and 30 min after awakening, no sex differences in awakening response
Bouma (2009) [41]	644	16.13±0.59 yrs	0 and 30 min after awakening	Saliva	Higher basal levels in girls, no difference in awakening responses
Bright (2014) [40]	47	12-24 months	0 and 30 min after awakening	Saliva	No sex differences; pubertal status not assessed
Dietrich (2013) [42]	1604	11.1±0.55 yrs	0 and 30 min after awakening	Saliva	AUCg and absolute cortisol values higher in girls, AUCi no sex differences
Fransson (2014) [31]	157	14-16 yrs	0, 30 and 60 min after awakening	Saliva	Higher CAR in girls
Hatzinger (2007) [43]	102	4.91±0.44 yrs	0, 10, 20 and 30 min after awakening	Saliva	Higher CAR in girls
Jones (2006) [14]	140	7-9 yrs	0 and 30 min after awakening	Saliva	CAR present in boys, not girls
Kuhlman (2015) [38]	121	12.8±2.3 yrs	0 and 45 min after awakening	Saliva	No sex differences; pubertal status not assessed
Martikainen (2013) [29]	252	8.1±0.3 yrs	0, 15 and 30 min after awakening	Saliva	Higher AUCg in girls, same increase and AUCi
Michels (2012) [18]	385	5-10 yrs	0, 30 and 60 min after awakening	Saliva	No sex differences; pubertal status not assessed
Morin-Major (2016) [28]	88	14.5±1.8 yrs	0 and 30 min after awakening	Saliva	Correlated to sex, higher CAR in girls
Osika (2007) [15]	84	9.9±0.55 yrs	0 and 15 min after awakening	Saliva	No sex differences; pubertal status not assessed
Pruessner (1997) [44]	42	11.16±1.99 yrs	On 3 days: 0, 10, 20 and 30 min after awakening	Saliva	Marginal differences: higher in girls
Susman (2007) [16]	111	Boys: 9, 11 or 13 yrs; girls: 8, 10 or 12 yrs	0, 20 and 40 min after awakening	Saliva	No sex differences; pubertal status not associated with reactivity
Tzortzi (2009) [17]	21	10-14 yrs	From waking: every 20 min until 3 hours after awakening	Saliva	No sex differences; pubertal status not assessed
Vanaelst (2013) [21]	355	5-10 yrs	0, 30 and 60 min after awakening	Saliva	No sex differences; pubertal status not assessed
Williams (2013) [27]	27	9.13±1.41 yrs	0 and 30 min after awakening	Saliva	No sex differences; pubertal status not assessed

However, no sex differences were found with regard to diurnal slope or evening levels. Netherton et al. (2004) [37] (n=129, age: 12.8±0.19 years) found higher morning cortisol levels in mid- to post-pubertal girls compared to boys, but no sex differences were found in evening cortisol levels. In pre- to early- pubertal children, no sex differences were found in either morning or evening cortisol levels. Contrastingly, Kuhlman et al. (2015) [38] (n=121, age: 12.8±2.3 years) reported no sex differences in cortisol levels at awakening or in linear decline, although girls showed more deceleration of the diurnal decline between dinner and bedtime than boys. Matchock et al. (2007) [39] (n=120, age: boys: 9, 11 or 13 years; girls: 8, 10 or 12 years) found an earlier cortisol peak in the morning in girls, and at pubertal stage 2 a lower morning cortisol levels in girls. However, although a pubertal stage effect was found, there were no sex differences in the AUCg.

TABLE 3. Summary of articles describing sex differences in protocolled social stress test similar or equal to the TSST-C

Author (year)	Sample size	Age	Sampling points	Medium	Results
Bae (2015) [36]	169 (81 controls)	10.8±1.8 yrs	8x (3 before, 5 after)	Saliva	No sex differences; pubertal status not associated with reactivity
Bouma (2009) [41]	644	16.13±0.59 yrs	5x (2 before, 3 after) (Groningen Social Stress test)	Saliva	Cortisol responses were stronger in boys
Bouma (2011) [46]	553	16.07±0.90 yrs	4x (1 before, 3 after) (Groningen Social Stress Test)	Saliva	Boys had higher cortisol levels on sample 2
De Veld (2012) [56]	158	10.61±0.52 yrs	7x (2 before, 5 after)	Saliva	Cortisol response stronger in girls
Dockray (2009) [48]	111	Boys: 9, 11 or 13 yrs; girls: 8, 10 or 12 yrs	5x, 2 before, 3 after	Saliva	No sex differences; age but not pubertal stage associated with reactivity in girls, no associations in boys.
Evans (2013) [45]	707	13.77±3.56 yrs	After each period/task, at the middle of the documentary and at the end of it (in fig. 2: 6 samples, 2 before, 4 during/after) (Social stress tests based on TSST)	Saliva	In children (7-12): lower cortisol reactivity in boys experiencing less emotional warmth Adolescents (13-20): no sex differences
Gunnar (2009) [58]	82	4 ages groups: 9 (9.79±0.16), 11 (11.57±0.15), 13 (13.55±0.46) 15 (15.55±0.47)	10x, 3 before, 7 after	Saliva	No sex differences, except higher cortisol reactivity in girls at age 13
Hostinar (2014) [52]	191	14.4±1.93 yrs	6x (2 before, 4 after) (TSST for groups)	Saliva	No sex differences; higher intercepts and greater anticipatory responses with increasing age, pubertal status not assessed
Hostinar (2015) [57]	81 (40 children, 41 adolescents)	Children: 9.97±0.52 yrs, adolescents: 16.05±0.39 yrs	4x (1 before, 3 after)	Saliva	Stronger response in 9-10 year old girls, no sex differences among adolescents
Ji (2016) [54]	135	Boys: 9, 11 or 13 yrs; girls: 8, 10 or 12 yrs	5x (2 before, 3 after)	Saliva	At wave 3 (each waves separated by 6 months): girls stronger reaction to stressor, no sex differences in recovery
Jones (2006) [14]	140	7-9 yrs	7x (3 before, 4 after)	Saliva	Anticipatory rise in both, further increment in girls
Kudielka (2004) [47]	31	12.1±0.3 yrs	5x, 1 before, 4 after	Saliva	No sex differences; pubertal status not assessed
Lu (2014) [60]	87	12.7±0.3 yrs	9x, not specified when	Saliva	More negative logAUCi in girls (less increase)
Martikainen (2013) [29]	252	8.1±0.3 yrs	7x (2 before, 5 after)	Saliva	Higher peak, AUCg and AUCi in girls
Martin (2011) [53]	40	16-18 yrs	7x (1 before, 6 after)	Saliva	No sex differences; pubertal status not assessed
Mrug (2016) [59]	84	13.36±0.95 yrs	3x, 1 before, 2 after	Saliva	Higher post-test cortisol and AUCi in girls
Peckins (2012) [50]	124	10.49±1.68 yrs; boys: 9, 11 or 13 yrs; girls: 8, 10 or 12 yrs	5x, 2 before, 3 after	Saliva	No sex differences; pubertal status not associated with reactivity
Portnoy (2015) [51]	446	11.92±0.59 yrs	4x, 1 before, 3 after	Saliva	No sex differences in AUCg; pubertal status not associated with reactivity
Raikkonen (2010) [55]	292	8.1±0.3 yrs	7x (2 before, 5 after)	Saliva	Boys lower than girls
Strahler (2010) [49]	62	6-10 yrs	4x, 1 before, 3 after	Saliva	No sex differences; pubertal status not assessed
Trickett (2014) [61]	303 maltreated, 151 control	Maltreated: 10.84±1.16 yrs; comparison: 11.11±1.15 yrs	6x (2 before, 4 after)	Saliva	Cortisol response blunted in girls compared to boys

TABLE 4. Summary of articles describing sex differences in pharmacological stress tests

Author (year)	Sample size	Age	Study protocol	Sampling points	Sampling medium	Results
Dahl (1992) [68]	25	10.3±1.6 yrs	CRH challenge: 1µg/kg iv in the late afternoon	9x, 3 before, 6 after	Blood	Greater peak in boys
Dorn (1996) [62]	20 control subjects	15.1±1.0 yrs	CRH challenge: 1µg/kg iv in the evening	12x, 6 before, 6 after	Blood	No sex differences; groups matched for pubertal status, effect not analyzed
Forest (1978) [63]	20 infants, 35 prepubertal children	Infants: 5-365 days, children: 1-12.6 yrs	ACTH test: 500µg/m^2 im at 8:00 and 20:00 on 3 days)	2x, 1 before, 1 after	Blood	No sex differences; pubertal status not assessed
Lashansky (1991) [64]	102	2 months – 17 yrs	ACTH test: 0.25mg iv in the morning	2x, 1 before, 1 after	Blood	No sex differences; decrease in stimulated cortisol levels with puberty, more pronounced in boys
Ross (1986) [65]	21	6-15 yrs	CRH challenge: 1µg/kg iv in the evening	7x, 2 before, 5 after	Blood	No sex differences; pubertal status not associated with reactivity
Stroud (2011) [67]	68	11.6±1.9 yrs	CRH challenge: 1µg/kg iv in the late afternoon	9-10x, 3 before, 6-7 after	Blood	Sex by Tanner differences: girls increases and boys decreases in cortisol with pubertal maturation, girls decreases and boys stable in reactivity. Boys larger peak change.
Tsvetkova (1977) [66]	31	4-14 yrs	ACTH test: 0.5mg im in the morning	2x, 1 before, 1 after	Blood	No sex differences; pubertal status not assessed

TABLE 5. Summary of articles describing sex differences in miscellaneous stress tests

	Author (year)	Sample size	Age	Study protocol	Sampling points		Results
0-1 year old	Davis (1995) [69]	36	30.99±8.09 hours	Neonatal Behavior Assessment Scale	5x, 1 before, 4 after test	Saliva	Higher reactivity in boys
	Eiden (2015) [70]	217	9 months	Laboratory Temperament Assessment Battery	4x, 1 before, 3 after test	Saliva	Cortisol increase in boys, not in girls
	Grunau (2010) [71]	32	4.2±1.0 months	Cortisol response after vaccination	3x, 1 before, 2 after	Saliva	No sex differences; pubertal status not assessed
1-7 years old	De Weerth (2013) [75]	42	68.0±4.3 months	CREST paradigm	6x (2 before, 4 after)	Saliva	No sex differences; pubertal status not assessed
	Gunnar (2010) [72]	151	3.81±0.23 years	Daycare attendance	2x/day on 2 days	Saliva	No sex differences; pubertal status not assessed
	Hatzinger (2007) [43]	102	4.91±0.44 years	MSSB	5x (2 before, 3 after)	Saliva	Higher reactivity in girls
	Kryski (2013) [76]	409	40.72±3.51 months	Matching task	6x (1 before, 5 after)	Saliva	No sex differences; pubertal status not assessed
	Mills (2008) [78]	214	4.14±0.24 years	Easy and difficult matching tasks	6x, 1 before, 5 after	Saliva	Further decreases in boys after initial decrease for both sexes
	Plusquellec (2011) [73]	376	18.85±0.74 months	Two unfamiliar situations (clown and robot)	2x, 1 before, 1 after	Saliva	No sex differences; pubertal status not assessed
	Spinrad (2009) [74]	84	54.07±0.97 months	Preschool Laboratory Assessment Battery	3x, 1 before, 2 after	Saliva	No sex differences; pubertal status not assessed
	Yong Ping (2014) [77]	94	29.9±1.1 months	Maternal separation	4x (2 before, 2 after)	Saliva	No sex differences; pubertal status not assessed

TABLE 5. Summary of articles describing sex differences in miscellaneous stress tests (Continued)

		Author (year)	Sample size	Age	Study protocol	Sampling points		Results
≥7 years old	Psychological stress	Daughters (2013) [82]	132	16.1±1.0 years	Behavioral Indicator of Resiliency to Distress	4x, 1 before, 3 after	Saliva	Boys: higher baseline, greater peak. No sex differences in AUCg.
		Hackman (2012) [79]	180	12-14 years	Parent-Adolescent Conflict Discusison	3x (2 before, 1 after)	Saliva	No sex differences; pubertal status not assessed
		Minkley (2012) [83]	93		Examination challenge (reproduction of knowledge, or transfer and problem-solving)	2x, 1 before, 1 after	Saliva	Not statistically significant, but higher increases in boys. More in reproduction of knowledge group, but also greater in transfer and problem-solving group.
		Zijlmans (2013) [80]	52	12.5±1.21 years	Social Evaluative Stress Test	7x, 1 before, 6 after	Saliva	Higher reactivity in boys
	Physical stress	Allen (2009) [84]	235	12.7±2.9 years	Laboratory Pain Tasks	Saliva: 3x, 1 before, 2 after Blood: 2x (after)	Saliva / blood	No sex differences; pubertal status not associated with reactivity
		Chiodo (2011) [90]	16	Boys: 14±0 years, girls: 13±1 years	Taekwondo competition	5x (2 before, 3 after)	Saliva	Lower overall values in girls, but higher peak.
≥7 years old	Physical stress	Covelli (2012) [85]	106	15.3±1.1 years	Cold water hand	2x, 1 before, 1 after	Saliva	No sex differences; pubertal
					immersion			status not assessed
		Frias (2000) [92]	48	13-17 years	Acute alcohol intoxication	1x (after); controls as reference	Blood	More pronounced increase in girls
		Gecgelen (2012) [86]	40	10.9-14.7 years	Rapid maxillary expansion	13x, 1 before, 3 after, and 9 during a period of treatment	Saliva	No sex differences; pubertal status not assessed
		Khilnani (1993) [87]	98	2-20 years	Elective surgery	2x, 1 before, 1 after	Blood	No sex differences; pubertal status not assessed
		Kuhlman (2015) [38]	121	12.8±2.3 years	Socially evaluated cold pressor test	7x (2 before, 5 after)	Saliva	No sex differences; pubertal status not assessed
		Lopez-Duran (2015) [89]	115	12.79±2.26 years	Socially evaluated cold pressor test	8x (2 before, 6 after)	Saliva	No sex differences; pubertal status not assessed
		Stupnicki (1995) [91]	29	Boys: 17.3±0.8, girls 16.4±0.6 years	Exercise	2x, 1 before, 1 after	Blood	Boys decrease in cortisol, girls increase in cortisol after exercise
		Yfanti (2014) [88]	97	89.73±15 months	Dental treatment	5x, 1 before, 4 after	Saliva	No sex differences; pubertal status not assessed

CAR

Eighteen studies (with the data of 3,549 subjects) described the CAR in children. Nine studies did not find differences between boys and girls,[15-18,21,26,27,38,40] although four of these [15-18] studied the CAR as part of the diurnal rhythm, and did not perform separate analyses for the CAR, with therefore limited data available on the CAR. Additionally, Michels et al. (2012) [18] (n=385, age: 5-10 years) and Vanaelst et al. (2014) [21] (n=355, age: 5-10 years) reported on the same cohort, and Osika et al. (2007) [15] (n=84, age: 9.9±0.55 years) only took samples between 0 and 15 minutes after awakening. Nine studies found significant differences in CAR between sexes, of which eight found a higher CAR in girls. Martikainen et al. (2013) [29] (n=252, age: 8.1±0.3 years) found a higher peak after awakening in girls, as well as a higher AUCg. However, the awakening

response (i.e., the peak value after awakening minus the value immediately after awakening) as well as the AUC increase was not significantly different between the sexes. This was also found by Bouma et al. (2009) [41] (n=644, age: 16.1±0.6 years) and Dietrich et al. (2013) [42] (n=1604, age: 11.1±0.6 years), who reported on the same cohort (albeit at different ages) and found higher morning cortisol concentrations in girls, but a similar response to awakening in boys and girls, manifesting as a higher AUCg in girls but a similar AUCi between sexes. Additionally, Bae et al. (2015) [36] (n=138, 10.7±1.7 years) found higher cortisol levels in girls at awakening and 30 minutes after awakening, although they did not find sex differences in the AUCg. Fransson et al. (2014) [31] (n=157, age: 14-16 years) and Hatzinger et al. (2007) [43] (n=102, age: 4.9±0.4 years) both found a higher CAR in girls, and Pruessner et al. (1997) [44] (n=42, age: 11.2±2.0 years) showed a tendency towards larger increases in girls compared to boys. Morin-Major et al. (2016) [28] (n=88, age: 14.5±1.8 years) found a correlation between the CAR and sex, with a higher CAR in girls. Contrastingly, Jones et al. (2006) [14] (n=140, age: 7-9 years) found the CAR to be absent in girls, but present in boys.

PROTOCOLLED SOCIAL STRESS TESTS SIMILAR OR EQUAL TO THE TSST-C

Twenty-one studies (with the data of 3,500 subjects) examined responses to standardized social stress tests. Eighteen used the TSST-C (validated in children aged ≥7 years), while three used other laboratory-based social stress tests that closely resemble the TSST-C [41,45,46]: the Groningen Social Stress Test (GSST) which consists of a 6-minute speech, a brief interlude and a subtracting task, and a psychosocial stress test which consisted of a mental arithmetic task, a public speaking task and a computer mathematics task. Eight studies, of which two studied the same cohort, did not find sex differences,[36,47-53] while 13 did find sex differences. Ji et al. (2016) [54] (n=135, age: boys: 9, 11 or 13 years; girls: 8, 10 or 12 years) reported on the same cohort as Dockray et al. (2009) [48] and Peckins et al. (2012) [50], who did not find sex differences. However, Ji et al. found that at wave 3, where each wave is separated by six months, girls had a stronger cortisol response to the stressor, although they did not find sex differences with regard to cortisol recovery. Raikkonen et al. (2010) [55] (n=292, age: 8.1±0.3 years) and Martikainen et al. (2013) [29] (n=252, age: 8.1±0.3 years) reported on the same cohort, and found a higher peak after stress and higher AUCs (both ground and increase) in girls, while no pre-test differences were found. De Veld (2012) [56] (n=158, age: 10.61±0.52 years) found a stronger cortisol response in girls. Jones et al. (2006) [14] (n=140, age: 7-9 years) found an anticipatory rise in cortisol in both sexes, but only an additional increase after the TSST-C in girls. Evans et al. (2013) [45] (n=707, age: 13.8±3.6 years) found that girls aged ≤12 years displayed higher cortisol reactivity to the psychological stress test, while sex differences were

not present in subjects aged 13-20 years. A similar result was found by Hostinar et al. (2015) [57] (n=81, age: 9.97±0.52 (children) and 16.05±0.39 (adolescents) years), who found a stronger cortisol response in girls at age 9-10, and no sex differences among the adolescents. Gunnar et al. (2009) [58] (n=82, age: 9, 11, 13 and 15 years) found a significantly higher AUCi in girls in response to the TSST-C at age 13, while no sex differences were found at ages 9, 11 and 15 years. Mrug et al. (2016) [59] (n=84, age: 13.4±1.0 years) found a higher cortisol 55 minutes post-test as well as a greater AUCi in girls. On the other hand, Lu et al. (2014) [60] (n=87, age: 12.7±0.3 years) found a significantly more negative logAUCi in girls, indicative of a smaller increase in cortisol in girls compared to boys after the TSST-C, and Trickett et al. (2014) [61] (n=151 controls, age 11.11±1.15 years) found a blunted cortisol response in girls compared to boys. Additionally, Bouma et al. (2009) [41] (n=644, age 16.1±0.6 years), who used the GSST, found lower cortisol responses in girls compared to boys, which was further specified in a study published by Bouma et al. in 2011 [46] (n=553, age: 16.07±0.90 years), who found lower cortisol levels in girls on the first sample after completing the GSST.

PHARMACOLOGICAL STRESS TESTS

Seven studies (with the data of 322 subjects) investigated cortisol responses to pharmacological ACTH or CRH. Five studies (3 with ACTH, 2 with CRH) did not find significant sex differences,[62-66] and two studies found a smaller cortisol increase in girls. Stroud et al. (2011) [67] (n=68, age: 11.9±1.9 years), who performed a CRH challenge with 1μg/kg human CRH, found a smaller increase from baseline in girls compared to boys for all Tanner pubertal stages. Additionally, sex-specific pubertal changes were observed, with a baseline cortisol that increased in girls and decreased in boys with advancing puberty. Moreover, girls showed decreases in reactivity/recovery rates (in μg/dL/min), as well as increases in total cortisol response (AUCg) and time to peak cortisol levels with pubertal maturation. Boys, on the other hand, showed little change in reactivity/recovery rates and no changes across puberty for the other parameters. Dahl et al. (1992) [68] (n=25, age: 10.3±1.6 years) also performed a 1μg/kg human CRH challenge, and found a smaller increase in cortisol concentration in girls compared to boys.

MISCELLANEOUS STRESS TESTS

Twenty-five studies (with the data of 3,004 subjects) performed a wide range of other stress tests.

Three studies were performed in infants aged <1 year (with the data of 285 subjects),[69-71] of which two found a lower cortisol reactivity in girls: Davis and Emory

(1995) [69] (n=36, age: 31.0±8.1 hours), who used the Neonatal Behavior Assessment Scale, and Eiden et al. (2015) [70] (n=217, age: 9 months), who used the Laboratory Temperament Assessment Battery.

Eight studies (with the data of 1,472 subjects) were performed in children aged 1-7 years, of which Six [72-77] found no sex differences. Hatzinger et al. (2007) [43] (n=102, age: 4.9±0.4 years) used the MacArthur Story Stem Battery, and found a higher reactivity in girls. Mills et al. (2008) [78] (n=214, age: 4.1±0.2 years) used easy and difficult matching tasks with standardized failure and success. They found decreases in cortisol concentrations in both sexes up to 15 minutes post-stressor, but only further decreases in boys.

Fourteen studies (with the data of 1,247 subjects) assessed stress in children aged ≥7 years using miscellaneous protocols. Four studies performed psychological stress tests: one found no sex differences [79], while three found lower reactivity in girls. Zijlmans et al. (2013) [80] (n=52, age: 12.5±1.2 years) used a computerized testing paradigm, the social evaluative stress test [81], containing elements of social evaluation, unpredictability and uncontrollability. A lower reactivity was found in girls. Daughters et al. (2013) [82] (n=132, age: 16.1±1.0 years) used the Behavioral Indicator of Resiliency to Distress (BIRD), and found no cortisol increase and slower cortisol decrease in girls, while there were no sex differences in AUCg. Minkley and Kirchner (2012) [83] (n=93, age: 17.9±0.1 years) used two knowledge tests aimed at testing ''reproduction of knowledge'' or ''transfer and problem-solving''. A lower reactivity was found in girls, although this was not statistically significant. Ten other studies assessed cortisol reactivity to physical stressors, of which seven did not find sex differences,[38,84-89] of which two reported on the same cohort.[38,89] Chiodo et al. (2011) [90] (n=16, age: boys: 14±0 years, girls: 13±1 years) used a Taekwondo competitions as stressor, and found lower overall values in girls, although they did exhibit a higher peak compared to boys. Stupnicki et al. (1995) [91] (n=29, age: 16-17 years) used physical exercise, and found a cortisol increase after physical exercise in girls, compared to a decrease in boys. Frias et al. (2000) [92] (n=48, age: 13-17 years) assessed cortisol reactivity after acute alcohol intoxication (AAI). Both boys and girls showed an increase in cortisol concentrations after AAI compared to controls, but this increase was more pronounced in girls, although this was not statistically tested.

DISCUSSION

In this systematic review, we found that sex differences in HPA axis reactivity are suggested to be present in childhood. In general, with regard to diurnal rhythm, the CAR and social stress tests, around 50% of the studies, notably the larger ones, found sex differences, of which approximately 80% found a more variable diurnal rhythm, a higher CAR, and/or a stronger cortisol response to social stress tests in girls, suggestive of a more variable HPA axis. We found no evidence for a sex difference in cortisol response after a pharmacological challenge, with only two out of seven studies reporting a higher cortisol response in boys. Findings from studies addressing sex differences in cortisol response after miscellaneous (social or physical) stress tests were inconsistent, due to different types of stressors applied.

In total, 12 out of 29 studies found a more variable diurnal rhythm in girls, while 2 found this in boys and 15 did not find sex differences. A higher CAR in girls was found in 8 out of 18 studies, although 1 study found a higher CAR in boys and 9 studies found no sex differences. Girls had a stronger cortisol response to social stress tests in 9 out of 21 studies, whereas boys had a stronger response in 4 studies and no sex differences were found in 8 studies. Therefore, although results are suggestive of a more responsive HPA axis in girls during childhood, these results must be interpreted with caution as the evidence is not unequivocal. However, the sample sizes of the studies that found sex differences were on average larger, while the studies that did not find sex differences more often had a sample size <100.

Our results differed considerably with findings from studies in adults. Notably, psychological stress studies in adults either found no gender difference or a more pronounced cortisol response in men.[4] This difference might be explained by gonadal hormones, more specifically estrogens. In childhood, as we have shown in this review, cortisol reactivity appears to be more pronounced in females. However, other research has shown that in adults, females were found to exhibit attenuated cortisol responses to stress, and males displayed a higher cortisol reactivity.[4] Consequently, it could be hypothesized that post-menopausal women once again show a stronger cortisol response to stress compared to men of the same age. Otte et al. (2005) [5], who performed a meta-analysis to evaluate and quantify age-related changes in cortisol response, found a three-fold higher increase in cortisol reactivity with aging in women compared to men. However, studies examining cortisol reactivity in elderly subjects are inconclusive with regard to gender differences.[93-96]

According to the Developmental Origins of Health and Disease (DOHaD) hypothesis, disease susceptibility arises early in development[1] and might be mediated by HPA axis (re)activity. Dysfunctional (hypo- or hyperreactive) HPA axis responses have previously been associated with cardiovascular disease risk.[97] In addition, more subtle differences in early HPA axis settings can also contribute to sex-specific disease risks throughout life.[10,98]

Sex differences in HPA axis reactivity might be due to interactions between the HPA- and HPG axes, and several mechanisms have been proposed. Estradiol has been shown to enhance, while testosterone inhibited CRH gene transcription in the hypothalamus.[9] In addition, estradiol has been found to sensitize the pituitary, thereby increasing the ACTH response, while progesterone seemed to oppose this effect.[9] Moreover, estrogen receptors (ERs) are widely expressed throughout the brain, especially in the limbic system. Although not unequivocal, the distribution of the ER subtypes α and β, which have opposing actions on the HPA axis,[99] is probably sex-dependent.[100] In rats, gender differences in the expression of ERs were already present early in life.[101] It is possible that sex differences in the balance and distribution of ERα and ERβ in the brain are already present before puberty as a result of priming [1] or genetics, which subsequently change after the onset of puberty. In addition, the sensitivity of the adrenal cortex to ACTH is suggested to be increased in young women,[9] while estrogens were found to increase the production of corticosteroid-binding globulin (CBG),[102] decrease glucocorticoid receptor (GR) expression and activitation[7] and lower hepatic clearance of cortisol by inhibition of A-ring reduction .[103] In contrast, testosterone was found to inhibit the release of ACTH, while progesterone possibly acts as a glucocorticoid antagonist.[9,47,104] However, estrogens seem to have different effects in (postmenopausal) women and men,[105-107] and ACTH responses to a TSST after two weeks of DHEA or placebo treatment was found to be equal for women treated with DHEA to those of men, but increased compared to women taking placebos.[4] These HPA/HPG axes interactions might explain why the sex differences in HPA axis reactivity that we found in children are not corroborated by studies in adults. Moreover, some of the included studies in this review took pubertal status into account,[13,24,30,33,34,36,37,39,45,48,50-52,64,65,67,84,95]. Although different (sex-specific) effects of pubertal status on cortisol reactivity were found, HPA/HPG axes interactions might nevertheless play a role in the possible sex-specific changes in HPA axis reactivity throughout puberty.

The different natures and effects of the applied stressors are something to take into account when assessing HPA axis reactivity. Different types of stressors activate

different levels along the HPA axis: standard ACTH tests stimulate the adrenals directly, while psychological tests are indirect stimuli of the adrenal cortex through activation of the limbic system. Moreover, the diurnal rhythm and CAR are largely controlled by the suprachiasmatic nucleus, which influences CRH release from the paraventricular nucleus.[108] Additionally, males seem to have a "fight or flight" reaction, with a stronger response when confronted with an achievement challenge (in which you can succeed or fail at a task), while women show a "tend or befriend" response, and therefore seem to be more sensitive to stress tests that incorporate social rejection or peer pressure.[82,109,110] This might be due to the previously mentioned HPA/HPG axes interactions, as well as possible sexually dimorphic site-specific GR and MR expression patterns in the brain.[2,81] Consequently, when designing a study, it is important to realize what type of stress and which level of the HPA axis is aimed to be tested. Subsequently, the effect of gender on that specific type of stressor should be taken into account. We recommend using standardized protocols, since gender-specific effects on HPA axis reactivity have been best described with regard to standardized stress protocols.

Additionally, comparing the results of the studies included in our systematic review was hampered by the fact that data were collected and presented in numerous ways. For the same reason, it was impossible to perform a meta-analysis. Moreover, only limited information was often provided, and it is therefore possible that (subtle) sex differences were not found. This was the case for all categories of HPA axis reactivity discussed in this review. In order to draw more precise conclusions concerning gender differences in HPA axis reactivity in childhood, we wish to argue using standardized protocols, as well as a standardized presentation of results for future studies on HPA axis reactivity. Seeman and Robbins (1994) [111] have defined stress resiliency as "the overall pattern of HPA response to challenge", which includes the rate of initial response, the magnitude of the response and the rate of recovery of the HPA axis. In order to be able to draw conclusions on all of these aspects, and to enable unbiased, quantitative comparisons, reporting data on HPA axis reactivity should take all of these aspects into account. This can be done by both reporting absolute cortisol values (e.g., minimum and maximum cortisol levels) as well as derived variables (e.g., time to peak/recover, delta cortisol, ascending/descending slopes and areas under the curve), preferably analyzing sex differences for all these parameters. This will allow a full appreciation and overview of the course followed by cortisol pre- and post-stressor.

Our review has several strengths and limitations. Our strengths lie in the systematic and extensive search performed, which has resulted in the inclusion of 81 studies. Our review is limited by the previously mentioned concerns, but also by the broad range in ages as well as the lack of (reliable) establishment of pubertal stage in the majority of the included articles. Although several studies mention an effect of age or pubertal status on cortisol reactivity,[13,22,24,30,33,34,36,37,39,45,48,50-52,54,57,58,64,65,67,84,95] findings are conflicting between the articles. Moreover, we ourselves were unable to draw any conclusions with regard to age or pubertal status, due to the heterogeneous ways of analyzing these effects as well as limited power within studies. Moreover, pubertal status was often assessed through self-report, which has poor reliability.[112] However, it is possible that the effect of age and/or pubertal status can partly explain our unequivocal conclusions regarding sex differences, as was previously suggested by Jessop and Turner-Cobb.[10] Aside from standardizing the collection and presentation of data, we therefore urge to also always take age and pubertal status into account. This is in line with a recent study in adults, which showed that adjusting for sex hormones significantly alters sex-specific cortisol profiles [113].

CONCLUSIONS

In conclusion, we found that gender differences in HPA axis reactivity appear to be present in childhood, suggestive of priming of the HPA axis during early development, although the evidence is not unequivocal. Overall, girls appear to have a more variable diurnal rhythm, a higher CAR, and a higher cortisol response to social stress tests. These differences are not in line with studies in adults, which might be due to changes in gonadal hormones during puberty impacting on HPA axis reactivity. We found various gender differences depending on the type of stressor applied, which stresses the importance of taking the nature of the stressor into account when designing a new study. Moreover, standardization of protocols and reports of results is warranted.

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ONLINE SUPPLEMENTARY FILE

1. Extracted data of studies included in the systematic review https://goo.gl/jwzB89

CHAPTER 12
GENERAL DISCUSSION

Nowadays in the Netherlands, more than 80% of very preterm (<32 wks GA) newborns survive due to large improvements in perinatal care [1]. Very preterm survivors are predisposed to poor growth, unfavorable body composition, cardiovascular diseases, and impaired neurodevelopment. Therefore long-term morbidity after very preterm birth becomes more important. Adaptation of HPA axis settings to very preterm birth is suggested to partly explain these unfavorable disease risks later in life (**Chapter 1**). Therefore, in this thesis we explored factors relevant to the early life morbidity and mortality of very preterm newborns that could influence permanent adaptation to early life stress: gender, a genetic predisposition to altered glucocorticoid sensitivity, antenatal glucocorticoid treatment, maternal perinatal stress and mother's milk glucocorticoids. Accordingly, with the data presented in this thesis, we gained some more insights into the consecutive sequelae of person-specific predisposition, early life conditions, and later life challenges that lead to long term risk for neurodevelopmental impairments and cardiovascular disease. Eventually, by getting a better view on these mechanisms, therapy regimes that are currently adequate for achieving the goal of survival could in the end be translated into appropriate therapy, that is adequate in early life while minimally harming the developmental outcomes in later life.

PERSON-SPECIFIC PREDISPOSITION

HPA axis functioning includes the maintenance of homeostasis by regulation of basal HPA axis activity, and adaptation to stressors by regulation of HPA axis reactivity. With regard to signal transduction, glucocorticoids have a high affinity for both mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs). MRs are mainly involved in basal HPA axis activity, while GRs, in coordination with MRs, are regulating HPA axis reactivity [2]. Inter-individual variation in the sensitivity of these receptors influences HPA axis functioning, and can thereby result in inter-individual variation in stress vulnerability.

In **Chapter 7**, we showed that genetic variations in the *GR* and *MR* resulted in a person-specific sensitivity towards endogenic glucocorticoids, i.e., reaction to the stressful event of very preterm birth, and exogenic glucocorticoids, antenatally administered.
In **Chapter 9 and 10**, we assessed the influence of gender on HPA axis functioning during childhood through a systematic review and meta-analysis of published literature, and showed that gender-specific differences in HPA axis activity and reactivity are already present during childhood. Thereafter, during puberty a gender-specific change in HPA axis activity was seen. This could not be analyzed with regard to HPA axis reactivity, due to the heterogeneity in stress protocols and the lack of data on

puberty. In summary, these data suggest that both gender and genetic predisposition can result in differences in stress vulnerability.

STRESS IN EARLY LIFE

When a child is born very preterm, it is exposed to an excessive amount of stress during a critical window of development, while the body's immaturity makes it difficult to respond adequately to these stressors. Indeed, in very preterm infants a high prevalence of relative adrenal insufficiency is seen in the first weeks of life, due to the immaturity of multiple levels along the HPA axis (**Chapter 1**). In addition, the ongoing process of third trimester neurodevelopment makes the brain vulnerable for stressful events [3]. Animal studies suggest that adaptation to stressful events during this critical developmental window may lead to alterations in HPA axis set point through an altered balance between *MR* and *GR* expression in the brain [2]. Replication of such experiments in humans is impossible, but such data provide working hypotheses on the process of stress adaptation in very preterm newborns. Accordingly, we assessed several indicators of early life stress, including hair glucocorticoid levels, breast-milk glucocorticoids, and early life growth.

In **chapter 2 and 3**, we explored the reliability of a novel strategy for assessment of intra-uterine glucocorticoid regulation. Whereas in adult hair, cortisol and cortisone levels are shown to offer a valid retrospective view on glucocorticoid exposure [4,5], we explored factors influencing neonatal hair glucocorticoids in utero and postpartum. In **chapter 2**, we found a positive association between gestational age and neonatal hair glucocorticoid levels, which seemed to represent the positive feedback loop between placental CRH, and maternal and fetal cortisol. In **chapter 3**, we found a negative association between maternal perinatal stress and neonatal hair glucocorticoid levels, which seemed to represent a downregulation of fetal HPA axis activity after overexposure to maternal glucocorticoids. Accordingly, these data suggest that newborn hair cortisol and cortisone levels could be an indicator of intra-uterine glucocorticoid regulation.

When own mother's milk is not available for very preterm newborns, pasteurized human donor milk is advised as the feeding of choice. Glucocorticoids in human milk might have the potential to provide beneficial effects on development of the preterm neonate. Therefore, we assessed variability in breast-milk glucocorticoid concentrations within and between mothers. In **chapter 5**, we found that breast-milk cortisol and cortisone concentrations were lower in mothers who deliver

very preterm. However, it was also seen that breast-milk cortisol and cortisone concentrations varied widely between and within mothers, which suggested that time of collection was an important influencing factor. Subsequently, we tested the existence of diurnal rhythmicity and found that breast-milk glucocorticoids follow the diurnal rhythm of maternal HPA axis activity. As a result, whether concentrations in preterm milk differ from that in term milk necessitates confirmation in a future study with a timed study design.
In **chapter 6**, we tested whether pasteurization of human milk is harmful to breast-milk glucocorticoids and determined that processing of human milk by donor milk banks does not affect milk cortisol and cortisone levels.

In **chapter 8**, the influence of fetal growth restriction and impaired early life growth on HPA axis development was analyzed, as a proxy for stressful intra-uterine and early life conditions. Cross-sectionally, at corrected term age, the infants that were born SGA and did not show catch up growth seemed to have lower cortisol levels as compared to their AGA counterparts that had grown conform expectations. These findings might reflect a suppression of neonatal HPA axis activity due to experienced stress.

LONG-TERM OUTCOMES

Stress reactions are important defense mechanisms, offering an advantage in terms of morbidity and mortality. However, adaptive responses to early life challenges may become non-adaptive if they overshoot or are a mismatch to later life challenges [6]. In 1986, Professor Barker published unique, epidemiological data that suggested a relation between early life stress, infant mortality and later life cardiovascular disease [7]. Nowadays, robust data support this Developmental Origins of Health and Disease (DOHaD) paradigm. Subsequently, the 'fetal cortisol hypothesis' was postulated as an explanation for increased fetal HPA axis activity and risk of later life cardiovascular disease. Briefly, this hypothesis suggested that an impaired activity of the plancental barrier enzyme 11βHSD type 2 results in intra-uterine overexposure to maternal glucocorticoids, and thereby increased fetal HPA axis activity. We have suggested that such programming mechanisms might also occur postnatally, at least in preterm infants (**Chapter 1**).

Accordingly in **chapter 7**, we found that genetic differences in glucocorticoid sensitivity can predispose very preterm survivors to long-term effects on cognitive and behavioral functioning after exposure to a single gift of synthetic glucocorticoids during a critical window of neurodevelopment.

In **chapter 8**, we showed that poor intrauterine and postnatal growth was associated with early life alterations in HPA axis activity in children born preterm, while at age 8 years HPA axis settings seemed to be recovered, as these did not differ from their well grown equivalent anymore. Since adaptation of the HPA axis is thought to underlie these changes, this could contribute to increased health risks later in life. Moreover with the data presented in **chapter 9**, it can be made plausible that development of the HPA axis takes place in a gender-specific manner, with a gender-specific evolution induced by puberty, thereby contributing to a gender-specific DOHaD.

STRENGTHS AND LIMITATIONS OF THIS THESIS

One of the strengths of this thesis is the development of new methodologies, specifically the LC-MS/MS assay to measure glucocorticoids in human milk. Although many studies have focused on the beneficial effects of nutritional contents of early life feeding, nutritive and non-nutritive, bioactive components of milk cannot be seen separately [8]. Accordingly, our data gave some more insights into the possible role of milk glucocorticoids, by showing the existence of a diurnal rhythm which correlated highly with maternal HPA axis activity. We believe that more attention should be paid to the non-nutritive signaling components of milk, in addition to the nutritional factors, and facilitated that by developing this new assay.

All studies that were presented in this thesis were observational, and therefore could be hampered by selection, performance and detection biases. Animal studies have the advantage that stress can be randomly assigned, environmental circumstances strictly controlled and *in vivo* effects determined at any time. Still, human studies are necessary to translate the hypotheses that were gained from those experiments to the very preterm newborn. When interpreting our observational data, it is important to take into consideration that we had to deal with uncontrollable factors like the amount of stress exposure (**chapter 3, 7 and 8**), timing of outpatient visit appointments (**chapter 2 and 3**), necessity to adhere to feeding times (**chapter 5**), selection by survival (**chapter 7**), attrition at follow-up (**chapter 7 and 8**), and the lack of standardization of study protocols and presentation of data (**chapter 9 and 10**). Among these biases, selection bias is the most difficult one. While the causes of preterm birth are complex, there might be an overlap between factors that cause individual variations in stress responses, and factors that lie in the causal pathway of being born preterm or surviving preterm birth. Many researchers therefore advocate to include a full term control group when studying (very) preterm newborns. Some

also discuss the possibility of selecting only 'healthy' very preterm newborns, e.g., excluding infants born small for gestational age (SGA) or who have developed bronchopulmonary dysplasia. However, this results in a selection of infants with lower risks of adverse outcomes, making it difficult to study effects of (standard) care in an unbiased way. Accordingly, in our studies we aimed to be as explorative as possible, and therefore applied no strict selection criteria within very preterm cohorts.

The impact of performance and detection biases in observational studies has previously been questioned by Vandenbroucke [9]. He advocated that unintended effects, such as those resulting from standard care, can be studied reliably within an observational study design, on the premise that study outcomes were not predictable at the time of allocation, and therefore were unrelated to intended effects. We therefore believe that the effects that were studied in **chapter 7, 9 and 10**, were studied in an unbiased way, i.e., long-term effects of antenatal glucocorticoid treatment in infants with a genetic predisposition to altered glucocorticoid sensitivity, and sex differences in HPA axis (re)activity.

Another challenge of observational studies is the sample size, which is 'offered by nature'. Therefore, it could have been too small or be substantially decreased due to attrition at follow-up, making it difficult to detect effects in clinically relevant subgroups, such as male infants (**Chapter 2, 3, 5, 7 and 8**) or very preterm newborns that were born SGA (**Chapter 7 and 8**). Nonetheless, post-hoc analyses revealed valuable working hypotheses that can be used in future studies, designed to study such groups of subjects. In addition, The International Committee of Medical Journal Editors (ICMJE) recently advocated for responsible data sharing, while they believe it is ethically right to use data as thorough as possible [10]. The advantages of combining data from several cohorts are: enlargement of power, and a reduction of biases due to the heterogeneity of subjects and allocated care. In this context, it should again be emphasized that data are collected and presented in standardized ways (see for a more extensive argumentation **chapter 10**). Moreover, the selection of subjects and classification of cohorts also needs more consistency. In previous trials both infants who were born very preterm and/or with a very low birth weight have been included (**chapter 7 and 8**). However, our research group has recently quantified the impact of such classification criteria on later-life outcomes [11].

To conclude, while the observational study designs that were used cannot rule out any form of bias, with our approach we tried to minimize it. When having the aim to explore unclarified mechanisms by use of such data it is important to present and

analyze the data as transparent as possible, enabling readers to interpret the results by themselves.

FUTURE PERSPECTIVES

The results reported in this thesis are too preliminary to be translated to tailor made approaches, titrated to the needs in early life while minimally harming later life outcomes. With that as our ultimate goal and the hypotheses that were generated with this thesis in mind, we recommend the following items to focus on in future research:

* To develop valid, reliable and easy to apply measurements of HPA axis functioning for very preterm newborns, to use as indication of adrenal functioning, prior to clinical symptoms of insufficiency.
  Nowadays, assessment of HPA axis activity in very preterm newborns is not common practice. Among other challenges, adrenal immaturity results in different steroid patterns that necessitates specialized assays [12,13]. In addition, isolated cortisol levels might not be a valid representative of glucocorticoid bioactivity in the very preterm newborn, while other substances also have the potential to activate the glucocorticoid receptor [14].

* To determine sex-specific differences in HPA axis functioning of very preterm newborns, with the aim to clarify part of the male disadvantage on short- and long-term morbidity and mortality [15-17].
  While our data indicated a gender-specific difference in HPA axis functioning of healthy children, we recommend to study gender-specific differences in glucocorticoid production and metabolism in very preterm newborns under both basal and stressful conditions.

* To assess the effect of early life interventions that support HPA axis functioning.
  Based on current research, hypotheses can be formulated on how HPA axis functioning can be supported over time, as secondary prevention of adverse later life outcomes. Plausible supportive effects can come from co-administration of a physiologic dose of hydrocortisone which is thought to refill depleted mineralocorticoid receptors. [18,19]. In addition, animal studies have suggested that enhanced maternal care after perinatal exposure to synthetic glucocorticoids, could result in a reduced risk of glucocorticoid-induced impairments in later life

[20,21]. Similarly in humans, kangaroo mother care is found to be associated with reductions in morbidity and mortality in low birthweight infants [22]. Furthermore, biochemical signals in mother's milk, which are suggested to offer the newborn information on environmental conditions, might support adaptation processes [8].

GENERAL CONCLUSIONS

By exploring different ways of assessing HPA axis functioning, we have identified several factors that have a significant impact on HPA axis development in very preterm newborns, including gender and early life growth. Moreover, we have shown that variations in HPA axis functioning, i.e., genetic predisposition to altered glucocorticoid sensitivity, can act as a mediator of adaptation. This knowledge needs to be engaged in current research in order to achieve improvement of short- and long-term outcomes.

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CHAPTER 12
SUMMARY

The aim of this thesis was to explore the influences of a genetic predisposition to altered glucocorticoid sensitivity, antenatal glucocorticoid treatment, maternal perinatal stress and mother's milk glucocorticoids on HPA axis functioning in very preterm newborns as well as their effect on adverse long-term outcomes.

In **Chapter 2 and 3,** we explored factors that influence neonatal hair glucocorticoid levels and their relation with maternal HPA axis activity, and showed that neonatal hair cortisol and cortisone levels could offer insights into intra-uterine glucocorticoid regulation.

In **Chapter 4, 5 and 6,** we described the development of a reliable LC–MS/MS assay to determine cortisol and cortisone in human breast milk, and explored variations in human milk glucocorticoid concentrations. Subsequently, by showing the existence of a diurnal rhythm in milk glucocorticoid levels that correlated highly with maternal HPA axis activity, we identified a plausible pathway through which biochemical signals from the mother could reach the neonate.

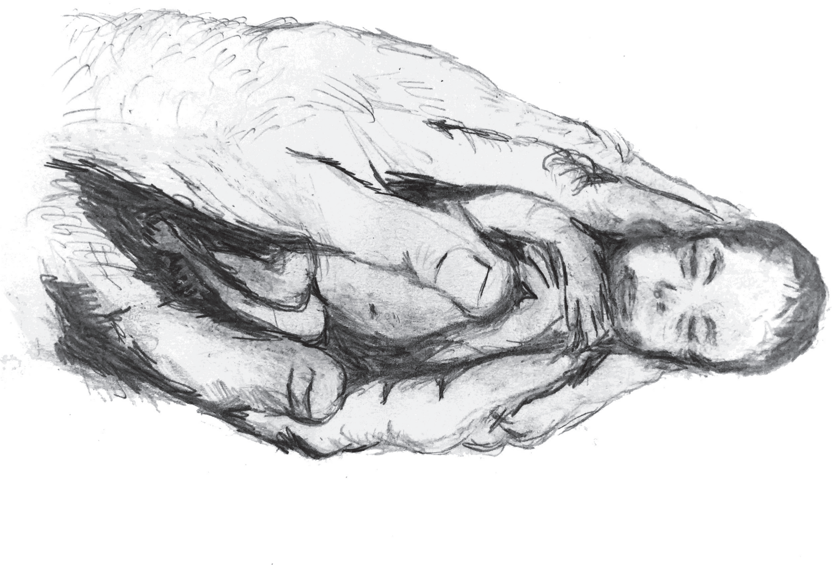
In **Chapter 7,** we found that genetic variation in glucocorticoid sensitivity could affect an individual's predisposition of long-term sequelae of early life stress. In young adults who were born very preterm, independent of antenatal glucocorticoid treatment, carriage of the R23K SNP (associated with decreased glucocorticoid receptor [GR] sensitivity) was associated with a higher IQ score and a more favorable parent-reported total problem behavior score; the -2G/C CC genotype (associated with increased mineralocorticoid receptor [MR] sensitivity) with a poorer IQ score; and the I180V SNP (associated with decreased MR sensitivity) with a more favorable internalizing behavior score. Interaction with antenatal glucocorticoid treatment resulted in more favorable total problem behavior scores for exposed R23K carriers, and poorer IQ scores for both exposed N363S (associated with increased GR sensitivity) and I180V carriers.

In **Chapter 8**, we explored the association between impaired early life growth and HPA axis development. Our data suggested that intra-uterine and early life conditions associated with poor growth resulted in a long-lasting suppression of neonatal HPA axis activity. Although, these effects had disappeared by age 8 years, they might still be a signal for adaptation of developmental pathways.

In **Chapter 9 and 10,** we showed that gender-specific differences in HPA axis activity and reactivity were already present during childhood, and changed during puberty. These findings highlight the importance of studying sexual dimorphism in the field of the DOHaD hypothesis.

To conclude, in this thesis we explored the effects of genetic predisposition, gender, early life conditions, and later life challenges that may lead to long term disease risk in

very preterm survivors. Moreover, more insights will be gained with future research, due to the efforts that we made on developing and validating new techniques to assess different indices of HPA axis functioning.



CHAPTER 13
NEDERLANDSE SAMENVATTING
- VOOR DE LEEK -

Het doel van mijn promotieonderzoek was het exploreren van de invloed van onderstaande factoren op de regulatie van stresshormonen bij veel te vroeg geboren kinderen op de korte en lange termijn: een genetische aanleg voor een verhoogde of verlaagde gevoeligheid voor de stresshormonen cortisol en (de inactieve tegenhanger) cortison, behandeling met stresshormonen van de moeder bij de verdenking op een zeer premature bevalling (na een zwangerschapsduur korter dan 32 weken), stress van de moeder tijdens de zwangerschap en in het kraambed, stresshormonen in moedermelk en geslachtsverschillen in het metabolisme van cortisol in de kindertijd. In het prille begin van het leven hebben zeer prematuur geboren kinderen frequent problemen met het genereren van een adequate (stress)reactie op gevaarlijke gebeurtenissen, zoals bloeddrukveranderingen en een beperkte voedingsinname. Dit is deels het gevolg van een nog onvolgroeide bijnier. Waar op vroege leeftijd vaak een tekort aan cortisol bij deze kinderen wordt gezien, worden op latere leeftijd juist tekenen van een overmatige cortisolproductie gezien, zoals een vetverdeling met vooral buikvet, een verhoogde bloeddruk en (tekenen van) suikerziekte. Het lijkt erop dat de zeer prematuur geborene om te overleven een overmatige stressreactie probeert te genereren. Deze adequate adaptatie in het begin van het leven lijkt, als de gevaren geweken zijn, te blijven bestaan tot later in het leven (zie **hoofdstuk 1**). Dit proces kan op de lange termijn de groei, lichaamssamenstelling, stofwisseling, neurologische ontwikkeling en uiteindelijk ook het risico op ouderdomsziekten beïnvloeden. Omdat de precieze mechanismen hierachter nog onduidelijk zijn en we daardoor deze kwetsbare pasgeborenen lastig kunnen ondersteunen in dit proces, zijn verschillende facetten van dit proces het onderwerp geweest van dit proefschrift.

Stresshormoonconcentraties zijn lastig te meten bij pasgeborenen. Dit heeft verschillende oorzaken, waaronder: het beperkte bloedvolume van een premature baby, het ontbreken van een gereguleerd dagritme in de productie van cortisol en de immaturiteit van de bijnieren resulterend in de productie van bijzondere componenten die speciale meetmethodes nodig maken. Onderzoek bij volwassenen laat zien dat concentraties van cortisol en cortison in haren inzicht geven in de blootstelling aan stresshormonen over een langere periode terug in de tijd: 1 cm. haar correspondeert met 1 maand terug in de tijd. In **hoofdstuk 2 en 3** hebben we daarom onderzocht of stresshormoonconcentraties ook in het haar van een pasgeborene te meten zijn en in hoeverre die beïnvloed zijn door de blootstelling aan stresshormonen van de moeder tijdens de zwangerschap. In **hoofdstuk 2** beschrijven wij dat hogere cortisolconcentraties in het haar van baby's wordt gevonden als de zwangerschapsduur langer is geweest. Dit past bij het gegeven dat gedurende een normale, voldragen zwangerschap cortisolconcentraties stijgen. In **hoofdstuk 3** vonden we een overeenkomst tussen ervaren stress van de moeder gedurende de

zwangerschap en in het kraambed en cortisolconcentraties in het haar van de baby. Onze bevindingen in deze hoofdstukken suggereren dat stresshormoonconcentraties gemeten in babyhaar kort na de geboorte, de blootstelling gedurende de tijd in de baarmoeder representeren en daarmee retrospectief inzicht kunnen geven in stresshormoon regulatie tijdens de zwangerschap.

Wanneer moedermelk van de eigen moeder (nog) niet beschikbaar is voor het zeer prematuur geboren kind, is gepasteuriseerde donormelk de voeding van eerste keus,. Stresshormonen die aanwezig zijn in moedermelk hebben de potentie om de kwetsbare prematuur te ondersteunen in zijn/haar ontwikkeling. Om hier meer inzicht in te krijgen onderzochten wij variabiliteit in stresshormoonconcentraties op verschillende momenten in melk van verschillende moeders. Allereerst hebben we een valide methode ontwikkeld om stresshormoonconcentraties in moedermelk te kunnen meten (**hoofdstuk 4).** In **hoofdstuk 5** beschrijven we hoe we in moedermelk het bestaan hebben ontdekt van een dagritme in stresshormoonconcentraties, die het ritme volgen van concentraties in het bloed van de moeder. Tevens beschrijven we dat stresshormoonconcentraties in de melk van moeders die zeer prematuur bevallen zijn, lager lijken dan die in de melk van moeders die na een voldragen zwangerschap bevallen zijn. Echter deze melk was op willekeurige tijdstippen afgenomen, wat maakt dat we deze bevinding opnieuw moeten testen in getimed afgenomen melk, alvorens hier iets over te kunnen zeggen. Vervolgens hebben we in **hoofdstuk 6** getest of pasteurisatie van donormoedermelk stresshormoonconcentraties beïnvloedt. Dit blijkt niet het geval. Kortom, in deze hoofdstukken beschrijven wij hoe een moeder via het geven van haar melk haar kind mogelijkerwijs zou kunnen helpen zich aan te passen aan de (gevaarlijke) omstandigheden na de geboorte.

Cortisol wordt niet alleen geproduceerd ten tijde van stress. Er wordt continu een (basale) aanvoer van cortisol vanuit de bijnier het bloed in gepompt wat ervoor zorgt dat de activiteit van verschillende processen in ons lichaam in balans is, zoals bloeddruk- en temperatuurregulatie, energietoevoer en ons immuunsysteem. Deze basale aanvoer van cortisol is gereguleerd middels een dag-/nachtritme, waarbij een piek bestaat in de ochtend en een daling in de avond en nacht. Daarnaast kan de bijnier door de hersenen worden aangestuurd om extra cortisol te produceren wanneer zich een stressvolle situatie aandoet. De hersenen en de bijnier communiceren middels signaalstoffen die binden aan receptoren om vervolgens een cel te activeren. De gevoeligheid van deze receptoren is vastgelegd in de genen. Individuele verschillen in de gevoeligheid van deze receptoren kunnen resulteren in individuele verschillen in stressgevoeligheid.

In **hoofdstuk 7** hebben we bij 344 jong volwassenen die zeer prematuur geboren zijn, onderzocht of neurologische uitkomsten op latere leeftijd verband houden met de genetische aanleg voor stressgevoeligheid. Wij vonden dat een genetische aanleg voor een verhoogde stressgevoeligheid geassocieerd was met lagere IQ scores en meer angstig, somber en teruggetrokken gedrag bij deze jongeren. Daarentegen was een genetische aanleg voor een verlaagde stressgevoeligheid geassocieerd met hogere IQ scores en minder probleemgedrag. Voorts hebben we gekeken of een stresshormoonbehandeling van hun moeder dit effect beïnvloedde. Deze stresshormoonbehandeling betreft een boost cortisol die wordt gegeven aan vrouwen die ervan worden verdacht zeer prematuur te bevallen, opdat de organen van de foetus versneld voor worden bereid op een leven buiten de baarmoeder. Waar de overlevingskansen van prematuren worden vergroot wanneer hun moeder deze behandeling heeft ondergaan, leek dit de lange termijn effecten te versterken.
In **hoofdstuk 8** hebben we bij 152 achtjarigen die zijn gevolgd vanaf hun zeer premature geboorte, aanwijzingen laten zien dat slechte groei in de baarmoeder en vroeg in het leven mogelijk resulteren in een onderdrukking van stresshormoonproductie gedurende het eerste levensjaar. Alhoewel deze effecten niet meer duidelijk naar voren kwamen op achtjarige leeftijd, kunnen wij niet uitsluiten dat de aanpassingen op de stressvolle start van het leven hun sporen nalaten in de gezondheid later in hun leven.
Men denkt dat geslachtspecifieke risico's op ziekte later in het leven onder andere veroorzaakt worden door geslachtsverschillen in stressgevoeligheid en stressreacties. Er wordt over het algemeen gedacht dat deze geslachtsverschillen pas na de puberteit aanwezig zijn. Er wordt bij een premature geboorte echter al gesproken over het zwakkere mannelijke geslacht, terwijl verschillen in geslachtshormonen dan nog nauwelijks een rol spelen. Wij hebben daarom onderzocht, op basis van een groot aantal studies bij gezonde kinderen, of geslachtsverschillen in stresshormoonactiviteit (**hoofdstuk 9**) en stressreactiviteit (**hoofdstuk 10**) ook al voor de leeftijd van 18 jaar lijken te bestaan. Gebaseerd op tientallen studies van verschillende grootte, uit verschillende landen, met kinderen van verschillende leeftijden, hebben wij aanwijzingen gevonden die suggereren dat geslachtsverschillen in stress(re) activiteit al voor de puberteit aanwezig zijn en tijdens de puberteit veranderen. Deze bevindingen benadrukken dat wanneer uitkomsten van ernstige prematuriteit worden onderzocht er altijd een onderscheid moet worden gemaakt tussen mannen en vrouwen.

CONCLUSIE

In dit proefschrift hebben we de effecten van stressgevoeligheid, geslacht en stressvolle omstandigheden in het begin van het leven onderzocht en hun mogelijke invloed op de ontwikkeling op de lange termijn van kinderen die een zeer premature geboorte overleefden. Bovendien hebben we technieken ontwikkeld en gevalideerd om stresshormoon-concentraties te meten in moedermelk en babyhaar, die toekomstige studies kunnen ondersteunen bij hun doel om de blootstelling aan stress te kwantificeren bij zeer prematuur geborenen.

ABBREVIATIONS
ACKNOWLEDGEMENTS
CURRICULUM VITAE

ABBREVIATIONS

11β-HSD	11β-HydroxySteroid Dehydrogenase
ACTH	Adrenocorticotropin Hormone
BMI	Body Mass Index
BPD	Bronchopulmonary Dysplasia
CAR	Cortisol Awakening Response
CBG	Corticosteroid-Binding Globulin
CRH	Corticotropin-Releasing Hormone
CUG	Catch-Up Growth
CYP	Cytochromes P450
DHEA(S)	Dehydroepiandrosterone (Sulphate)
DM2	Diabetes Mellitus type 2
ELBW	Extremely Low Birth Weight
GA	Gestational Age
GC	Glucocorticoid
GR	Glucocorticoid Receptor
HPA axis	Hypothalamus-Pituitary-Adrenal axis
HELLP	Hemolysis, Elevated Liver enzymes, and Low Platelet
IUGR	Intrauterine Growth Restriction
LC–MS/MS	Liquid Chromatography–tandem Mass Spectrometry
MR	Mineralocorticoid Receptor
SNP	Single Nucleotide Polymorphism
NICU	Neonatal Intensive Care Unit
VLBW	Very Low Birth Weight
VUMC	VU University Medical Center

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DANKWOORD

- IN GELEENDE WOORDEN -

Joost & Martijn	De beste leraren zijn degene die je laten zien waar te kijken, maar niet vertellen wat te zien - Alexander Trenfor
Hans v. Goudoever	Once you have tasted the sky, you will forever look up - Leonardo Da Vinci
Harrie Lafeber	Onderwijs is niet het vullen van een vat, maar het ontsteken van een vuur - Wiliam Buttler Yeats
Annemieke Heijboer	Faith is taking the first step even when you don't see the whole staircase - Martin Luther King
Endolab	Hakuna Matata
Co-auteurs	Either write something worth reading or do something worth writing - Benjamin Franklin
Jacqueline Cloos	Als we wisten wat we deden, heette het geen onderzoek - Albert Einstein
Jos Twisk	Common sense is just a matter of statistics - George Orwell
Bronovo – KIN	Every adventure requires a first step - Alice in Wonderland
Onderwijscie	Het onderwijs is te belangrijk om alleen aan leraren over te laten - Henk Vredeling
De JOK	"Maak je geen zorgen. Je moet alleen maar glimlachen en de mensen antwoord geven en daarna kunnen we weer gaan spelen", zei prinses Amalia tegen prinses Ariane (2009).
Secretariaat KIN	Humor en geduld zijn de kamelen waarmee je door alle woestijnen kunt gaan – Phil Bosmans
Kamer 027	Behind every successful person is a substantial amount of good coffee - Leon Truong
PK4X	May your choices reflect your hopes, not your fears - Nelson Mandela
Stagiaires	Kostbaar is de wijsheid die door ervaring wordt verkregen - Robert Ascham
Katja	No one is you and that is your power - Joyce
Jonneke	Niet alle "i-tjes" zullen een punt hebben - Jonneke
Charlotte	I have never met a strong person with an easy past - Atticus
Sophie VvZ.	A smile is the prettiest thing you can wear
Fatma	Een goede daad verrichten is makkelijk; de gewoonte ontwikkelen om dat altijd te doen niet - Aristoteles

Kim	Leven en laten leven – mama Kim
Jolice	Stop at never – Ryan Hall, Asics
Lisan	Surround yourself with people who believe in your dreams - Roy Bennett
Lon	Leef met de moed als gids en het geluk als gezellin – Cicero
Sophie K.	Je doet wat je bent. Je bent niet wat je doet – Eddy van Wessel
Chantal	You can find inspiration in everything. If you can't, look again! - Paul Smith
Nilse	Travel the world. Fill your suitcase with treasures. They all have a story that becomes a memory for life
DoMaBe	Dans alsof er niemand kijkt - Rumi
BV de OMAFIETS	Ik ben blij dat ik leef, en geen vliegje in mijn oog heb - Toon Hermans
Glow yoga	You have the right to fail and it may even work out.
Dokters vd Wereld	Educatie is het krachtigste wapen dat we kunnen gebruiken om de wereld te veranderen - Nelson Mandela
Vivian & Harald	If you can't hide it, paint it red - Omdenken
Agnes Abesiga	What can you do to promote world peace? Go home and love your family - Mother Theresa
Esmée & Ramon	Everybody is a genius. But if you judge a fish by its ability to climb a tree, it will live its whole life believing that it is stupid - Albert Einstein
Willem, Annemiek & Mark	Je gevoel weet altijd wat het beste voor je is
Usha	De beste vriend zal waarschijnlijk de beste echtgenoot krijgen, omdat een goed huwelijk op een talent voor vriendschap berust – Friedrich Nietzsche
Rens & Jolien	Don't grow up. It's a trap - Jonah Lake
(Over)oma Merks	Als ik niet eigenwijs ben, dan leef ik niet meer - oma
Opa vd Voorn	Impossible is just an opinion - Paulo Coelho
Oma vd Voorn	Give. Even when you only have a little - Buddha
Pap & Mam	Talent bepaalt wat je kan bereiken. Inzet of je het bereikt - Lou Holtz
Tom	From the day that I met you I stopped feeling afraid. In your arms I feel safe - Chef's Special
Hannah	"What day is it?" "It's today", squeaked Piglet. "My favorite day", said Pooh

CURRICULUM VITAE

PERSONALIA

Achternaam:	Metselaar - van der Voorn
Voornamen:	Bibian
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Burgerlijke staat:	Getrouwd met Tom moeder van Hannah

OPLEIDING

Nov - Dec 2017	**APLS en NLS, SSHK**
Juli 2014 – Juli 2017	**Promotie-onderzoek** Afdeling kinderendocrinologie, VUMC
Jan 2015 - Sept 2015	**Basis Kwalificatie Onderwijs (BKO)**: Certificering didactische bekwaamheid wetenschappelijk onderwijs
2005-2012	**Master's Degree Geneeskunde** VU, Amsterdam

WERK – ONDERWIJS

Mei 2015 – Jan 2017	**Docent Cursus Regressietechnieken** EPIDM, VUMC, Amsterdam, in samenwerking met Prof. Dr. Jos Twisk
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2014 –2016	**Wetenschappelijke stage begeleider** van 3 Honours Program studenten en 2 master studenten, VU, Amsterdam

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Juni 2012 – Mei 2013	**ANIOS Kindergeneeskunde** Bronovo, Den Haag
Juli 2007 – Juli 2009	**Doktersassistente Huisartsenpraktijk Insulindeweg** Amsterdam-Oost

PUBLICATIES

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OVERIGE ACTIVITEITEN

April 2016 – heden	**Vrijwilliger Dokters van de Wereld** Amsterdam. Project Operatie Glimlach
Mei 2015 – Mei 2017	**Voorzitter 'Jonge Onderzoekers Kindergeneeskunde'** (JOK) VUMC – AMC
2011 en 2013	**Tropencoschap (10 wkn, 2011) en werk bezoek (6wkn, 2013) Kagondo, Tanzania** Kinderafdeling en 'Out Patient Department' (OPD)
2011	**Vrijwilliger Sterkamp** Sterkampen staan voor ontwikkeling en zelfvertrouwen door vriendschap en plezier onder pedagogische en psychologische begeleiding voor kinderen van 8 tot 13 jr
2004 – 2010	**Softbal speelster, nationaal topniveau** Positie: pitcher
2005	**Big League World series softball, Michigan, VS** Deelname aan dit WK als pitcher

"Mijn leer is niet bedoeld om aan te nemen of in te geloven, maar als richtlijn om zelf de werkelijkheid te onderzoeken en je de ware aard ervan te realiseren. Zoals een goudsmit zijn goud zou testen door het te verhitten, te wrijven en te krassen, op dezelfde wijze moeten jullie mijn woorden onderzoeken en niet alleen aanvaarden uit eerbied voor mij."

- Buddha