PRENATAL STRESS AND CHILD DEVELOPMENT:

A FOCUS ON PHYSICAL OUTCOMES AND STRESS REACTIVITY

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Chapter 1

Introduction

General introduction

Growing empirical evidence in both animals and humans supports the notion that the environment in utero can influence fetal development, with often long term consequences for the offspring's development of health and psychological functioning. This is also known as 'fetal programming' (Lucas, 1991; Seckl and Meaney, 2004; van den Bergh et al., 2005). Animal studies show, for example, that offspring of mothers who were stressed during pregnancy display more cognitive impairments, increased levels of anxiety and depressive behavior, and alterations in immune and metabolic functioning (Glover et al., 2010; Pryce et al., 2011; Weinstock, 2011). Also in humans there is ample evidence that high levels of maternal prenatal stress (PNS) are related to a broad range of child outcomes, including health (e.g. lower birth weight, immune and metabolic dysfunction), behavior (e.g. emotional problems and ADHD symptoms), and cognitive functioning (e.g. delayed mental development) (e.g. Entringer, 2013; Sandman et al., 2011; Tarabulsy et al., 2014; van den Bergh and Marcoen, 2004).

Although relations between PNS and child outcomes have been studied extensively, much is still unknown. This thesis aims to further increase our understanding about this subject by pursuing three research aims that have received less attention previously. First, PNS is usually measured with maternal self-reports and via physiological stress indicators, which are mainly cortisol concentrations. However, empirical studies on the relations between maternal prenatal cortisol concentrations and child outcomes are methodologically diverse, and their results are mixed. The **first aim of this thesis** is therefore to systematically review the literature in order to clarify what has been found in earlier studies regarding relations between maternal prenatal cortisol and child outcomes. Second, most studies on the relation between PNS and infant health are focused on birth outcomes (e.g. birth weight, gestational age, APGAR scores) and the first year of life (e.g. Beijers et al., 2010; Dunkel et al., 2012; Entringer, 2013; Shapiro et al., 2013). Research on long-term associations is scarce. Hence, the **second aim of this thesis** is to obtain more insight in the persistent and/or long-term associations of PNS with child outcomes by following children into middle childhood. Finally, the **third research aim** is to gain more insight in mechanisms that may underlie/explain relations between PNS and child outcomes. The focus will be on a mechanism that has recently been put forward in this context, namely the gut-brain axis, and more specifically the infant intestinal microbiota (Beijers et al., 2014). In the next three sections of this introductory chapter the three research aims will be clarified and described in more detail. The outline of this thesis is presented at the end of the chapter.

Maternal prenatal cortisol and child outcomes

During pregnancy women can experience stress due to both severe/extreme stressors (e.g. environmental disasters, terrorist attacks) and to milder forms of psychosocial stress (e.g. daily hassles, fear of giving birth). Studies show that the exposure to both types of stressors is related to child psychological and health outcomes (Berkowitz et al., 2003; Glynn et al., 2001; Huizink et al., 2007; King et al., 2012; Kingston et al., 2012; Lederman et al., 2004; Raikkonen et al., 2011; van den Bergh et al., 2005). For example, newborns of mothers who were pregnant during the World Trade Center disaster had a lower birth weight than newborns of mothers who did not experience this disaster (Lederman et al., 2004), and 8-month-old infants from mothers with high levels of pregnancy-specific anxiety had lower cognitive and mental abilities than infants from mothers who experienced low levels of anxiety during pregnancy (Buitelaar et al., 2003).

Anxious, stressed and/or depressed feelings can arise when an imbalance between demands from the environment and individual resources exists. Coping with stressors requires organisms to adapt to the environment by modifying their physiological and psychological systems, a process called allostasis (McEwen and Wingfield, 2003). Physiological systems that are activated in reaction to stressors are the sympathetic nervous system (SNS) and the Hypothalamic Pituitary Adrenal axis (HPA-axis). The SNS releases epinephrine and norepinephrine, and triggers the fast fight-or-flight response to stress (Gunnar and Quevedo, 2007). Activation of the HPA axis results in release of the corticotrophin-releasing hormone (CRH) by the hypothalamus, which in turn activates the release of adrenocorticotrophin hormone (ACTH) by the pituitary gland (Stratakis and Chrousos, 1995). Finally, ACTH stimulates the adrenal cortex to secrete cortisol, a glucocorticoid, into the bloodstream. Cortisol is the end product of the HPA axis and closes the negative feedback loop by inhibiting the release of CRH (Gunnar and Quevedo, 2007). Glucocorticoids are a major subclass of steroid hormones that are involved in several essential bodily functions, regulating metabolic, cardiovascular, immune and behavioral processes (Lundberg, 2005), and not surprisingly, glucocorticoid receptors are widely distributed through the brain and peripheral tissues. This makes the HPA axis, including cortisol, an essential part of our physiology. Because extremely high or chronically high cortisol concentrations are detrimental for physical and mental health (e.g. Petrowski et al., 2010; Segerstrom and Miller, 2004), it is important to study cortisol concentrations in studies focusing on stress. Determination of cortisol concentrations in saliva is a reflection of biologically active cortisol in blood, and is a reliable and non-invasive method for studying human cortisol concentrations (Lundberg, 2005; Nicolson, 2008). Therefore, salivary cortisol is often used in scientific research as an indicator of physiological stress, also during gestation.

During pregnancy not only the mother is affected by increasing concentrations of cortisol in periods of stress, but also fetal development may be affected in at least three ways. *First*, maternal cortisol can cross the placenta (Gitau et al., 1998). The fetus is protected

from extremely high levels of cortisol by the protective barrier function of the placenta, which enzymatically inactivates most of the cortisol into its inactive form cortisone (Beneditksson et al., 1997; Murphy et al., 2006; Seckl, 1997). However, although most of the cortisol is inactivated, elevations in maternal cortisol can still double the fetal cortisol levels (Gitau et al., 1998, 2001). *Second*, the placenta secretes the hormone CRH (Petraglia et al., 1987), and in contrast to the above described negative feedback loop of the HPA axis, this feedback loop is positive for both mother and fetus (Mazjoub and Karalis, 1999). Through the umbilical vein, placental CRH enters the fetus, stimulating the fetal HPA axis, and the secretion of ACTH and cortisol. Fetal cortisol reaches the placenta through the umbilical arteries and stimulates placental CRH secretion, completing the fetal positive feedback loop. Also, the production of placental CRH is stimulated by maternal adrenal cortisol, which results in increasing concentrations of maternal cortisol and placental CRH during gestation, completing the maternal positive feedback loop. Both feedback loops finally result in increased cortisol concentrations of mother and fetus. Extremely high elevations in cortisol concentrations can in this way affect the fetal central nervous system development (Meaney et al., 1996). *Third*, in response to the increased cortisol concentrations of the mother, the uteroplacental blood flow may be reduced (Levine et al., 2016), which may lead to fetal growth restriction. For the above reasons, elevations of maternal cortisol concentrations are a potentially important mediator between stress experienced by the mother during pregnancy and fetal, and later child, development.

If maternal cortisol concentrations are indeed a mediator, then there should be evidence from empirical studies that there are links between maternal prenatal cortisol concentrations and child outcomes. The designs of previous studies on the relations between maternal prenatal cortisol concentrations and child outcomes are diverse, and the results are mixed. Systematic reviews of these empirical studies are lacking, but would help understand the complex interactions between the maternal HPA axis and child development. The **second chapter** of this thesis is such a systematic review that summarizes the empirical findings on the associations between maternal prenatal cortisol concentrations and child outcomes. Results are subdivided in physical/health, cognitive/motor, psychological/behavioural, and cortisol outcomes.

Longitudinal associations between maternal prenatal stress and child outcomes

As mentioned earlier, empirical studies show that PNS (reported and/or assessed with stress hormones) is related to health outcomes in offspring. For example, studies show that infants from mothers with high levels of PNS have a lower birth weight (Dunkel et al., 2012; Shapiro et al., 2013) as well as more respiratory, general, and skin illnesses and more frequent antibiotic treatments during the first year of life (Beijers et al., 2010). As also evident from the systematic

review reported in Chapter 2, most of the studies that examined the relation between PNS and offspring health outcomes focused on birth outcomes and early life. The Developmental Origins of Health and Disease (DOhAD) hypothesis states that fetal exposure to developmental risk factors in utero (e.g. malnutrition) conditions the risk of disease throughout life: in infancy, childhood, and adulthood (Gluckman and Hanson, 2004). This suggests that the effects of PNS may be persistent or even progressive over time. In the **third chapter** of this thesis this question is addressed by following up a sample of children whose health across the first year after birth has been related to maternal PNS (Beijers et al., 2010). In the present study the same children's health was followed until middle childhood (age 6) and examined in relation to PNS.

Animal studies have shown that maternal prenatal stress also affects the offspring's HPA axis functioning. For example, offspring of stressed rat and primate mothers often have an increased HPA axis reactivity (Welberg and Seckl, 2001). HPA axis functioning characterized by hypercortisolism may be related to worse health and wellbeing. However, few empirical studies examined this association in human offspring, and previous studies focused especially on the first year of life. For example, Davis et al. (2011) found that elevated maternal cortisol concentrations during pregnancy are related to higher cortisol responses in newborns in response to a heel-stick. Tollenaar et al. (2011) showed that reported pregnancy-specific anxiety is related to increased infant cortisol responses to a bathing session at 5 weeks, and decreased cortisol responses to a vaccination at 8 weeks and a maternal separation at 12 months. The latter study shows that there is a relation between PNS and cortisol reactivity during the first year of life, but that infant age and/or nature of the stressor possibly determines whether the relation is with high vs low cortisol reactivity. Also, HPA axis functioning develops/changes over time (Gunnar and Quevedo, 2007; Simons et al., 2015). To detect gradual change of HPA axis functioning over time, it is important to examine HPA axis reactivity and its association with PNS at several time points during development. Such research is hampered, however, by the fact that an effective stress paradigm for young children (from toddlerhood till age seven) is lacking, resulting in a gap of stress reactivity research around early childhood. For older children and adolescents, stress paradigms are available, such as the Trier Social Stress Test for Children (TSST-C; Buske-Kirschbaum et al. 1997). A problem with these tests, however, is that they require multiple experimenters, adding significantly to the costs of studies using them. Hence, to examine the relation between PNS and child stress reactivity longitudinally, effective and cost efficient stress paradigms for (young) children and adolescents are needed. The present thesis reports on the development of two such paradigms.

At different ages, different situations or stimuli elicit cortisol reactivity. For example, cortisol in babies is elicited in reaction to a bath or a vaccination (Jansen et al., 2010), and cortisol increases after a public speaking task can be seen in children age seven and above (Gunnar et al., 2009). The **fourth chapter** of this thesis describes the development of a new stress paradigm and its effectiveness in increasing cortisol concentrations in five- and six-year-olds. This stress paradigm is based on elements proven to be effective in increasing cortisol

in older children and adults, namely: social evaluation, unpredictability, and uncontrollability (Dickerson and Kemeny, 2004; Gunnar et al., 2009). In addition, Gunnar et al. (2009) suggest that failing publicly on a task that children believe even younger children could complete successfully is also stressful. Therefore, this element was also included in the stress paradigm. The **fifth chapter** of this thesis presents a newly developed stress paradigm specifically designed for adolescents and reports on its effectiveness in increasing cortisol concentrations. In this test the social evaluation is focused on personal characteristics and comparisons with peers. Furthermore, this new paradigm is aimed to be more cost efficient than existing public speaking tasks, in which several experimenters are needed to carry them out.

With the creation of an effective stress paradigm for five-to-six-year-olds, it became possible to follow up the sample of children whose stress reactivity across the first year after birth had already been related to maternal PNS (Tollenaar et al., 2011). Hence, in the **sixth chapter** the same children were followed up at age 6 and their stress reactivity to this new social evaluative stressor paradigm was examined in relation to PNS.

PNS and child outcomes: infant intestinal microbiota as an underlying mechanism?

Although – as indicated earlier – maternal prenatal cortisol is a potential underlying mechanism in the relation between PNS and child outcomes, it is likely that it acts in concert with other mechanisms. An indirect mechanism that may be involved in the links between PNS and child outcomes is the infant intestinal microbiota.

The intestinal microbiota is a complex, balanced ecosystem, consisting of more than 10^14 microbes living in the intestinal tract (Savage, 1997; Whitman et al., 1998). One of the microbiota's major functions, also called the 'barrier-effect', is to protect humans from colonization and invasion by harmful species in the gut (Veal and Desager, 2009). Also, the intestinal microbiota has many important roles, such as the maturation of the human immune system and gastro-intestinal tract, digestion, and metabolism. A late acquisition of intestinal bacteria or a reduced complexity of the microbiota may delay immune maturation (Adlerberth and Wold, 2008). In sum, the intestinal microbiome functions as an 'organ' that has an important influence on human health. So, imbalances in the microbial composition may lead to distortions in the physiological functions of the microbiota, in turn potentially resulting in increases in host morbidity.

There are indications that the bacterial colonization of the infant gut already starts in utero (Ardissone et al., 2014; DiGiulio, 2012). After delivery and in the first months of life, the infant's colonization of the gut continues at a rapid pace. Microbes originating from the mother and the environment can be found in the infant's gut, and the intestinal microbiota closely resembles that of an adult at around three years of age (Bergstrom et al., 2014). There

are several factors that can influence the colonization process around birth and in the first years of life, such as mode of delivery, genetic background, environment (e.g. pets and house dust, siblings), (breast)feeding, and use of antibiotics (Best et al., 2015; de Weerth et al., 2013). Distortions in the intestinal microbiota contribute to a wide range of diseases, including the risk of diarrheal illness, food allergy, inflammatory diseases (atopic diseases and inflammatory bowel disease), irritable bowel syndrome, obesitas and diabetes (Sekirov et al., 2010).

In rhesus monkeys, PNS was found to affect the colonization of the infants' gut (Bailey et al., 2004). Infant monkeys of mothers who experienced stress during late pregnancy had lower levels of protective bacteria (Bifidobacteria and Lactobacilli) in their intestinal microbiota. The function of these bacteria is to inhibit the attachment of pathogens to intestinal cells. Furthermore, they produce antibacterial substances that kill or slow the replication of enteric pathogens. Subsequently, these monkeys are less protected against infection, which heightens their probability of contracting diseases. Indeed, the infant monkeys of mothers who were stressed showed more health problems than the infants of non-stressed mothers (Bailey et al., 2004). One way in which PNS could affect an infant's intestinal microbiota and proneness for disease is, as mentioned above, the transfer of high levels of maternal cortisol through the placenta. This in turn could increase fetal cortisol concentrations, resulting in higher infant basal and reactivity concentrations of cortisol after birth. Heightened infant cortisol concentrations could affect intestinal immune cells. Furthermore, cortisol can change the permeability of the gut, and disrupt the barrier function, possibly affecting the gut microbiota (Cryan and Dinan, 2012). Theoretically, since rhesus monkeys are similar to humans in fetal development and maturational state at birth, findings similar to those of Bailey et al. (2004) may also be expected in humans. To date there are no human studies replicating this research. The **seventh chapter** of this thesis presents the findings of the very first human study examining the relations between PNS and infant intestinal microbiota and health.

Thesis outline

Next to this introductory chapter (chapter 1), the thesis consists of a systematic review and five empirical studies, which are presented in six chapters (chapters 2-7; see Table 1). Three of the empirical studies examined the relation between maternal prenatal stress and child outcomes; these three studies used data from the BIBO (Dutch acronym for Basal Influences in Baby Development) study. The BIBO study is an ongoing longitudinal study in which 193 mothers and their children were recruited and followed from pregnancy on. The two empirical studies reporting the development and effectiveness of new stress paradigms were based on different populations. The stress paradigm for five-to-six-year-olds was tested in a study population of 42 five- and six-year-old children from regular primary schools in Nijmegen and surroundings. The study population used for the development of the stress paradigm for

adolescents consisted of 52 adolescents from five primary and three secondary schools in Nijmegen. More details about the three populations used in the present thesis are presented in the separate chapters. The final chapter (chapter 8) consists of a summary of the thesis, conclusions, and general discussion.

Table 1. Thesis Outline

Chapter	Maternal prenatal stress measure	Child outcome measure	Assessment age	Type of study
2	Cortisol	- Health - Cognitive/motor - Psychological/behavioral - Cortisol reactivity		Systematic review
3	Cortisol Reported stress	Health	Age 1-6	Empirical, longitudinal
4		Cortisol reactivity (to newly developed stress paradigm)	Age 6	Empirical, test development
5		Cortisol reactivity (to newly developed stress paradigm)	Age 10-15	Empirical, test development
6	Cortisol Reported stress	- Cortisol reactivity - Behavior (gazing towards social evaluative threat)	Age 6	Empirical
7	Cortisol Reported stress	- Intestinal microbiota - Health	0-4 months	Empirical, longitudinal

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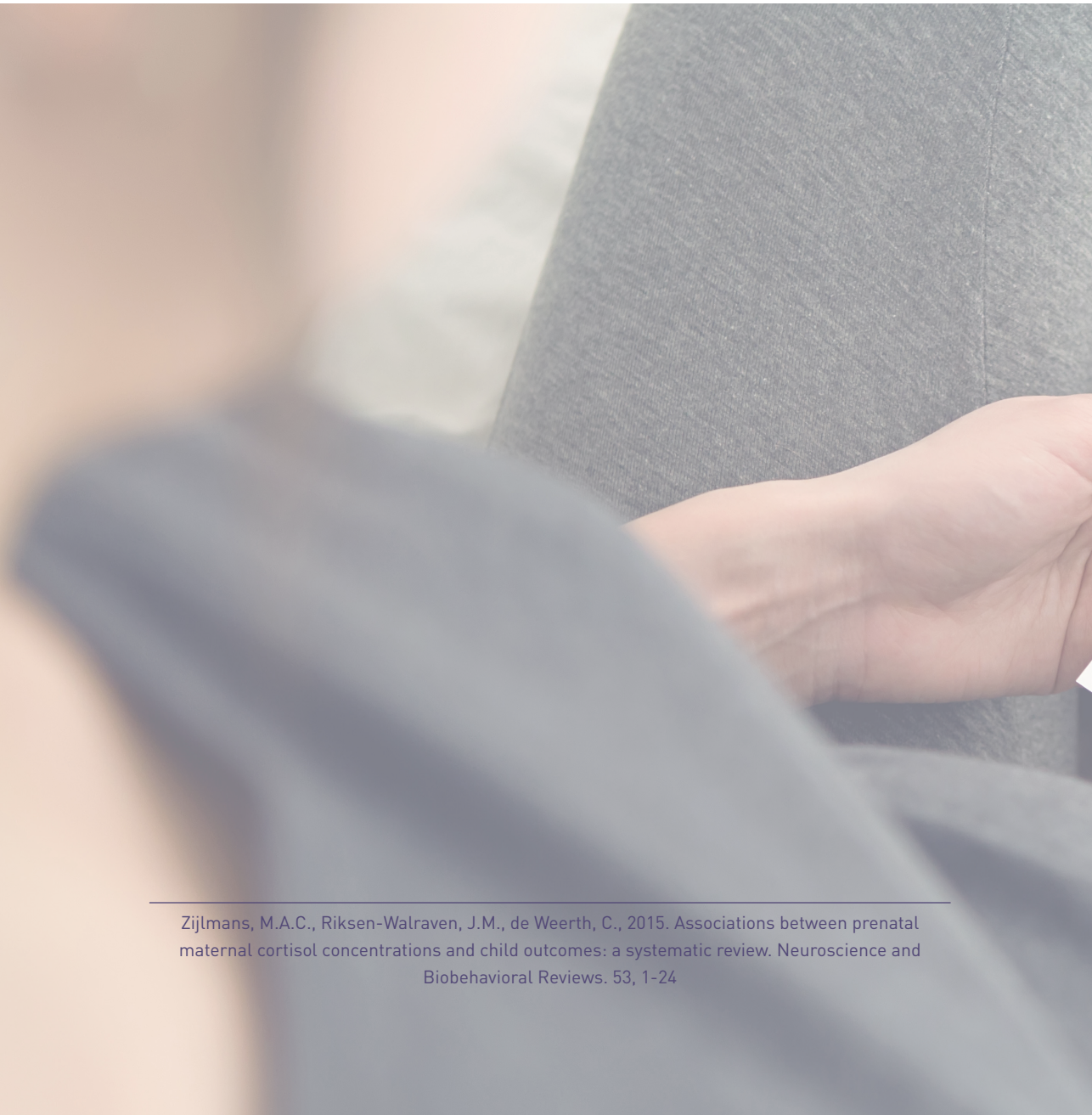

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Chapter 2

Associations between Maternal Prenatal Cortisol Concentrations and Child Outcomes: A Systematic Review

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ABSTRACT

A frequently proposed mechanism underlying the link between maternal prenatal stress/anxiety and child outcomes is heightened concentrations of maternal cortisol. In this systematic review, empirical findings on associations between maternal prenatal cortisol concentrations and child outcomes (physical/health, cognitive/motor, psychological/behavioral, and cortisol) are summarized. The number of empirical studies that find significant associations between maternal prenatal cortisol and child outcomes is small, but the majority of the studies that do find associations show that maternal cortisol is related to altered child outcomes (e.g. more physical/health problems, lower cognitive/motor development, more psychological/behavioral problems, and higher child cortisol concentrations). Inspection of the studies reveals possible critical gestational periods for maternal cortisol to affect different child outcomes.

The heterogeneity in study designs and cortisol assessment methods makes drawing strong conclusions premature. However, the fact that most studies did not find significant associations suggests that maternal cortisol may not to be the sole or even main underlying mechanism in the relation between maternal prenatal stress/anxiety and child outcomes. Limitations of the reviewed studies are discussed, and directions for future research and reporting strategies are provided.

INTRODUCTION

There is growing empirical evidence that the intrauterine/fetal environment can influence fetal development, possibly even having long-term consequences for the offspring's development and the development of pathophysiology. This phenomenon is known as prenatal programming (Lucas, 1991; Seckl and Meaney, 2004; van den Bergh et al., 2005). Barker pioneered the field, by proposing the 'fetal origins of adult disease hypothesis'. This hypothesis was based on Barker's observations that lower birth weight predicted a higher risk of coronary diseases in adulthood (Barker and Martyn, 1992; Barker, 1998; Barker et al., 2010). From then on, there has been an increasing interest in the relations between the prenatal environment and the postnatal development of the offspring. A specific area of interest has been that of the relations between maternal prenatal stress and fetal and child development, not only focusing on infant health outcomes, but also on behavioral development. In rodents and non-human primates, maternal prenatal stress has been found to affect offspring learning, anxiety and social behavior (Kofman, 2002). Also, in humans, there is growing evidence that maternal prenatal stress is related to altered birth outcomes (e.g. preterm delivery, lower birth weight) and to altered developmental outcomes for the child (e.g. delayed motor development, cognitive and behavioral disorders) (Mulder et al., 2002).

Prenatal stress can be caused by exposure to both severe/extreme stressors and milder forms of psychosocial stress during pregnancy. Examples of severe stressors are the exposure to (environmental) disasters, such as an earthquake, the Canadian Ice Storm, the World Trade Center disaster, and the Chernobyl disaster. Several studies have shown that the experience of maternal stress due to these extreme stressors was related to worse birth outcomes (Berkowitz et al., 2003; Lederman et al., 2004; Glynn et al., 2001) and worse performance on cognition, behavior, motor development, and health outcomes of their children (King et al., 2012). Furthermore, the offspring had a higher risk for developing symptoms of depression and symptoms of attention deficit hyperactivity disorder during adolescence (Huizink et al., 2007). Examples of milder forms of stress are for example daily hassles (DiPietro et al., 2004), and pregnancy specific anxiety, such as fear of having a handicapped child (Mulder et al., 2002). Evidence for a link between these forms of prenatal stress and altered infant outcomes, e.g. infant cognitive, behavioural and psychomotor developmental problems, was shown in several review studies (van den Bergh et al., 2005; Kingston et al., 2012; Räikkönen et al., 2011). Also maternal prenatal depression was related to infant outcomes, both short-term (e.g. increased distress after delivery, disrupted sleep) and long term (e.g. disruptive social behavior; as reviewed by Suri et al., 2014). Furthermore, prenatal depressive symptoms were found to be linked to infant negative reactivity (Davis et al., 2007), child externalizing difficulties and a decreased (verbal) IQ (Barker et al., 2011; Evans et al., 2012). On the other hand, there are also studies showing only a weak negative relation between maternal prenatal stress, anxiety and depression and infant cognitive development (Keim et al., 2011) or even a positive

influence of moderate prenatal distress on increased motor, mental and specific language development (Keim et al., 2011; DiPietro et al., 2006). A study of Sandman et al. (2012) found that mothers who experienced congruent levels of depression pre- and postnatally, had infants with increased motor and mental development during their first year of life, even when the levels of depression were relatively high. These apparent contradictory findings may be due to the nature of the maternal stress as well as to the effects of stress being different for different outcomes.

HPA axis/circadian rhythm

One of the major proposed physiological mechanisms underlying the relation between maternal prenatal stress and altered outcomes in children is hormonal, namely through the actions of glucocorticoids secreted by the Hypothalamic Pituitary Adrenal (HPA) axis and the placenta (Cottrell and Seckl, 2009). In humans and many other species, the HPA axis is activated in reaction to both physical and psychological stress. The hypothalamus releases corticotrophin releasing hormone (CRH), which in turn stimulates the pituitary gland to release adrenocorticotrophin hormone (ACTH) (Stratakis and Chrousos, 1995). ACTH activates the adrenal cortex to release cortisol into the bloodstream, which is the end product of the HPA axis. Cortisol, in turn, inhibits the release of CRH, closing the negative feedback loop (Gunnar and Quevedo, 2007). When stressors are plentiful, cortisol concentrations in blood may rise, becoming even chronically heightened (Gunnar and Quevedo, 2007). These heightened concentrations of cortisol, when occurring during pregnancy, are thought to constitute the basis of an important mechanism by which prenatal maternal stress may produce altered outcomes in the offspring. However, the results of empirical studies examining the links between maternal prenatal cortisol concentrations and outcomes in the offspring are conflicting. The aim of this review is to give an overview of these studies, and to examine whether there are critical periods in gestation during which the fetus may be more susceptible to heightened maternal cortisol concentrations.

Changes in maternal HPA axis during pregnancy

During pregnancy the maternal HPA axis shows substantial changes. The placenta is a major producer of peripheral CRH during pregnancy, which is produced from about 8-10 weeks of gestation (Petraglia et al., 1996; Mulder et al., 2002) and is similar to that secreted by the hypothalamus (Mazjoub and Karalis, 1999; Challis et al., 1995). However, the regulation of the hypothalamic CRH and placental CRH is tissue specific. Instead of the inhibitory influence of the glucocorticoids on the hypothalamic CRH, placental CRH is stimulated by glucocorticoids in a positive feedback loop (Economides et al., 1987; Goland et al., 1988). This in turn results in progressive increases in CRH during pregnancy (Riley and Challis, 1991; Warren and Silverman, 1995; Liu and Rebar, 1999), reaching levels that are a thousand times higher in pregnant women

compared to non-pregnant women. Furthermore, ACTH levels increase during pregnancy, but rise more slowly than the CRH levels (Goland et al., 1994; Perkins et al., 1995).

Daily cortisol secretion increases from week 25 of pregnancy to reach around 2 fold the cortisol of non-pregnant women in the third trimester (Allolio et al., 1990). Despite these increases in cortisol concentrations, the circadian rhythm of cortisol, with higher levels in the morning and lower levels in the afternoon and evening, is maintained (Allolio et al., 1990; Kirschbaum and Hellhammer, 1994; Kirschbaum and Hellhammer, 1989; de Weerth and Buitelaar, 2005b). Glucocorticoids, including cortisol, are essential for the development and maturation of fetal organs before birth (Murphy et al., 2006; Mulder et al., 2002). However, prolonged exposure to heightened maternal cortisol concentrations could have detrimental and long-term effects on fetal growth and organ development (Field and Diego, 2008; Weinstock, 2005). This is in line with studies on fetal exposure to synthetic glucocorticoids, which are often administered to pregnant woman at risk for premature delivery to promote lung development. This prenatal administration of glucocorticoids can also have other effects on infant development. For example, preterm infants treated with antenatal betamethasone showed blunted cortisol responses to a stressor during the first weeks of life (Davis et al., 2004; Davis et al., 2006).

Mechanisms through which elevations in maternal cortisol may affect the fetus

The mechanisms through which heightened levels of stress and cortisol in the pregnant mother may affect the fetus are only partly understood (Gitau et al., 2001). Three potential mechanisms have been suggested. *First*, maternal cortisol can cross the placenta (Gitau et al., 1998). To protect the fetus from extremely high levels of maternal cortisol the placenta has a protective barrier function; 50-90% of the hormone is inactivated by the placental dehydrogenase enzyme 11β-HSD2, which converts cortisol to biologically inactive cortisone (Benediktssson et al., 1997; Murhpy et al., 2006; Seckl, 1997). Hence, cortisol in fetal blood reaches levels that are 10-20% of maternal levels of cortisol (Murphy et al., 2006). Although the fetal cortisol concentrations are lower compared to maternal cortisol concentrations, elevations in maternal cortisol can still double the fetal cortisol concentrations. Around 30-40% of the variance in fetal cortisol during weeks 13-35 of pregnancy has been found to be the result of maternal cortisol concentrations (Gitau et al., 1998; Gitau et al., 2001). Moreover, recent research has shown that maternal anxiety is negatively correlated with the activity of 11β-HSD2, possibly leading to a higher exposure of the fetus to maternal cortisol in highly anxious mothers (O'Donnell et al., 2012). Maternal cortisol reaching the fetus is thought to possibly influence the fetal development (Jacobson and Sapolsky, 1991; Seckl and Holmes, 2007). Animal studies have shown that fetal exposure to extreme cortisol concentrations alters neural development, resulting in deleterious effects on fetal HPA axis and affecting hippocampal development (Coe et al., 2003; Schmitz et al., 2002). This in turn results in negative influences on the postnatal development (Charil et al., 2010). These and similar effects may also be present in humans.

The *second* potential mechanism by which maternal cortisol could affect the fetus is by placental secretion of CRH (Petraglia et al., 1987). It has a fetal and maternal feedback loop, which are both *positive* instead of negative (Mazjoub and Karalis, 1999), as described above for the HPA axis. Placental CRH enters the fetal circulation through the umbilical vein, stimulating the fetal HPA axis, and resulting in the secretion of ACTH and cortisol. Additionally, further placental CRH secretion is stimulated by cortisol that reaches the placenta through the umbilical arteries, thereby completing the fetal positive feedback loop (Majzoub and Karalis, 1999). Furthermore, adrenal cortisol stimulates the production of placental CRH (Cheng et al., 2000), resulting in increasing concentrations of maternal cortisol and placental CRH during gestation (Glynn and Sandman, 2011), completing the maternal positive feedback loop. Summarizing, placental secretion of CRH results in positive maternal and fetal feedback loops and increased cortisol concentrations, which can affect fetal central nervous system development (Meaney et al., 1996), altering also the fetal HPA axis (Huizink et al., 2004).

Third, the fetus may be affected indirectly by increased levels of maternal cortisol because in response to increased secretion of maternal cortisol and catecholamines the uteroplacental blood flow may be reduced (Mulder and Visser, 1987). In turn, this reduced blood flow may lead to fetal growth restriction (Huizink et al., 2004; Mulder et al., 2002).

The present study

As we have seen, evidence exists for links between maternal prenatal stress, both in severe and milder forms, and child outcomes. A frequently proposed underlying mechanism in these links involves heightened concentrations of maternal cortisol. This review summarizes the empirical findings on associations between maternal prenatal cortisol concentrations and child outcomes, subdivided in physical/health, cognitive/motor, psychological/behavioral, and cortisol outcomes. Furthermore, several studies showed that there are critical gestational periods in which the fetus is more susceptible to maternal cortisol concentrations. Class et al. (2011) showed that especially month 5 and 6 were related to adverse birth outcomes, and Glynn et al. (2001) showed that the effects of early stress on gestational age were more visible than those of stress in later pregnancy. Kleinhaus et al. (2013) found that the third month of gestation was an especially vulnerable period associated with increased levels of affective disorders in offspring. It is plausible that the vulnerable period differs for the diverse outcomes because different brain regions develop during different stages (Glover et al., 2010; Harris and Seckl, 2011). Therefore, this review will also attempt to identify potentially critical gestational periods for the different outcomes in which the fetus is more susceptible to maternal cortisol concentrations.

METHOD

This review was carried out following the PRISMA guidelines for systematic reviews (Moher et al., 2009; Liberati et al., 2009). The databases PUBMED, MEDLINE, PSYCHINFO and WEB of SCIENCE were used for a literature search until April 2013. The following search terms were used: (cortisol) AND (pregnancy OR prenatal) AND (infant OR child OR offspring OR outcome) and search limits were set to English language and human studies. The results of the literature search were transported to Endnote X5.01 (Thomson Reuters [Scientific] Inc, Carlsbad, CA) and the duplicate results removed.

The remaining papers were then filtered by reading the titles, the abstracts (checked independently by the first and last author) and the full articles. Exclusion criteria were: study not in English; does not include human subjects; includes a clinical group without a control group; does not measure maternal prenatal cortisol; concerns teenage pregnancies; focuses on prenatal child outcome variables; reviews or case reports; does not report empirical data; not peer reviewed; cortisol measured in placenta or cord. Three authors were contacted to ask for four papers that were not available online.

The outcome variable was defined as physiological and psychological measures in infants and children. This variable was of a broad nature to ensure that an ample variety of possible associations of maternal prenatal cortisol and child outcomes were included.

The remaining papers were reviewed and the following data were extracted: authors, year of publication, independent variable(s), biological material in which cortisol was measured, timing of cortisol measurement (weeks of pregnancy), child age, outcome variable(s) and instrument, statistical analyses, confounders and results. Based on the outcome variables, the papers were subdivided into four main categories: physical/health, mental/motor development, psychological/behavior, and child cortisol. For every main category the bibliography of the most recent paper was checked to identify further eligible papers.

The study selection process is illustrated in Figure 1.

Identified studies

The selection process resulted in a total of 28 papers (Beijers et al., 2010; Bolten et al., 2011; Buitelaar et al., 2003; Buss et al., 2012; D'Anna-Hernandez et al., 2012; Davis et al., 2007; Davis et al., 2010; Davis et al., 2011; Davis et al., 2012; Diego et al., 2009; van Dijk et al., 2012; Ellman et al., 2008; Erickson et al., 2001; Goedhart et al., 2010; Gutteling et al., 2004; Gutteling et al., 2005a; Gutteling et al., 2005b; Gutteling et al., 2006; Gutteling et al., 2007; Huizink et al., 2003; Mercer et al., 2006; Rondó et al., 2010; Rondó et al., 2011; Ruiz et al., 2001; LeWinn et al., 2009; de Weerth et al., 2003; Rothenberger et al., 2011; Tollenaar et al., 2011). The selected studies were published between 2001 and 2012.

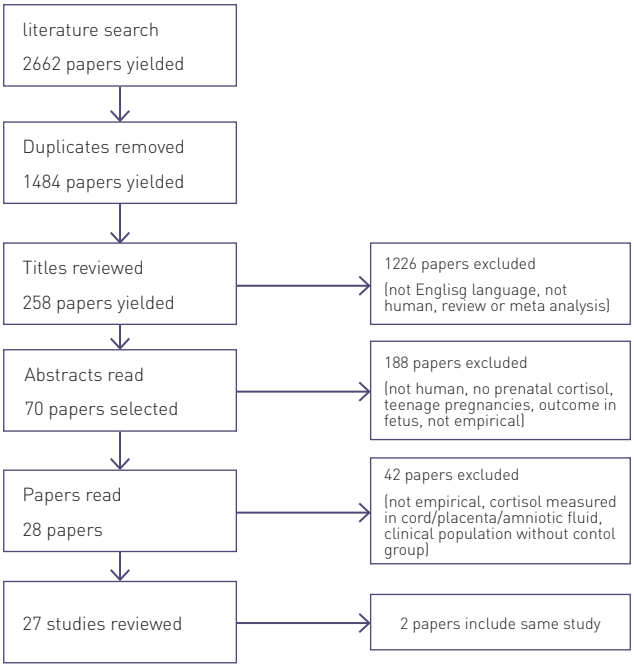

Figure 1. Flowchart of study selection process.

Heterogeneity of maternal prenatal cortisol and outcome measures

The measures of maternal prenatal cortisol varied in timing of day (morning, evening, composite scores of two or more cortisol measurements), gestational week, and biological material in which cortisol was measured (blood, saliva, urine). Furthermore, the outcome measures were diverse in nature and children's ages ranged from birth to 7.5 years. This variety of measurements of the independent and dependent variables rendered the data unsuitable for conducting a meta-analysis, and therefore, a systematic review of the papers was carried out instead.

Subdivision of results

Because of the heterogeneity of the outcome measures, we classified the studies in four main categories; 1. Physical/health outcomes, 2. Cognitive/motor outcomes, 3. Psychological/behavioral outcomes, 4. Cortisol outcomes. Furthermore, to examine if there are critical gestational periods in which the fetus is more susceptible to maternal cortisol concentrations, we subdivided the studies based on weeks of gestation in which cortisol was measured. Because maternal cortisol starts to rise from week 25 week of pregnancy on, we decided to subdivide the results into two main categories; first and second trimester (early and midgestation, <27 weeks) and third trimester (late gestation, ≥27 weeks).

Proportion of significant analyses

Most papers that examined the relation between maternal prenatal cortisol and infant outcomes conducted several analyses. To control for the number of analyses that were conducted, the proportion of significant analyses was calculated. Furthermore, to control for the quality of the papers, we repeated the same analyses on a subset of papers that were considered of high quality. We defined 'high quality' papers as papers that carried out multivariate analyses and included two or more confounding variables in their analyses.

Maternal prenatal cortisol and physical/health outcomes in child

Overview results

Table 1 shows a summary of the empirical studies on the relation between maternal prenatal cortisol and physical/health outcomes.

Early and mid-gestation: weeks <27

A total of nine papers examined the relation between maternal prenatal cortisol in early and mid-gestation and physical outcomes such as the gestational age, physical maturation and size of the child.

Three papers examined the relation between maternal prenatal cortisol and birth weight. Lower infant birth weight was related to a higher CAR (Bolten et al., 2011) and higher morning cortisol values (Goedhart et al., 2010), but not to the slope of diurnal decline (D'Anna-Hernandez et al., 2012). Higher morning cortisol concentrations were not only correlated with lower birth weight, but also with an increased risk of being small for gestational age (Goedhart et al., 2010). The CAR was not related to birth body length or head circumference (Bolten et al., 2011) and pregnancy morning cortisol was not associated with body mass index (BMI), waist-to-height ratio (WHtR) or fat mass index (FMI) in 5-year-old children (van Dijk et al., 2012). One paper included afternoon cortisol samples and showed that higher afternoon cortisol concentrations at 15 and 19 weeks were significantly related to decreased infant physical and neuromuscular maturation at birth (Ellman et al., 2008).

With regard to infant gestational age as an outcome measure, findings are also mixed. Higher morning cortisol concentrations and serum cortisol concentrations (time of day of serum samples not reported) were associated with lower gestational age at birth (Diego et al., 2009; Mercer et al., 2006). In contrast, two other papers found no relation between prenatal serum cortisol (time of day not reported) and gestational age (Erickson et al., 2001; Ruiz et al., 2001). Overall, these papers suggest that higher maternal morning cortisol in early to mid-pregnancy is related to lower infant birth weight, being small for gestational age, and lower gestational age. However, the results of one of the two papers that showed a relation between prenatal

stress and gestational age may be driven by the inclusion of depressed women in the study group. Furthermore, higher maternal afternoon cortisol is related to decreased physical and neuromuscular maturation. Maternal cortisol seems to be unrelated with birth body length and head circumference, and with later BMI, WHtR and FMI.

Late gestation: weeks ≥ 27

Eight papers examined the relation between cortisol concentrations in late pregnancy and infants' physical and health outcomes. A higher CAR and lower diurnal cortisol slope in week 35 of pregnancy were related to lower birth weight (Bolten et al. 2011; D'Anna-Hernandez et al. 2012). However, this relation was not found when cortisol was measured in week 28 of pregnancy (D'Anna-Hernandez et al., 2012). Furthermore, a higher 35-week CAR was related to a smaller birth body length, but unrelated to birth head circumference (Bolten et al., 2011). In contrast, one paper included afternoon cortisol samples and showed that higher afternoon cortisol concentrations at 31 weeks were significantly related to increased infant physical and neuromuscular maturation at birth (Ellman et al., 2008).

The papers with gestational age as an outcome measure showed mixed findings. Erickson et al. (2001) found a negative relation between prenatal cortisol (time of day not reported) measured in weeks 27-37 and gestational age, with mothers delivering preterm showing higher cortisol concentrations than those delivering full term. However, in other papers gestational age was not found to be related to serum maternal prenatal cortisol (time of day not reported) and cortisol decline over the day in weeks 27-35 of pregnancy (D'Anna-Hernandez et al., 2012; Ruiz et al., 2001).

Three papers examined the relation between prenatal cortisol and children's health. In the first, the infants' health was assessed in the first 12 postnatal months. A smaller cortisol decline over the day was related to more respiratory illnesses, and higher evening cortisol was (marginally) related to more general and skin illnesses (Beijers et al., 2010). However, these cortisol variables were not related to digestive illnesses. In the other two papers, higher maternal morning cortisol was related to two markers of altered health: a larger systemic vascular resistance (SVR) and lower artery elasticity in 5- to 7-year-old children (Rondó et al., 2010; Rondó et al., 2011).

Overall, these papers show that higher maternal cortisol concentrations and smaller decline of cortisol over the day in late pregnancy are related to lower birth weight and shorter birth length. With respect to gestational age, while two papers found no relation with maternal cortisol, a third paper found that higher maternal cortisol was related to lower gestational age. Finally, higher cortisol concentrations in late pregnancy showed relations with altered child health: more respiratory and skin illnesses, and larger SVR and lower artery elasticity

Confounders

From the 12 papers on the relation between maternal prenatal cortisol and physical/health outcomes, eight papers controlled for potential confounders (early/midgestation: Bolten et al., 2011; Goedhart et al., 2010; van Dijk et al., 2012; Ellman et al., 2008; Diego et al., 2009. Late gestation: Bolten et al., 2011; Ellman et al., 2008; Beijers et al., 2010; Rondó et al., 2010; Rondó et al., 2011).

Four out of these eight papers reported the results both with and without controlling for confounders. Two papers showed that the significant relation between maternal prenatal cortisol and infant physical/health outcomes became non-significant after controlling for confounders (birth weight and small for gestational age: Goedhart et al., 2010; fat mass index: van Dijk et al., 2012). The other two papers showed no effects of confounders on the significant results (systematic vascular resistance: Rondo et al., 2010; artery elasticity: Rondo et al., 2011).

Four papers presented only the results with confounders included (Bolten et al., 2011; Ellman et al., 2008; Diego et al., 2009; Beijers et al., 2010). After controlling for confounders, three papers found a significant relation between maternal prenatal cortisol in early/midgestation and physical/health outcomes (birth weight: Bolten et al., 2011; physical and neuromuscular maturation: Ellman et al., 2008; gestational age: Diego et al., 2009). Two papers found a significant relation between maternal prenatal cortisol in late gestation and physical health outcomes after controlling for confounders (birth weight and body length: Bolten et al., 2011; respiratory and skin illnesses; Beijers et al., 2010).

Four studies did not control for confounding variables in their analyses (including both early/midgestation and late gestation: D'Anna-Hernandez et al., 2012; Mercer et al., 2006; Ruiz et al., 2001; Erickson et al., 2001). One of these studies found a relation between maternal prenatal cortisol during early/midgestation and gestational age (Mercer et al., 2006), and one found a significant relation between maternal prenatal cortisol measured during late gestation and birth weight (D'Anna-Hernandez et al., 2012).

Measurement of cortisol

From the 12 papers on the relation between maternal prenatal cortisol and physical/health outcomes, six papers used blood samples (Ellman et al., 2008; Erickson et al., 2001; Goedhart et al., 2010; Mercer et al., 2006; Ruiz et al., 2001; Van Dijk et al., 2012), five papers used saliva samples (Beijers et al., 2010; Bolten et al., 2011; D'Anna-Hernandez et al., 2012; Rondó et al., 2010, Rondó et al., 2011), and one paper used urine samples (Diego et al., 2009) as *biological material* to measure cortisol concentrations. There was no clear relation between the different biological materials that were used and the outcome variables.

With regard to *sampling days*, the papers using blood and urine samples measured cortisol concentrations only once, whereas four of the five studies using saliva samples used mean values of sampling on two or three days.

Furthermore, the *timing* of saliva sampling differed between papers. Samples were taken in the morning, afternoon and evening, and composite scores were used in the analyses as well (decline over the day, slope of diurnal decline, AUCgCAR). In the papers using blood samples, the timing of sampling is not always reported. Significant relations were found for all sampling times with the outcome variables (see Table 1 for details), therefore no conclusions can be drawn about one sampling moment over the other.

Proportion of significant analyses

Most papers that examined the relation between maternal prenatal cortisol and infant physical/health outcomes conducted several analyses. To control for the number of analyses that were conducted, the proportion of significant analyses was calculated. In total, as can be seen in Table 5, 36.7% of the analyses showed significant results, with approximately equal proportions for analyses including maternal prenatal cortisol measured before (32.4%) and after week 27 of pregnancy (42.3%). When controlling for the quality of the studies, around half of the papers were considered of higher quality and could be included in these secondary analyses (11 out of 23 papers). Results showed that the proportion of significant analyses remained almost equal; 40% of the total analyses from the high quality papers were significant; 33% of the analyses before week 27, and 46.7% after week 27.

Conclusions for physical/health outcome studies

To conclude, the reported significant associations between maternal prenatal cortisol and children's physical/health outcomes were all in the same direction. Higher cortisol concentrations were related to lower birth weight, shorter gestational age, and more health problems. However, after controlling for potential confounders the evidence for the association between prenatal cortisol and birth weight becomes scarce. Furthermore, the majority of the papers on the relation between prenatal cortisol and gestational age did not find a significant association, and the few papers that did find one did not control for confounding variables. Three papers on infant health controlled for many confounders and found maternal prenatal cortisol related to more infant health problems (e.g. more respiratory and skin illnesses, and larger SVR and lower artery elasticity). These three papers only included prenatal cortisol samples measured during late gestation, and not during early and midgestation.

Maternal prenatal cortisol and cognitive/motor outcomes in child

Overview results

Table 2 shows the results of the empirical findings of papers on the relation between maternal prenatal cortisol and cognitive/motor outcomes in the child.

Early and mid-gestation: weeks <27

In total, five papers examined the relation between maternal prenatal cortisol in early and mid-gestation and the cognitive and psychomotor development of children. Two papers examined the relation between maternal prenatal cortisol and infants' mental development during the first year of life. Mental development includes the infants' cognitive abilities through sensory-perception, knowledge, memory learning and problem solving, and the development of early language (Lowe et al., 2012). The daily mean maternal prenatal cortisol samples in early pregnancy (weeks 15-17) were not related to infant mental development at 3 and 8 months of age (two papers on same study: Buitelaar et al., 2003; Huizink et al., 2003). However, Davis et al. (2010) found that lower maternal cortisol in early afternoon in week 15 of pregnancy predicted accelerated mental development during the first year of life, which resulted in enhanced cognitive functioning at 12 months of age. The latter relation was not found for cortisol measured in weeks 19 and 25 of pregnancy (Davis et al., 2010). Furthermore, no effect was found of prenatal maternal morning cortisol on learning and memory functioning in 6-year-old children (Gutteling et al., 2006).

The papers on the relation between cortisol and mental development addressed above, also examined the association of prenatal cortisol and psychomotor development at 3 and 8 months of age. The results showed that cortisol was not related to psychomotor development (Buitelaar et al., 2003; Davis et al., 2010; Huizink et al., 2003). There was also no significant effect of maternal prenatal morning cortisol on child mixed handedness (Gutteling et al., 2007).

In summary, maternal cortisol in early pregnancy was unrelated to infant mental development at 3 and 8 months of age, but it was related to mental development at 12 months of age. It may be possible that the effects of cortisol during early pregnancy are only visible in older children, but more research is needed in order to determine whether the results of this paper can be replicated and possibly extended to older ages. Finally, no evidence was found that prenatal cortisol in early and mid-gestation is related to psychomotor development.

Late gestation: weeks ≥27

Five papers examined the relation between maternal prenatal cortisol in late gestation and the cognitive and psychomotor development of children. Two papers examined the relation between prenatal cortisol measured in late gestation and infant mental development. Higher morning cortisol in week 37-38 of gestation was related to lower mental development at 3 months of age. However, this association was not significant at 8 months of age (two papers on same study: Buitelaar et al., 2003; Huizink et al., 2003). Davis et al. (2010) found that higher early afternoon maternal cortisol in week 37 predicted accelerated mental development during the first year of life, which resulted in enhanced cognitive functioning at 12 months of age. However, this association was not found for prenatal cortisol measured in week 31 of pregnancy. LeWinn et al. (2009) examined the association between maternal serum cortisol (weeks 31-36; time

of sampling not recorded) and IQ in 7 year olds and showed that higher maternal cortisol was related to a lower IQ. This association was most pronounced for verbal IQ, and not significant for performance IQ (LeWinn et al., 2009). In line with the finding in early and mid-gestation, no relation between morning cortisol measured in late pregnancy and learning and memory functioning at age 6 were found (Gutteling et al., 2006). Importantly, the study of Davis et al. (2010) also examined if the trajectory of cortisol secretion during pregnancy was related to infant mental development. They found that low concentrations of maternal cortisol during early pregnancy and elevated concentrations during late pregnancy were related to enhanced infant mental development, suggesting that the pattern of cortisol secretion during pregnancy is even more predictive than cortisol concentrations measured at specific gestational weeks.

Regarding the association between prenatal cortisol and psychomotor development, one study found that high morning and daily mean maternal cortisol in late pregnancy was related to lower psychomotor development at 3 and 8 months of age (two papers on same study: Buitelaar et al., 2003; Huizink et al., 2003), whereas another paper did not find an association between early afternoon cortisol and psychomotor development during the first year of life (Davis et al. 2010). Furthermore, no significant effects of prenatal morning cortisol on child mixed handedness were found (Gutteling et al., 2007).

In conclusion, there is some evidence that higher cortisol during late pregnancy is related to lower mental developmental functioning at 3 months and 12 months of age, and lower verbal IQ at age 7. Regarding the effects on psychomotor development, the findings are mixed.

Confounders

The reported papers on the associations between maternal prenatal cortisol and children's mental and motor development were all carried out controlling for potential confounders. The following confounders were included in most of the analyses: maternal smoking and use of alcohol during pregnancy, social economic status, birth weight, gestational age, maternal age, child sex, maternal work status, and postnatal maternal stress.

Measurement of cortisol

From the five papers on the relation between maternal prenatal cortisol and infant cognitive and motor outcomes, one paper used blood samples (LeWinn et al., 2009), and four papers used saliva samples (Buitelaar et al., 2003; Davis et al., 2010; Huizink et al., 2003; Gutteling et al., 2007; Gutteling et al., 2006) as *biological material* to measure cortisol concentrations. With regard to *sampling days*, all papers measured the cortisol concentrations on a single day.

With regard to the *timing* of the cortisol measures, most of the papers used early morning samples (Buitelaar et al., 2003; Huizink et al., 2003; Gutteling et al., 2006; Gutteling et al., 2007), two in combination with a composite score of the mean value of seven samples during the day (Buitelaar et al., 2003; Huizink et al., 2003), one paper used early afternoon samples

(Davis et al., 2010), and the paper that used blood sampling did not report the sampling time (LeWinn et al., 2009). Results were found with early morning and early afternoon samples (see Table 1 for details).

Proportion of significant analyses

As can be seen in Table 5, 30.8% of the total analyses were significant. For maternal prenatal cortisol measured before week 27 of pregnancy 20.0% were reported as significant, after 27 weeks, 34.5% were significant. All studies could be classified as high quality, so no analyses were carried out to control for the quality of the studies.

Conclusions for cognitive/motor outcome studies

To conclude, evidence for the relation between maternal cortisol in early gestation and infant cognitive development is scarce. Only one study found a significant association between lower prenatal cortisol in early pregnancy and accelerated mental development at 12 months of age. Furthermore, no relation between maternal cortisol in early and mid-gestation and psychomotor development was found. More associations were found between cortisol measured in late pregnancy and child mental development. Fetuses exposed to higher maternal cortisol in the third trimester showed lower cognitive functioning at 3 months of age, and at 7 years of age. In contrast, one study found that higher maternal cortisol in the third trimester of pregnancy was related to accelerated mental functioning at 12 months of age. Additionally, the results of one paper suggest that the trajectory of change in cortisol during pregnancy may be more predictive of cognitive functioning than cortisol concentrations in specific weeks of pregnancy (Davis et al., 2010). The findings on the relation between late pregnancy maternal prenatal cortisol and psychomotor development were also mixed. One study found that higher cortisol was related to lower psychomotor development at 3 and 8 months of age, whereas another study did not find this relation.

Maternal prenatal cortisol and psychological/behavioral outcomes in child

Overview results

Table 3 shows the empirical findings of the papers on the relation between maternal prenatal cortisol and psychological/behavioral outcomes in the child.

Early and mid-gestation: weeks <27

In total, eight papers examined the relation between maternal prenatal cortisol in early and mid- gestation and psychological/behavioral outcomes in children. These papers can be subdivided into two main categories; maternal reports of children's temperament and behavior problems, and children's observed behavior.

Six papers examined the association between maternal prenatal cortisol during early and mid-gestation and children's temperament and behavioural problems (maternal reports) and most of these papers did not find a significant association. Early afternoon cortisol was not related to the children's temperament scales 'negative reactivity' (extent to which infants display startle or distress in response to sudden changes in stimulation or novel or surprising stimuli) at 8 weeks (Davis et al., 2007), morning and daily mean levels of maternal cortisol were not related to 'difficult behavior' and 'inadaptability' at 3 and 8 months (Buitelaar et al., 2003), and not related to 'restless/disruptive behavior' and 'irritability' at 27 months (Gutteling et al., 2005b). Furthermore, Gutteling et al. (2005b) did not find a relation between morning and daily mean levels of maternal cortisol and maternal reports of child's internalizing, externalizing and total behavior problems at 27 months of age. Two papers examined the association between prenatal cortisol and behaviour problems in 7 year old children. Davis et al. (2012) found that elevated average maternal gestational cortisol during pregnancy (overall mean over three trimesters of pregnancy) was associated with higher levels of child anxiety reported by the mother. Children with anxiety ratings within the borderline and clinically significant range were twice as likely to have been exposed to higher maternal cortisol during gestation. However, there was no significant gestational timing effect of cortisol. The paper of Buss et al. (2012) found that mothers with higher afternoon levels of cortisol in week 15 of pregnancy reported more affective problems in their 7 year old daughters. In this paper, the maternal cortisol was also related to larger right amygdala volumes in girls. These associations did not exist for boys and for cortisol measured in the second trimester of pregnancy.

The remaining three papers assessed the relation of maternal prenatal cortisol and observed behavior. Maternal prenatal late morning cortisol was not related to observed 'affective reactivity' at 5 months (Rothenberger et al., 2011). Furthermore, morning and daily mean prenatal cortisol was unrelated to observed behavior at 3 and 8 months (exploration, goal directedness, and test-affectivity; Buitelaar et al., 2003). Davis et al. (2011) found that prenatal afternoon maternal cortisol in weeks 13-14 was not associated with newborn children's observed behavioral state, i.e. quiet sleep, active sleep, quiet awakeness/drowsy, awake and alert, awake and fussy, and crying, during a stressor (heel stick), but was associated with higher children's observed behavioral arousal during recovery of this stressor.

Summarizing, no evidence exists for the relation between cortisol in early and mid-gestation and children's temperament. There is some evidence that higher maternal prenatal cortisol in early and mid-gestation is related to more maternally reported behavior problems in 7 year old children (girls) and more observed arousal during recovery of a stressor in newborn infants.

Late gestation: weeks ≥27

With respect to temperament, Davis et al. (2007) found that higher early afternoon maternal cortisol in late gestation (weeks 30-32) is related to more maternally reported child 'negative reactivity' at 8 weeks of age. Furthermore, children of mothers with high awakening cortisol concentrations during pregnancy had higher scores on 'emotion' (more crying and agitation during the day) and 'activity' (frequency of being excited/active) in week 7, but not in week 18 (de Weerth et al., 2003). However, maternal morning cortisol and daily mean level of cortisol measured in late gestation was unrelated to maternal reports of 'difficult behavior' and 'inadaptability' at 3 and 8 months (Buitelaar et al., 2003) and maternally reported 'restless, disruptive behavior' and 'irritability' at 27 months (Gutteling et al., 2005b). Prenatal morning and daily mean levels of cortisol were also unrelated to maternally reported internalizing, externalizing and total behavioural problems at 27 months (Gutteling et al., 2005b), and afternoon maternal prenatal cortisol was unrelated to affective problems at age 7 (Buss et al., 2012).

In studies looking at links between maternal cortisol and observed children's behavior, de Weerth et al. (2003) found that higher prenatal awakening cortisol in late pregnancy was related to more observed crying, fussing and negative facial expressions during several bathing sessions in the first 20 weeks of life. Maternal afternoon cortisol was not related to the arousal during a stressor and during recovery from this stressor of newborn infants (Davis et al., 2011). Furthermore, maternal late morning cortisol measured in late gestation was unrelated to observed 'affective reactivity' at 5 months (Rothenberger et al., 2011) and morning and daily mean prenatal cortisol were unrelated to observed behavior at 3 and 8 months (exploration, goal-directedness, and test-affectivity; Buitelaar et al., 2003)

Summarizing, the relation between prenatal cortisol during late pregnancy and temperament is found in 7- and 8-week-old children. No associations were found between maternal cortisol and parental reports of problem behavior at older ages. With respect to the association between maternal prenatal cortisol and observed behavior, in the first months of life one paper found an association between high maternal prenatal cortisol and more negative observed behavior, and one did not.

Confounders

Four out of eight papers reported the results with inclusion of potential confounders (Davis et al., 2007; Buitelaar et al., 2003; Davis et al., 2012; Buss et al., 2012), two out of eight papers reported the results both with and without controlling for confounders (Gutteling et al., 2005; Davis et al., 2011) and two did not include confounders (Rothenberger et al., 2011; de Weerth et al., 2003). The two papers that did find a significant association between maternal cortisol measured in early and mid-gestation and infants' temperament and behaviour did control for confounders, i.e. maternal current psychological state, child's sex, age at testing (Davis et al., 2011; Buss et al., 2012). With regard to studies during late gestation, the paper on the

association between prenatal cortisol and infant negative reactivity controlled for maternal postnatal psychological state (Davis et al., 2007). However, the paper that found a significant association between high maternal prenatal cortisol and more temperamental emotionality and activity, and more negative observed behavior, did not control for potential confounders (de Weerth et al., 2003).

Measurement of cortisol

From the eight papers on the relation between maternal prenatal cortisol and infant psychological and behavioral outcomes, the *biological material* used to measure cortisol concentrations was saliva in seven papers (Buss et al., 2012; Davis et al., 2007; Davis et al., 2012; de Weerth et al., 2003; Gutteling et al., 2005b; Rothenberger et al., 2011; Buitelaar et al., 2003) and blood in one paper (Davis et al., 2011).

With respect to *sampling days*, six of the papers sampled on a single day, and two papers used the mean value of two or three days (de Weerth et al., 2003; Rothenberger et al., 2003).

With regard to the *timing* of the cortisol measures, one of the papers used awakening samples (de Weerth et al., 2003), one paper used early morning samples (Gutteling et al., 2005b; Buitelaar et al., 2003), one paper used late morning samples (Rothenberger et al., 2011), and four papers used early afternoon samples (Buss et al., 2012; Davis et al., 2007; Davis et al., 2011; Davis et al., 2012). Several researchers used composite scores, namely the mean cortisol value of seven samples during the day, the AUC over weeks 15-28 and 28-37 of gestation, and the mean of samples measured at each trimester of pregnancy. Significant results were found for awakening samples, early afternoon samples, and the mean of samples measured at each trimester (see Table 3 for details).

Proportion of significant analyses

Of all the analyses, 12.0% were significant (see Table 5). For maternal prenatal cortisol measured before week 27 of pregnancy 6.7% were reported as significant, after 27 weeks, 13.8% were significant. When controlling for the quality of the studies, five papers were considered as high quality and could be included. Results showed that the proportion of significant analyses remained almost equal (12.8%). Interestingly, the period with the most significant analyses changed; 11.8% of the analyses were significant before week 27 of pregnancy, and 5.0% after week 27 of pregnancy.

Conclusions for psychological/behavioral outcome studies

To conclude, no evidence exists for the relation between cortisol in early and mid-gestation and children's temperament. Two papers did find a significant association when cortisol was measured during late gestation. Children from mothers with higher cortisol in late pregnancy showed higher levels of negative reactivity and emotion and activity.

Regarding the association between maternal prenatal cortisol and observed behavior and maternal reports of children's psychological problems, the evidence is also scarce. One paper found that higher maternal prenatal cortisol during early and mid-gestation was related to more affective behavior problems in 7-year-old children (girls), and one found an association between high maternal prenatal cortisol during late pregnancy and more negative observed behavior during the first weeks of life.

Maternal prenatal cortisol and cortisol outcomes in child

Overview results

Table 4 shows the empirical findings of the relation between maternal prenatal cortisol and infant cortisol outcomes.

Early and mid-gestation: weeks <27

Three papers examined the relation between maternal prenatal cortisol during early and mid-gestation and infant cortisol responses to a stressor. Davis et al. (2011) found that high maternal afternoon cortisol in weeks 20-27 predicted a larger increase in infant cortisol in response to a heel stick. This relation was strongest for maternal cortisol measured in week 25 of gestation. Additionally, maternal cortisol was related to elevated infant cortisol at the recovery of the heel-stick. Furthermore, higher maternal morning cortisol was related to higher cortisol concentrations in reaction to a vaccination in four-year-old children (Gutteling et al., 2004), and higher levels of cortisol during the first day at school of five-year-old children (Gutteling et al., 2005a).

Late gestation: weeks ≥27

Two papers examined the relation between maternal prenatal cortisol during late gestation and infant's cortisol response to a stressor. In line with the finding in early gestation, elevated maternal afternoon cortisol was associated with a larger increase in infant cortisol in response to the heel stick. However, maternal prenatal cortisol was not associated with the recovery of this stressor (Davis et al., 2011). Another paper examined the association of prenatal maternal evening cortisol and cortisol decline over the day and infant cortisol responses to four stressors during the first year of life. The maternal prenatal cortisol did not predict infant cortisol reactivity on the stressors (Tollenaar et al., 2011).

Confounders

Three of the four reported papers included confounders in the analyses, i.e. time of saliva sampling, age, gender, birth weight, prenatal smoking and alcohol use (Gutteling et al., 2004; Gutteling et al., 2005; Tollenaar et al., 2011). The paper of Davis et al. (2011) did not include possible confounding variables.

Measurement of cortisol

From the four papers on the relation between maternal prenatal cortisol and infant cortisol as an outcome variable, the *biological material* to measure cortisol concentrations was blood in one paper (Davis et al., 2011), and saliva in three papers (Gutteling et al., 2004; Gutteling et al., 2005a; Tollenaar et al., 2011)

With respect to *sampling* days, three of the four papers sampled at one day (Gutteling et al., 2004; Gutteling et al., 2005; Davis et al., 2011), and one paper used the mean cortisol concentration of two consecutive days (Tollenaar et al., 2011).

With regard to the *timing* of the cortisol measures, two papers used the morning samples (Gutteling et al., 2004; Gutteling et al., 2005a), one paper used afternoon samples (Davis et al., 2011), one paper used evening cortisol concentrations (Tollenaar et al., 2011), and two papers used the mean of the samples during the day (Gutteling et al., 2004; Gutteling et al., 2005a). The paper of Tollenaar et al. (2011) used both the raw evening sample and a composite score; the decline over the day. Significant relations were found for early morning and afternoon samples (see Table 4 for details).

Proportion of significant analyses

As can be seen in Table 5, 42.9% of the total analyses were significant. For maternal prenatal cortisol measured before week 27 of pregnancy 63.6% were reported as significant, after 27 weeks 20.0% was significant. All studies were of high quality, so additional analyses to control for the quality of the studies were not carried out.

Conclusions for cortisol outcome studies

Especially in early and midgestation, higher maternal cortisol was associated with higher cortisol responses to stressors in young children.

CONCLUSIONS, DISCUSSION AND DIRECTIONS FOR FUTURE RESEARCH

General conclusions

Numerous papers have found links between maternal prenatal stress and (negative) child outcomes. In these papers, prenatal stress was mostly measured with the use of questionnaires, and the authors frequently proposed heightened concentrations of maternal cortisol as the underlying mechanism linking maternal reports of stress/anxiety and child outcomes. To investigate whether there is evidence supporting this mechanism, we systematically reviewed empirical findings on associations between maternal prenatal cortisol concentrations and child outcomes. The results showed that 76% of the statistical analyses performed in the reviewed papers did not find a significant association between maternal cortisol and child

outcomes (only 57 out of a total of 237 statistical analyses -24%- were significant). Additional analyses on the subset of high quality studies produced virually identical results; 70.5% of the analyses were non-significant (38 out of a total of 129 statistical analyses were significant). However, most of the papers that did find significant associations found these in the expected direction: higher levels of maternal cortisol during pregnancy were related to altered child outcomes in the form of poorer physical/health outcomes (e.g. lower birth weight, shorter birth length, more respiratory and skin illnesses, and larger SVR and lower artery elasticity), lower cognitive/motordevelopment, more psychological/behavioral problems, and higher child cortisol concentrations.

We also examined whether critical gestational periods in which the fetus is more susceptible to maternal cortisol concentrations could be identified. No differences between gestational periods were found for the associations between maternal prenatal cortisol and physical infant outcomes (birth weight, gestational age). The associations between maternal prenatal cortisol and offspring health outcomes were only measured during late gestation, so it is not clear if cortisol concentrations during early/midgestation are also related to more health problems. For both infant cognitive development and child psychological/behavioral problems most evidence was found when maternal prenatal cortisol was measured during late gestation. In contrast, most of the papers on the association between maternal prenatal cortisol and child cortisol found significant relations when maternal cortisol was measured during early/midgestation. These findings show that for different child outcomes there may be different critical gestational periods in which the fetus is more susceptible to maternal cortisol concentrations.

Physical/health outcomes

Inspection of the papers finding a significant association between maternal prenatal cortisol and physical/health outcomes shows that higher maternal prenatal cortisol was related to lower birth weight/length, lower gestational age and more health problems.

The negative association between maternal prenatal cortisol and *birth weight/length* is in line with several studies that showed that the administration of synthetic corticosteroids to pregnant women was associated with lower birth weight. This suggests that although glucocorticoids are necessary for fetal growth, high levels of glucocorticoids may be detrimental for the fetus (Bloom et al., 2001; Ellman et al., 2008; French et al., 1999). Furthermore, a possible mechanism in the association between prenatal cortisol and lower birth weight could be that under stressful conditions, the fetus has to compete with the mother for resources required for coping and survival, leading to fetal growth restriction (Stearns 2005).

However, when the studies on the association between prenatal cortisol and birth weight/length that were included in the present review controlled for *gestational age*, the evidence for the association became scarce. This could mean that the gestational length was shorter for

the infants that were exposed to higher maternal cortisol concentrations, subsequently leading to lower birth weight/length. This is in line with previous findings showing that exposure to maternal stress hormones is associated with preterm delivery and shorter gestational periods (Sandman et al., 2006). Sandman et al. (2006) showed that high concentrations of cortisol in early pregnancy were related to elevations in the placental corticotrophin releasing hormone (CRH), and concluded that CRH is a mediating factor in the relation between prenatal maternal cortisol and gestational length. However, this is not in line with the findings from the present review. We found that the majority of the papers did not find a significant association between maternal prenatal cortisol and gestational age, suggesting that infants that were exposed to higher levels of maternal prenatal cortisol are not necessarily born at an earlier gestational age. This may be due to the fact that the women that were included in the papers were of healthy and non-clinical samples. These women may not reach the high cortisol concentrations needed for labor-inducing signals. Nonetheless, these results pinpoint the importance of controlling for gestational age when studying associations between maternal prenatal cortisol and birth weight/length, as it can be an important confounding variable.

The three papers that examined the relation between prenatal cortisol and children's *health* found that higher maternal prenatal cortisol was correlated with to more infant health problems (e.g. more respiratory and skin illnesses, and larger SVR and lower artery elasticity). However, to further unravel the association between prenatal cortisol and infant health more empirical studies are needed in this area.

The measurement of cortisol in the different papers showed large variability in biological material, diurnal timing of sampling, the use of raw vs composite scores, and sampling on single vs several days. The relatively small number of papers does not permit us to draw conclusions about the possible relations between cortisol measurement characteristics and child outcomes.

Cognitive/motor outcomes

The results of the papers with *cognitive development* as an outcome measure were not all in the same direction. While some papers found no relation between prenatal cortisol and cognitive development, others found that higher prenatal cortisol was related to lower cognitive development, and yet another showed a relation in the opposite direction.

In the three early/midgestation papers, one reported a positive relation between maternal prenatal cortisol and infant *cognitive development*, while the others found no links between both. During early and midgestation the placental barrier enzyme (11β-HSD2) protects the fetus from maternal cortisol (Duthie and Reynolds, 2013). This could explain why increases in maternal cortisol in this period of pregnancy are apparently not strongly related to fetal cognitive development.

Most evidence for a relation between maternal prenatal cortisol and infant cognitive development was found when maternal prenatal cortisol was measured around week 37 of pregnancy. Of four papers, two papers found that higher levels of maternal cortisol were related to lower infant cognitive development, one to higher, and one found no relation. During this third trimester of pregnancy, there is a decline in placental 11β-HSD2 activity (Murphy et al., 2006) which results in an increase in fetal exposure to cortisol. This increase in exposure to cortisol is necessary for fetal organ maturation (Howerton and Bale, 2012). However, while moderate increases in glucocorticoids facilitate brain development and behavioral regulation, excessive exposure to glucocorticoids causes neural degeneration (Kapoor et al., 2006; Scaccianoce et al., 2001). Especially the limbic system is sensitive to glucocorticoids. The hippocampus is part of the limbic system and plays a central role in cognition, behavior and memory (Andrews and Matthews, 2003). The hippocampus develops during gestation and the anatomy begins to be similar to the adult hippocampus by 18 to 20 weeks of gestation (Kier et al., 1997). Although in humans little is known about the ontogeny of glucocorticoid receptors in the brain, a study by Noorlander et al. (2006) showed that the receptors are present in the fetal hippocampus from week 24 of gestation on. Hence, the increased exposure to cortisol could result in negative effects on brain and behavioral development, explaining the results of the two papers that found a relation with lower cognitive development.

The paper that found positive effects on cognitive development (Davis et al., 2010) had similar populations and methodology than the other three papers, with the exception that the infants were tested at 12 months instead of the 3-8 months, or 7 years in the remaining papers. More research and replication studies are necessary to further investigate this apparent contradiction to the other studies.

For infant *motor development* as an outcome measure, most papers did not find an association between maternal prenatal cortisol and infant motor development. An explanation for this could be that the brain areas that are involved in motor development are not that vulnerable for glucocorticoids (Davis et al., 2010).

Psychological/behavioral outcomes

A few papers (2 out of six, 7% of the total analyses) showed a relation between higher maternal prenatal cortisol during early and midgestation and more maternal *reported behavior problems* (affective problems) of infants. This finding is in line with the results of animal studies showing that primates that were exposed to high levels of glucocorticoids were more irritable and showed more disturbance behavior in response to novel situations (Schneider et al., 1992). This could be due to glucocorticoids affecting the development of neural systems, including brain limbic regions, which are regions involved in regulating fear and behavioral inhibition (Matthews 2002; Andrews and Matthews, 2003). Although the development of the glucocorticoid

receptors is species-specific, and little is known about the ontogeny of the receptors in humans (Matthews 2002), there are studies in humans that are in line with these animal studies. Trautman et al. (1995) showed that children that were exposed to dexamethasone during early gestation - because they were at risk for congenital adrenal hyperplasia - showed higher levels of shyness and emotionality, less sociability and more internalizing and total behavioral problems (Trautman et al., 1995). Furthermore, Buss et al. (2012) found a significant relation between maternal prenatal cortisol during early gestation and a larger right amygdala volume in girls at age 7 (Buss et al., 2012). The amygdala is part of the limbic region and is involved in the regulation of fear, depression and anxiety. These results suggest that already in early gestation maternal glucocorticoids may have long-lasting effects on offspring brain and behavior development.

However, the majority of the papers on the link between maternal prenatal cortisol during early and midgestation and children's behavior/psychological problems did not find a significant relation. This could be explained by the difficult translation from animal to human research. While in the animal models artificial glucocorticoids were given during pregnancy, the human studies included in this review looked at pregnancy concentrations of naturally produced cortisol. In the healthy populations that mostly participate in these longitudinal studies, the cortisol concentrations may not reach extremely high levels and therefore not be comparable to those of the animal models. Furthermore, the ontogeny of the glucocorticoid receptors in the brain is species dependent (Matthews 2002), which makes it difficult to compare animal and human studies.

Although evidence for the link between maternal prenatal cortisol and children's psychological/behavioral problems is scarce, more evidence is found when cortisol is measured in late pregnancy (14% of the analyses). This is in line with the findings on the association between prenatal cortisol and infant cognitive development. Again, it could be that increases in cortisol concentrations affect fetal brain development during the last trimester of pregnancy. That this is especially true for late pregnancy could be caused by the decreased activity of the 11β-HSD2 enzyme (Murphy et al., 2006), leading to more exposure to cortisol in the fetus. However, in the high quality studies, more evidence is found when cortisol is measured in early pregnancy (11.8% of the analyses) than in late pregnancy (5% of the analyses).

Cortisol outcomes

All three papers on the relation between maternal prenatal cortisol during early and midgestation and child cortisol (accounting for 64% of the total analyses) showed that higher prenatal cortisol concentrations are related to higher cortisol concentrations in the child. During late gestation, one of two papers (accounting for 20% of the total analyses), found a significant positive association.

During fetal development the HPA axis is developing, and is susceptible to environmental influences. The key limbic regulator in the HPA axis is the hippocampus, a brain region which is particularly sensitive to glucocorticoids during development (Harris and Seckl, 2011) From 24 weeks of gestation on, the cortisol receptors (mineralocorticoid and glucocorticoid) are present in the hippocampus, making this period of gestation a susceptible period to excessive levels of cortisol (Noorlander et al., 2006). Furthermore, as described before, the activity of the placental 11β-HSD2 enzyme decreases in the second half of gestation, hence protecting the fetus less from maternal cortisol (Murphy et al., 2006). This suggests that especially during late gestation the fetus would be more susceptible to maternal cortisol. However, in our review the apparent effects of maternal cortisol are visible during both early/midgestation and late gestation. A possible explanation for the relation found between maternal cortisol in early/midgestation and infant cortisol could be the heritability of the HPA axis functioning. During early/midgestation maternal cortisol has not yet increased as a result of pregnancy, and therefore the individual differences in maternal genetically based HPA axis functioning may be more clearly visible. This advocates studying genetic factors related to HPA axis functioning in the relation between prenatal maternal physiological stress responses and child HPA axis functioning. In the second half of pregnancy cortisol concentrations increase to such high levels that a ceiling effect may impede detection of individual differences.

General limitations and recommendations

In this section we discuss general limitations of the papers included in this systematic review, and make recommendations for future studies. In Table 6. the limitations and recommendations are summarized.

Confounders

The majority of the papers included confounders in the analyses. However, because of the heterogeneity of the confounders that were included in the analyses, it is hard to compare studies. To increase the comparability of future studies, presenting the results for the analyses with and without confounders is recommended (Simmons et al. 2011). In Table 7. the most important confounders per outcome variable are presented, so that future studies may include these in a standard fashion in their research designs and analyses.

Moderators

A possible explanation for not finding more associations between maternal prenatal cortisol and infant outcomes is that variables that moderate the associations are often not taken into account. We suggest including *fetal sex* as a moderator when examining the relations between maternal prenatal cortisol concentrations and infant outcomes. A recent review article provided evidence that female fetuses may be more susceptible to the effects of prenatal cortisol and placental CRH for the development of affective problems (Sandman et al., 2013). This could also explain why Buss et al. (2012) only found significant results for the association between prenatal cortisol and affective problems and right amygdala volume in girls. Also, DiPietro et al. (2011) observed that women who were pregnant of male fetuses had higher cortisol concentrations from week 24 to week 30 of pregnancy. After gestational week 30 the results were the opposite: mothers who were pregnant of female fetuses showed higher cortisol concentrations compared to women who carried male fetuses. These results highlight the importance of including fetal sex as a possible moderator in future research.

Another potential moderator that may be interesting to consider in future research is the *placental functioning* of the 11β-HSD2 enzyme. If the placental barrier functioning is not working optimally, the fetus will be less protected from maternal cortisol concentrations. A study by O'Donnell et al. (2012) showed that the placental 11β-HSD2 enzyme expression can be related to maternal anxiety. Mothers who were more anxious during pregnancy showed decreased 11β-HSD2 expression. This reduced barrier function of the placenta probably leads to higher exposure of the fetus to maternal cortisol. This highlights that not only high levels of maternal cortisol concentrations could result in increased fetal levels of cortisol, but also variations in placental functioning could be related to individual variation in fetal cortisol exposure. This underlines the importance of including the placental barrier functioning to cortisol as a possible moderator in future research on the relation between maternal prenatal cortisol and child outcome.

Heterogeneity of the cortisol measures

One of the main limitations of the literature on the associations between maternal prenatal cortisol and child outcomes is the heterogeneity of the cortisol measures. This heterogeneity lowers the comparability and makes it hard to draw general conclusions. Regarding the measurement of maternal prenatal cortisol, studies use different numbers of sampling days (e.g. 1, 2 or 3 days), different sampling times during the day, and are not consistent in the use of morning, afternoon or evening values. Furthermore, studies vary in their choice of composite scores (e.g. decline over day, CAR, or total output AUC) and in the way in which they create composite scores. For example, the CAR can be calculated as the difference score between awakening and 30 minutes after awakening, or as the area under the curve with the wakening sample as baseline. Other studies include the ratio between the sample taken 30 minutes after awakening and the awakening sample (Adam and Kumari, 2009). Similarly, the cortisol decline

over the day is measured as the evening sample minus the wakening sample, or as the evening sample minus the 30 minutes post awakening sample (Adam and Kumari, 2009). Next to the CAR and the decline over the day, the AUC can provide unique information about the overall cortisol secretion during the day. The AUC is not strongly correlated with the diurnal slope and can be calculated in two ways, namely by including or by excluding the CAR (Adam and Kumari, 2009; Pruessner et al., 2003).

One way in which researchers can increase the comparability of their studies despite using different sampling times and composite scores, is by always including the raw mean values and standard deviations of the cortisol assessments. Next to reporting these raw values, the correlations between these values and the outcome measures should be reported to give more insight and increase the comparability between studies. Harville et al. (2007) showed that a single measure of cortisol has a low correlation (0.1) with the AUC of 15 measures of cortisol during a day. They recommend, with the goal of reducing the financial costs and increasing the compliance of participants, collecting cortisol samples 5 times during a day (wakening, wakening + 30 minutes, 11.00 am, 5.00 pm, 9.00 pm) or using 2 time points (30 minutes post-awakening, and 9:00pm) on three days. Both measurement options would be highly correlated with the AUC based on 15 assessments during one day. However, because there is intra-individual variation in cortisol values (in pregnant women especially for the morning value, Harville et al., 2007), we recommend measuring cortisol values on at least two different days. Hellhammer et al. (2007) suggest measuring the cortisol rise after awakening on two workdays, because this would reflect trait cortisol concentrations, whereas weekend days would represent state levels. In sum, for future research on the relation between the basal maternal cortisol values and child outcomes, cortisol should be measured on at least two different days, and at least at two time points (e.g. 30 minutes post-awakening, 9:00pm), and raw values should always be reported.

Maternal cortisol was measured using different biological material. Blood, saliva, and urine (one paper) were used in the papers with physical/health outcomes. The papers using other outcome variables used mainly salivary cortisol measures. There was no clear relation between the different biological materials that were used and the outcome variables. Although free cortisol in saliva is 10-35% lower than it is in blood, salivary cortisol is highly correlated to unbound free plasma cortisol levels (Levine et al., 2007; Nicolson et al., 2008). However, because corticosteroid binding globulin (CBG) levels rise during weeks 10-20 of gestation (Levine et al., 2007), the relation between total blood concentrations and salivary cortisol concentrations is non-linear during pregnancy (Hellhammer et al., 2009). This reduces the comparability of pregnancy studies using blood cortisol with those using saliva cortisol.

Gestational timing

To examine if there are susceptible gestational periods for the relation between prenatal maternal cortisol and the outcome variables, most papers measure cortisol during several

trimesters and perform the same analyses for the different gestational intervals. By Doing this, they are missing the opportunity of studying how patterns of cortisol secretion throughout pregnancy may be related to child outcome. Only a few studies also take pregnancy changes in cortisol secretion into account in their analyses (Davis et al., 2010; Davis et al., 2011). The HPA axis shows substantial changes during pregnancy, and Davis et al. (2010), for example, found that low concentrations of maternal prenatal cortisol during early pregnancy in combination with high concentrations of maternal cortisol during late pregnancy were related to enhanced infant cognitive development. In addition, they found that an accelerated increase in cortisol during gestation was related to higher cognitive development. Hence, the slope of the maternal cortisol concentrations over pregnancy was more predictive than the separate cortisol concentrations of early or late pregnancy. These authors suggest that it may be trajectory of change in maternal prenatal cortisol that is the most predictive of the infant development.

Until there are more studies that have examined several relevant gestational intervals in the same cohort, and use all cortisol measurements in one analysis (e.g. trajectories with variants of growth curves), our knowledge about the gestational timing effects of maternal cortisol must be considered incomplete.

Statistical analyses

Another limitation that makes it hard to compare studies is that different statistical analyses are used to study the relation between maternal prenatal cortisol and child outcomes (e.g. ANOVA, regression analyses, multilevel models). For analyzing repeated measures of cortisol we suggest the use of multilevel models. The advantages of these analytical models are that different sources of variance can be separated, within subject associations can be analyzed, and the method does not exclude cases that have some missing data points (Kudielka et al., 2012). When using a composite measure of maternal prenatal cortisol as a predictor, regression analyses can be used. In these analyses confounders can be included, and the specific contribution of specific confounders in the variation of the outcome variable can be analyzed (Tabachnick and Fidell, 2013). For analyzing trajectories of change in cortisol concentrations during pregnancy we recommend to use growth curve analyses (Davis et al., 2010; Davis et al., 2011).

A related problem is that of the large number of statistical analyses that are performed in each study (in the papers in this review a mean of 8.5 analyses per paper). This greatly increases the chance of obtaining false positive results (Simmons et al., 2011). Although the relatively limited research in the field of maternal prenatal cortisol to date has often necessarily pushed researchers to assume a more exploratory approach to data analysis, future studies should take measures to diminish the number of analyses and hence the danger of false positive results. This can be done by setting up a statistical analysis plan beforehand, and by using multivariate analysis techniques. Furthermore, more conservative p-values can be used to indicate statistical significance (e.g., $p<0.01$) or use a correction for family-wise error (e.g.,

Bonferroni correction). Statistical reporting should consist of the results of these planned, main analyses (with and without confounders, see 4.6.1), and eventually, of a priori contrasts and posthoc exploratory analyses that were undertaken after carrying out the original statistical plan. A clear distinction between confirmatory hypothesis testing and exploratory analyses should be present in the text.

Maternal prenatal cortisol: mediator between prenatal stress and infant outcomes?

The results of this systematic review provide weak evidence for a relation between maternal prenatal cortisol and children's psychological/behavioral problems (12% of the analyses were significant). The relations between maternal prenatal cortisol and other outcome variables (health, cognitive development, infant cortisol) appear to be stronger. However, whether maternal prenatal cortisol is the main mediator in the often found relations between maternal psychosocial prenatal stress and infant outcomes is as yet unclear. In several of the papers included in this review, correlations were found between reported maternal prenatal stress/anxiety and cortisol concentrations (positive: Diego et al., 2009; positive or non-significant: Beijers et al., 2010; Tollenaar et al., 2011; negative or non-significant: Davis et al., 2010). However, the majority of the analyses of the papers that were included in this review did not find a significant association between maternal prenatal stress, anxiety and depression measured with questionnaires, and maternal prenatal cortisol concentrations (Beijers et al., 2010; Bolten et al., 2011; D'Anna Hernandez et al., 2012, Davis et al., 2007; Davis et al., 2010; Davis et al., 2011; Davis et al., 2012; Goedhart et al., 2010; Gutteling et al., 2006; Gutteling et al., 2007; Ruiz et al., 2001; Tollenaar et al., 2011). This is in line with a recent study of Voegtline et al. (2013) which found that there is no association between self reported maternal psychological distress and well-being with salivary cortisol measured after week 24 of gestation. This highlights the importance of being cautious in suggesting maternal prenatal cortisol as the sole or even main mediator in the relation between self-reports of maternal stress/anxiety and infant outcomes. The question then is how the often found links between maternal reports of prenatal stress/anxiety and psychological and behavioral problems in children can be explained if not through cortisol? One possibility is that mothers who report experiencing high stress/anxiety during pregnancy may be biased in their self-reports, or in their reports of their children's behavior, or even in both. Another possibility could be the infant's genetic inheritance of the anxious behavior of their mothers. Furthermore, perceived stress could influence a mother's lifestyle during pregnancy (e.g. sleeping and eating behavior), and this in turn could influence fetal development (Monk et al., 2013; Hung et al., 2013). In sum, although it is often suggested that maternal prenatal cortisol is an important mediator between prenatal perceived stress and child outcomes, the results of the present review do not offer strong support for this

hypothesis. In this context it is important to note that we base this conclusion on maternal cortisol as measured with the markers used in the reviewed papers (e.g. CAR, diurnal slope, AUC). We cannot exclude the possibility that other markers of cortisol physiology may be more strongly linked to maternal psychological stress/anxiety and hence more relevant for fetal programming processes. For example, in a recent study of Kane et al. (2014) the relation between the trajectory of changes in cortisol concentrations during pregnancy were examined in relation with pregnancy anxiety. These authors found that higher mean levels of pregnancy anxiety from mid to late pregnancy was related to steeper increases in cortisol secretion over pregnancy. This highlights the importance of taking other cortisol markers into account. Possible additional markers that may be interesting to explore in the future may be maternal cortisol reactivity to daily or laboratory stressors, maternal hair cortisol concentrations and maternal nocturnal cortisol concentrations.

Although from this review we may conclude that maternal prenatal cortisol need not be the sole or even main underlying mechanism in the relation between maternal reported prenatal stress and child outcomes, there are indications that maternal prenatal cortisol is related to certain (altered) child outcomes. This, together with the limitations of the reviewed papers, leads us to further conclude that the link between maternal prenatal cortisol and child outcomes merits attention in future studies. Nonetheless, research including other potential mechanisms underlying the link between maternal prenatal stress/anxiety and child outcomes, as well as the inclusion of important confounding variables and promising moderator variables, is highly recommended. These types of future studies will give us more insight in how and when maternal prenatal cortisol may affect fetal development, helping us further unravel the complex mechanisms between maternal prenatal stress/anxiety, maternal prenatal cortisol, and child outcomes.

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Table 1. Characteristics and results of studies on the association between maternal prenatal cortisol and infant physical/health outcomes

Reference	Independent variable, biological material	Timing: Mean weeks of pregnancy (SD)	Child's age: Mean (SD)	Dependent variables, Instrument.	Number of participants included in analyses	Statistical analyses, confounders	Results
Beijers et al. 2010	Evening cortisol: 9:00 pm, mean 2 days. Decline over the day: waking minus 9:00 pm, mean 2 days Material: Saliva	37 weeks (9.4 days)	12 months (-)	Respiratory, digestive, general, and skin illnesses and complaints and antibiotic sum 12 months. Instrument: Classification of primary care, monthly interviews.	169	Multiple hierarchical regression model. Confounders: duration of breastfeeding, child care, nr of siblings, daily hassles postpartum, state anxiety postpartum	Respiratory illnesses related to smaller cortisol decline β=-.27, p<.01 General illnesses related to higher evening cortisol β=.14, p<.10 Skin illnesses related to higher evening cortisol β=.235, p<.01 Digestive and antibiotic use: n.s. related to cortisol.
Bolten et al. 2011	AUCg CAR: awakening, 30, 45 and 60 minutes after awakening. Material: saliva	Weeks 13-18 (-) Weeks 35-37 (-)	Birth (-)	Birth weight, body length, head circumference.	70	Multiple hierarchical regression analyses Confounders: maternal age, parity, pre-pregnancy BMI, smoking, infant's sex, gestational age, prenatal distress, perceived stress.	Birth weight related to lower CAR early pregnancy β=-.29, p<.05 and late pregnancy β=-.30, p<.01 Body length not related to CAR early pregnancy and related to lower CAR late pregnancy β=-.28, p<0.05 Head circumference n.s. related to CAR in early and late pregnancy

D'Anna-Hernandez et al. 2012	Slope diurnal decline: 30 min after awakening – 4:00pm. Mean of 3 days. Material: saliva	Week 17.3 (1.8) Week 28.1 (1.5) Week 34.3 (1.4)	Birth (-)	Infant birth weight, gestational age at delivery. Derived from: chart review.	55	Correlation analyses Confounders: -	Lower infant birth weight related to lower diurnal slope, in late pregnancy r=-.29, p=.05 n.s. related in early pregnancy or midgestation. Gestational age n.s. related to diurnal slope in all periods.
Diego et al. 2009	Midmorning Material: urine	Week 19.0 (0.8)	Birth (-)	Gestational age at birth. Derived from: medical charts.	40 depressed 40 non-depressed	Stepwise regression analyses Confounder: prenatal depression	Significant relation between maternal prenatal cortisol and gestational age at birth after controlling for the effects of prenatal depression b=-.008, p<.001.
Ellman et al. 2008	Afternoon Material: blood	Week 15.10 (1.22) Week 19.20 (0.94) Week 24.85 (0.97) Week 31.00 (0.88).	8.9 hr after birth (7.94 hr).	Physical and neuromuscular maturation. Instrument: New Ballard Maturation Score.	158	Multiple linear regression model. Confounders: infant sex, infant's age at examination, parity, caesarian-section delivery, maternal age at delivery, ethnic/ racial categories.	Week 15 + 19: increases cortisol significantly related with decreases in infant physical and neuromuscular maturation, with (β=-.44, p=.000, β=-.185, p=0.026) and without controlling for length of gestation (β=-.409, p=0.00, β=-.139, p=0.047). Week 25: n.s. Week 31: positive significant relation without controlling for gestational length (β=.179, p=0.04).

Reference	Independent variable, biological material	Timing: Mean weeks of pregnancy (SD)	Child's age: Mean (SD)	Dependent variables, Instrument.	Number of participants included in analyses	Statistical analyses, confounders	Results
Erickson et al. 2001	- Material: blood.	Week 16 (-) Weeks 27-37 (-)	Birth (-)	Gestational age at birth.	59 preterm 166 term	Wilcoxon signed rank sum test. Confounders: -	<24 weeks: no significant difference between term and preterm birth 27-37 weeks: cortisol concentrations were higher in preterm than in the term group (p=0.001).
Goedhart et al. 2010	Sample ranged from 08:00 to 19:25h. Estimated cortisol value at 08:00-09:00h and at gestational age of 40-83 days. Material: blood.	Week 13 (-)	Birth (-)	Birthweight and small for gestational age (SGA). Derived from: Youth Health Care registration at Public Health Service.	2810	Birthweight: linear regression analyses. Confounders: gestational age, infant gender, ethnicity, maternal age, parity, BMI, smoking SGA: logistic regression analyses. Confounders: ethnicity, maternal age, BMI, smoking.	Birthweight: maternal cortisol negatively associated with birthweight β=-.35, p<.001. After controlling confounders n.s. SGA: only univariately, an increasing cortisol concentration was associated with increasing risk for SGA (OR = 1.00, p=.027).
Mercer et al. 2006	- Material: blood.	Week 22-24 (-)	Birth (-)	Gestational age: recurrent spontaneous preterm birth (rSPBs), isolated preterm birth (iSPBs), recurrent term births (rTBs).	46 rSPBs 92 iSPBs (32 current and 60 prior iSPBs) 92 rTBs.	Wilcoxon rank-sum test or Kruskal-Wallis test. Confounders: -	Maternal cortisol was significantly different between groups. Median levels increasing with worsening pregnancy outcomes (p=.001).

Rondó et al. 2010	Mean of 9 measurements: 3 days between 8:30 and 09:00 am. Material: saliva	Weeks 35.97 (4.85).	Range 5 years and 4 months -7 years and 6 months	Systemic vascular resistance (SVR). Instrument: HDI/Pulse Wave CR-2000 Research CardioVascular Profiling System.	130	Spearman correlation and Multivariate linear regression analysis. Confounders: maternal BMI, BMI-z score, birth weight, age and gender of the children.	SVR: positive correlation between maternal cortisol and SVR (r=0.23, p=0.01). Significant positive association between maternal cortisol and SVR (β=18.04, p=0.043), controlling for confounders.
Rondó et al. 2011	Mean of 9 measurements: 3 days between 8:30 and 09:00 am. Material: saliva	Weeks 35.97 (4.84).	Range 5 years and 4 months -7 years and 6 months	Large artery elasticity index (LAEI). Instrument: recording radial artery pulse wave.	130	Univariate and multivariate linear regression analysis. Confounders: birth weight, age, BMI, and HDL-c of the children.	Negative statistically significant associations between LAEI and maternal cortisol by univeriate and multivariate analysis (β=-0.05, p=0.02).
Ruiz et al. 2001	- Material: blood.	4 measurements assigned to 5 periods: Weeks 15-19 (-) Week 20-22 (-) Week 23-26 (-) Week 27-30 (-) Week 31-35 (-)	Birth (-)	Preterm Labor (PTL) Preterm birth (PTB) Gestational age Derived from: chart review of outpatient and delivery records.	76	Two-way analysis of variance (ANOVA). Post hoc student t-test. Correlations. Confounders: -	No significant difference between the PTB, PTL, and term groups on cortisol concentrations at any of the gestational periods. Cortisol concentrations at any gestational stage did not correlate with gestational age at birth p>.69.

Reference	Independent variable, biological material	Timing: Mean weeks of pregnancy (SD)	Child's age: Mean (SD)	Dependent variables, Instrument.	Number of participants included in analyses	Statistical analyses, confounders	Results
Van Dijk et al. 2012	Cortisol concentrations were standardized by regressing time of day and gestational age at sampling. Estimated cortisol value at the median gestational age of assessment and at time of day between 8:00-9:00 am were calculated. Material: blood.	Median 13 weeks; IQR 11-15	Age 5.6 years (0.4)	BMI WHtR (waist to height ratio) FMI (Fat Mass Index)	1320	Linear regression analyses + interaction with sex. Confounders: maternal age, pre-pregnancy BMI, educational level, smoking, alcohol consumption, hypertension, primiparity, ethnicity, exclusive breastfeeding.	Cortisol was in the crude nor the adjusted model significantly associated with BMI or WHtR. Higher maternal cortisol was independently associated with marginally higher FMI in girls, but marginally lower FMI in boys

n.s. = not significant

AUCg = area under the curve with respect to the ground

CAR = cortisol awakening response

IQR = interquartile range

Table 2. Characteristics and results of studies on the association between maternal prenatal cortisol and infant cognitive and motor outcomes

Reference	Independent variable, biological material	Timing: Mean weeks of pregnancy (SD)	Child's age: Mean (SD)	Dependent variables, Instrument.	Number of participants included in analyses	Statistical analyses, confounders	Results
Buitelaar et al. 2003	Early morning cortisol concentration (08:00 a.m.) and mean cortisol concentration of seven samples during the day between 08:00 a.m. and 08:00 p.m.). Material: saliva	Weeks 15–17 (-) Weeks 27-28 (-) Weeks 37–38 (-)	3 and 8 months after birth (mean and SD not reported)	Mental development (MDI) and psychomotor development (PDI). Instrument: Bayley Scales of Infant Development (BSID)	170	MANCOVA, MANOVA, multiple regression analyses. Confounders: gestational age at birth, birth weight and the postnatal stress and depression level of the mother	High cortisol (8 am) in late pregnancy related to lower MDI scores at 3 months of age ($F = 6.38$, $P < 0.05$) and lower PDI scores at both 3 and 8 months of age ($F = 9.15$, $P < 0.005$; and $F = 9.38$, $P < 0.005$). Linear negative effect of cortisol determined at 8 a.m. in late pregnancy on the MDI -scores at 3 months ($F = 7.19$, $\beta = -0.31$, $R2 = 0.10$, $P < 0.01$) and the PDI scores both at 3 and 8 months of age ($F = 5.16$, $\beta = -0.28$, $R2 = 0.14$, $P < 0.01$ and $F = 7.08$, $\beta = -0.28$, $R2 = 0.08$, $P < 0.01$).

Reference	Independent variable, biological material	Timing: Mean weeks of pregnancy (SD)	Child's age: Mean (SD)	Dependent variables, Instrument.	Number of participants included in analyses	Statistical analyses, confounders	Results
Davis et al. 2010	Sample taken at early afternoon. Material: saliva.	Week 15.1 (0.89) Week 19.1 (0.93) Week 25.4 (0.95) Week 30.8 (0.69) Week 36.6 (0.61)	3.1 months (0.27) 6.2 months (0.30) 11.9 months (0.30)	Mental Developmental Index (MDI) and Psychomotor Development Index (PDI). Instrument: Bayley Scales of Infant Development	125	Hierarchical linear modeling (HLM). Confounders: infant GA at birth, maternal race, birth order, prenatal medical risk, postnatal measures of psychological distress.	Lower maternal cortisol in week 15 and higher maternal cortisol in week 37 predicted accelerated MDI across first postnatal year, resulting in enhanced cognitive functioning at 12 months of age. Prenatal cortisol in weeks 19, 25, 31 not related to MDI. A larger slope of maternal cortisol during gestation related to higher MDI scores (ΔR^2=0.06, β=0.24, t=2.8, p<0.01) No cortisol measures related to PDI.
Huizink et al. 2003	Early morning cortisol concentration (08:00 a.m.) and mean cortisol concentration of seven samples during the day between 08:00 a.m. and 08:00 p.m.). Material: saliva.	Weeks 15–17 (-) Weeks 27-28 (-) Weeks 37–38 (-)	at 3 and 8 months after birth (mean /SD not reported)	Mental Developmental Index (MDI) and Psychomotor Development Index (PDI). Instrument: Bayley Scales of Infant Development	170	MANCOVA Confounders: gestational age at birth, birth weight, and the postnatal stress and depression level of the mother.	High cortisol in late pregnancy related to lower MDI scores at 3 months of age (F = 6.38, P < 0.05) and lower PDI scores at both 3 and 8 months of age (F = 9.15, P < 0.005; and F = 9.38, P < 0.005). Cortisol in early and mid-pregnancy did not show overall significant effects on infant development.

Gutteling et al. 2007	Samples at 08:00 a.m. Material: saliva	Weeks 15-17 (-) Weeks 27-28 (-) Weeks 37-38 (-)	6.7 years (0.7 years).	Handedness. Instrument: children were observed while they pretended to perform activities.	110	Logistic regression analyses. Confounders: maternal smoking and use of alcohol, social economic status, birth weight, gestational age, maternal age, maternal mixed-handedness, paternal mixed-handedness, child's sex.	N.s. effects of maternal cortisol on child mixed-handedness were found.
LeWinn et al. 2009	Time of day at which samples were taken was not recorded. Material: serum	Week 31-36 (-)	7 years	Childhood IQ; total, verbal, performance. Instrument: Wechsler Intelligence Scale for Children (WISC).	832	Linear regression models. Confounders: maternal work status, maternal education, child race and sex, maternal age, single motherhood.	Children with cortisol exposure in the highest quintile had full scale IQ scores 2.78 points lower than those in the lowest quintileof exposure. This association was most pronounced for the verbal subscale. No significant relations were observed between cortisol concentration and performance IQ. Across outcomes, the addition of covariates slightly reduced the magnitude of the effect estimates. Within sibling pairs: being in the highest quinitile of exposure was associated with lower verbal IQ scores.

Reference	Independent variable, biological material	Timing: Mean weeks of pregnancy (SD)	Child's age: Mean (SD)	Dependent variables, Instrument.	Number of participants included in analyses	Statistical analyses, confounders	Results
Gutteling et al. 2006	Samples at 08:00 a.m. Material: saliva	Weeks 15-17 (-) Weeks 27-28 (-) Weeks 37-38 (-)	6.7 years (8.4 months)	Learning and memory functioning. Instrument: Test of Memory and Learning (TOMAL).	112	Hierarchical multiple regression analyses. Confounders: IQ, child's gender, maternal prenatal smoking, postnatal stress.	No significant associations were found for maternal prenatal cortisol concentration in each period and learning and memory functioning.

n.s. = not significant

Table 3. Characteristics and results of studies on the association between maternal prenatal cortisol and infant psychological and behavioral outcomes.

Reference	Independent variable, biological material	Timing: Mean weeks of pregnancy (SD)	Child's age: Mean (SD)	Dependent variables, Instrument.	Number of participants included in analyses	Statistical analyses, confounders	Results
Buss et al. 2012	Cortisol concentrations were adjusted for time of day (mean 2:00 PM). 3 cortisol measures: 1. cortisol at 15 wk gestation. 2. AUC wk 15-28. 3. AUC wk 28-37. Material: saliva	15 weeks (1.0 wk) 19 weeks (0.8 wk) 25 weeks (0.9 wk) 31 weeks (0.9 wk) 37 weeks (0.7 wk)	7.5 years (0.9 y)	Affective problems. Instrument: Child Behaviour Checklist (CBCL) Amygdala volume Hippocampus volume	65	Regression models. Confounders: presence of obstetric complications, duration of gestation, birth weight percentile, maternal depression at child follow-up, and child sex, age at testing, and handedness.	Girls: Higher maternal cortisol at wk 15 related to larger right amygdala volumes (1.06 ± 0.46; P = 0.02, q=0.025), but n.s. related to left amygdala volume or right or left hippocampus volume. Boys: maternal cortisol concentrations at 15 wk not associated with left or right amygdala volume. Trend for higher maternal cortisol in wk 15 being associated with smaller left (-1.18 ± 0.61, p=0.06) and right hippocampal volume (-1.13 ± 0.66, P = 0.09). Second and third trimester cortisol n.s. related to child brain volumes. Higher maternal cortisol at wk 15 related to more affective problems among girls, but not in boys. Second and third trimester cortisol n.s. related to affective problems.

Reference	Independent variable, biological material	Timing: Mean weeks of pregnancy (SD)	Child's age: Mean (SD)	Dependent variables, Instrument.	Number of participants included in analyses	Statistical analyses, confounders	Results
Davis et al. 2007	Early afternoon (mean 14:20, SD=1.5 hours) Material: saliva	Week 19.1 (0.8 wk) Week 24.9 (0.84 wk) Week 30.8 (1.0 wk)	8 weeks (2.1)	Negative reactivity. Instrument: Infant Behavior Questionnaire (IBQ, fear subscale).	247	Partial correlation. Confounder: time of sampling Hierarchical regression. Confounders: postnatal maternal psychological state, average prenatal maternal depression.	Maternal cortisol in late gestation (wk 30-32), but n.s. earlier in pregnancy, related to infant negative reactivity (partial r_{243}=0.20, p<.01). After controlling for maternal postnatal psychological state, maternal cortisol (ΔR^2=0.05, β=.24, t=3.3, p<.01) independently predicted report of infant negative reactivity.
Davis et al. 2011	Sample taken in the afternoon (range 13:19 to 13:30). Material: plasma	Week 15.1 (0.9) Week 19.3 (1.0) Week 25.4 (0.9) Week 30.9 (0.69) Week 36.5 (1.2)	23 hrs 47 min (4 hrs 13 min)	Behavioural response (arousal) to stressor: response delta + recovery delta. Instrument: observation during heel stick.	116	Hierarchical linear modeling (HLM). Confounders: sociodemographic factors (marital status, race/ethnicity, education, household income), medical risk (prenatal medical risk, parity, maternal age, mode of delivery), infant factors (gestational age, birth weight, sex, Apgar score, postnatal age), aspect heel stick procedure. Only time of maternal sample collection included in analyses	Infant behavioral state during the stressor was n.s. associated with maternal prenatal cortisol. Pattern of maternal cortisol across gestation predicted infant behavioral state during the recovery period: elevated maternal cortisol concentrations at early pregnancy (especially weeks 13-14) were associated with higher infant behavioral arousal during recovery.

Davis et al. 2012	Mean of the samples collected in early afternoon (mean ranged from 14:17 to 14:46 across the three assessments). Material: saliva	Week 19.3 (0.8) Week 25.0 (0.8) Week 31.0 (0.8)	7.3 years (0.8)	Anxiety problems. Instrument: Child Behaviour Checklist (CBCL).	178	Hierarchical linear regression analyses and logistic regression. Confounders: gestational age at birth, maternal current psychological state, maternal education, child sex.	Elevated average maternal gestational cortisol was associated with higher child anxiety (β=0.16, $p<0.05$; $F_{4,167}$=6.7, $p<0.05$). N.s. gestational timing effects of cortisol exposure were observed. Children with anxiety ratings within borderline/clinically significant range were twice as likely to have been exposed to higher maternal cortisol during gestation (odds ratio=2.1, 95% conf interval = 1.1-3.9, $p<0.05$).
de Weerth et al. 2003	Mean of 2 awakening sample (range 07:30 and 10:00). Regressed against time of day. Median residuals = dividing subjects into high en low cortisol groups. Material: saliva	Week 36 (-) Week 37 (-)	Behavior: Week 1, 3, 5, 7, 18, 20 (-) Temperament: week 7, 18 (-)	Behavior. Instrument: observations during bath. Temperament: emotion, adaptation, activity. Instrument: Infant Characteristics Questionnaire (ICQ).	17	Mann-Whitney U-tests. Confounders: -	Crying, fussing, negative facial expression and no vocalization were significantly different between high and low prenatal cortisol group. Infants from the higher cortisol group were more irritable and showed more negative affect during the bathing sessions than those from the lower cortisol group. Differences were largest in weeks 1-7. Infants from the high prenatal cortisol group had significantly higher scores on emotion and activity in week 7, but not in week 18. No difference in adaptation.

Reference	Independent variable, biological material	Timing: Mean weeks of pregnancy (SD)	Child's age: Mean (SD)	Dependent variables, Instrument.	Number of participants included in analyses	Statistical analyses, confounders	Results
Gutteling et al. 2005b	Early morning cortisol concentration (08:00 a.m.) and mean cortisol concentration of seven samples during the day between 08:00 a.m. and 08:00 p.m.).	Weeks 15-17 (-) Weeks 27-28 (-) Weeks 37-38 (-)	27 months (-)	Temperament (restless/ disruptive behavior and irritability). Instrument: Infant Characteristics Questionnaire. Attention regulation. Instrument: third component of the Bayley Scales of Infant Development. Internalizing problems, externalizing problems, total problem behavior. Instrument: Child Behaviour Checklist (CBCL).	103	Logistic regression. Confounders: maternal age, smoking behavior and alcohol use during pregnancy, SES, prenatal risk factors, perinatal factors, postnatal factors, and gender.	Maternal cortisol concentration, both with and without psychological measurements, proved to be unrelated to temperament and behavioural problems.
Rothenberger et al. 2011	Cortisol measured on 3 consecutive days between 11 am and 1 pm. Material: saliva	Each trimester.	5 months (-)	Temperament (affective reactivity). Instrument: observation.	104	Mann-Whitney U-test and univariate ANOVA. Confounders: -	Intercorrelation cortisol and affective reactivity n.s. in each trimester. No difference between 'high and no' affective reactivity in cortisol during pregnancy.

Buitelaar et al. 2003	Early morning cortisol concentration (08:00 a.m.) and daily mean cortisol concentration of seven samples during the day between 08:00 a.m. and 08:00 p.m.). Material: saliva	Weeks 15–17 (-) Weeks 27-28 (-) Weeks 37–38 (-)	3 and 8 months after birth (-)	Temperament (difficult behavior and inadaptability) Instrument: infant Characteristic Questionnaire (ICQ). Behavior (exploration, goal-directedness, and test-affectivity). Instrument: observation, third component of the Bayley scale.	170	MANCOVA, MANOVA, multiple regression analyses. Confounders: gestational age at birth, birth weight and the postnatal stress and depression level of the mother	No relation between cortisol and infant temperament or observed behavior.

AUC = area under the curve

n.s. = not significant

Table 4. Characteristics and results of studies on the association between maternal prenatal cortisol and infant cortisol.

Reference	Independent variable, biological material	Timing: Mean weeks of pregnancy (SD)	Child's age: Mean (SD)	Dependent variables, Instrument.	Number of participants included in analyses	Statistical analyses, confounders	Results
Gutteling et al. 2004	Daycurves of cortisol measured every 2h between 8 AM and 8 PM. Material: saliva	Weeks 15-17 (-)	4 years and 9 months (0.66).	Cortisol response to stressor. Instrument: 5 saliva samples during day of vaccination.	24	Multilevel Analysis calculations (Hierarchical Linear Modeling). Confounders: time of sampling on the day of vaccination, gender.	Higher maternal prenatal early morning cortisol was related to higher concentrations of children's cortisol in reaction to the vaccination.
Gutteling et al. 2005a	Daycurves of cortisol measured every 2h between 8 AM and 8 PM. Material: Saliva	Weeks 15-17 (-)	5.31 years (0.50)	Cortisol response to stressor. Instrument: 4 saliva samples on first day at school.	29	Multilevel analysis (Hierarchical Linear Modeling). Confounders: time of sampling, age and gender.	Children of mothers with higher levels of prenatal morning cortisol showed higher levels of cortisol as compared to children whose mothers had lower levels of prenatal morning cortisol.
Tollenaar et al. 2011	Evening cortisol concentrations (21:00h) and decline over the day (waking – 21:00h). Mean of two consecutive days Material: saliva.	37.4 weeks (1.4)	4.7 weeks (0.7) 9.5 weeks (3.0) 22 weeks (1.2) 54 weeks (2.9)	Cortisol responses to 4 stressors. Instrument: saliva samples during 1.Bathing, 2.Vaccination, 3.Still face procedure, 4.Strange Situation Cortisol reactivity: peak minus baseline.	173	Multiple Hierarchical Regression Analyses. Confounders: all variables that explained at least 1% of the variation in infants' cortisol reactivity.	The mothers' circadian salivary cortisol measures did n.s. predict infant cortisol reacitivity on any of the tasks.

Davis et al. 2011	Sample taken in the afternoon (range 13:19 to 13:30). Material: plasma	Week 15.1 (0.9) Week 19.3 (1.0) Week 25.4 (0.9) Week 30.9 (0.69) Week 36.5 (1.2)	23 hrs 47 min (4 hrs 13 min)	Cortisol response + recovery to heel stick. Instrument: three saliva samples during heel stick.	116	Growth curve analyses. Conf ounders: sociodemographic factors (marital status, race/ ethnicity, education, household income), medical risk (prenatal medical risk, parity, maternal age, mode of delivery), infant factors (gestational age, birth weight, sex, Apgar score, postnatal age), aspect heel stick procedure. Only time of maternal sample collection included in final analyses.	Trajectory of maternal cortisol associated with infant cort response: elevated maternal cortisol between week 21-35 was associated with a larger increase in infant cortisol in response to the heel-stick. High maternal cortisol in weeks 20-27 predicted elevated infant cortisol at the recovery assessment. The strongest association between maternal prenatal cortisol and infant cortisol response was at 25 gestational weeks.

n.s. = not significant

Table 5. Percentage significant analyses per outcome variable, subdivided in early/midgestation (<27 weeks) and late gestation (≥27 weeks).

Infant outcome		<27 weeks	≥27 weeks	Total
Health	Nr studies	13	10	23
	Nr analyses	34	26	60
	Nr sign analyses	11	11	22
	% sign analyses	32.4	42.3	36.7
Cognitive/motor development	Nr studies	6	9	15
	Nr analyses	10	29	39
	Nr sign analyses	2	10	12
	% sign analyses	20.0	34.5	30.8
Psychology/behavior	Nr studies	11	10	21
	Nr analyses	30	87	117
	Nr sign analyses	2	12	14
	% sign analyses	6.7	13.8	12.0
Infant cortisol	Nr studies	5	3	8
	Nr analyses	11	10	21
	Nr sign analyses	7	2	9
	% sign analyses	63.6	20.0	42.9

nr = number
sign = significant

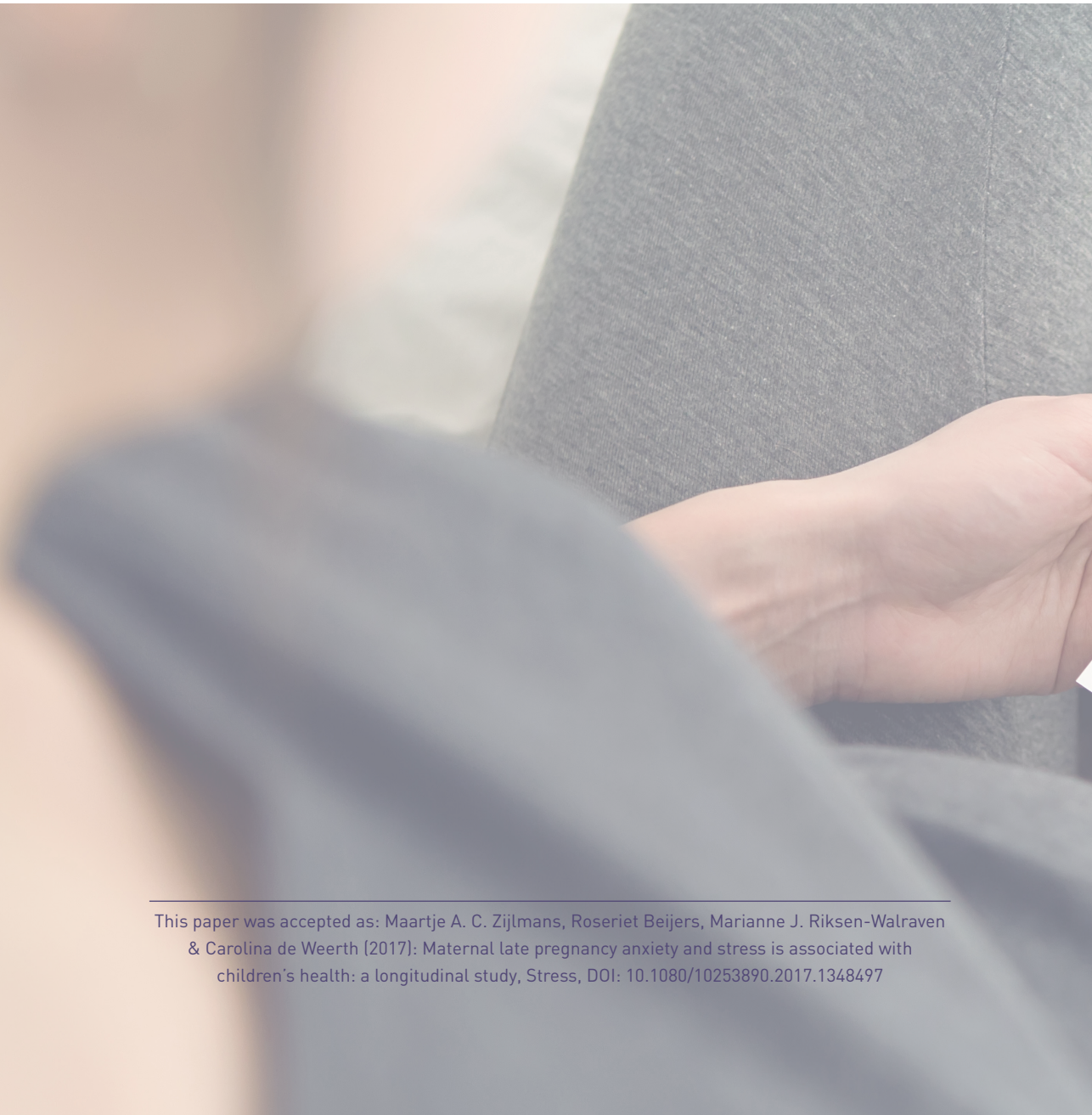
Table 6. General limitations of reviewed empirical studies and recommendations for future studies on the link between maternal prenatal cortisol and child outcomes.

Limitations	Recommendations
Not clear if confounders affect the results	Always include important confounders in the statistical analyses and report their contribution in the analyses.
Moderators are not included in the statistical analyses	Include moderators such as fetal sex, and placental functioning
Heterogeneity of measurement of independent variable:	
- different sampling times of prenatal cortisol	For measurement of diurnal rhythm: sampling of cortisol measures on at least 2 days, at least 2 time points (wakening+30 minutes and 9pm)
- variation in use of composite scores (decline, CAR, AUC)	Use of composite scores depends on research question. But when using composite scores, also report the correlations between the raw cortisol values and the outcome variables so studies can be compared.
Large number of statistical analyses increases chance of false positive results	Set up statistical analysis plan beforehand.
	Use of conservative p-value (e.g. $p<0.01$)
	Use of correction for family-wise error (e.g. Bonferroni correction).
Variation in use of statistical methods	For repeated measures of cortisol: multilevel analyses or growth curve analyses
	For composite scores of cortisol: regression analyses
Difficult to compare studies due to lack of information	Always report means/SDs of raw maternal prenatal cortisol data
Not always clear if the relation between maternal prenatal cortisol and child outcomes differs between gestational periods.	Include assessments for at least 2 different trimesters in each study
Unclear if cortisol is mediator between maternal stress/anxiety and child outcomes	Include other possible mechanisms, next to maternal cortisol, in the study design.

Table 7. Confounders that have been found to be significantly related to the outcome variables.

	Confounders
Physical/health outcomes:	
- Health	Child care attendance, duration of breastfeeding
- Birth weight/length	Child sex, gestational age, parity, pre-pregnancy BMI, maternal age
Cognitive/motor outcomes	Maternal ethnicity, parental educational level, maternal age, prenatal smoking and alcohol use, gestational age, birth weight, birth order, prenatal medical risk, postnatal maternal stress, postnatal maternal depression, child IQ
Psychological/behavioral outcomes	Prenatal smoking, maternal education, maternal age, child sex, gestational age, birth weight, maternal current psychological state (i.e. postnatal anxiety and depression)
Cortisol outcomes	Maternal education, prenatal smoking and alcohol use, maternal age, parity, child sex, delivery mode, birth weight, breastfeeding, postnatal maternal anxiety, attendance to child care

Note. The information in this table is based on the reviewed papers and is therefore not exhaustive. Future studies may find additional potentially relevant confounders in their studies.



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Chapter 3

Maternal Prenatal Anxiety and Stress is Associated with Children's Health: a Longitudinal Study

Maartje A.C. Zijlmans, Roseriet Beijers, J. Marianne Riksen-Walraven & Carolina de Weerth

ABSTRACT

Maternal prenatal anxiety and stress (PNS) have been positively associated to physical health problems in offspring in the first year of life. Whether these associations are transient, persistent, or even progressive over time, is as yet unknown. The goal of the present study is to investigate associations between PNS and child health from 18 months till age 6.

Mothers were recruited in late pregnancy, and had uncomplicated, singleton pregnancies without physical health problems. At week 37 of pregnancy, mothers reported on their PNS by means of questionnaires, and provided saliva for determination of circadian cortisol concentrations. Children's illnesses in the preceding year were assessed with the use of maternal reports at 30, 48, 60, and 72 months. Antibiotic use was obtained from medical records between one and six years of age.

Multilevel models (N=174) showed a positive relation between maternal prenatal general and pregnancy-specific anxiety during late pregnancy and offspring respiratory illnesses and symptoms. Interaction effects with time indicated that more PNS was related to more illnesses until toddlerhood, but not later in life. Furthermore, maternal prenatal cortisol concentrations were related to child digestive illnesses. A steeper maternal cortisol decline over the day was related to more child digestive illnesses, until around three years of age. Finally, children of mothers who suffered more from daily hassles during pregnancy received more antibiotics between one and six years of age. PNS was not related to general and skin illnesses.

Summarizing, this study showed that PNS was associated with children's respiratory and digestive illnesses till the age of 3.0-3.5 years. Additionally, more PNS was related to more prescribed antibiotics between one and six years. These findings point in the direction of possible effects of PNS persisting beyond the first year of life and into toddlerhood, but disappearing at older ages.

INTRODUCTION

Prenatal exposure to maternal anxiety and stress (PNS), as reported by the mother or assessed with stress hormones, is associated with more physical health problems in offspring. Higher levels of reported PNS are associated with preterm birth, lower birth weight (Shapiro et al., 2013; Dunkel Schetter and Tanner, 2012), obesity, and metabolic dysfunction (Entringer, 2013), and more illnesses and antibiotic treatments (Beijers et al., 2010). Higher PNS, as assessed with maternal prenatal cortisol concentrations, is also associated with offspring health (Zijlmans et al., 2015), including lower birth weight (D'Anna-Hernandez et al., 2012; Bolten et al., 2011; Goedhart et al., 2010), shorter gestational age (Diego et al., 2009; Mercer et al., 2006; Erickson et al., 2001), more infant respiratory and skin illnesses (Beijers et al., 2010), and larger systemic vascular resistance and lower artery elasticity (Rondó et al., 2011; Rondó et al., 2010).

However, most studies on the relation between PNS and physical health in offspring focused on early life. Whether the associations are transient, persistent, or even progressive over time, is as yet unknown. The Developmental Origins of Health and Disease (DOhAD) hypothesis states that environmental exposures in utero condition the risk of disease throughout life: in infancy, childhood, and adult life (Gluckman and Hanson, 2004). Furthermore, life course models suggest that health development is a dynamic process, beginning before conception and continuing throughout life (Halfon et al., 2014). This suggests that PNS could influence the health development of children already in utero and that the effects may be long-lasting. The goal of the present study is to investigate associations between PNS and child health throughout the first 6 postnatal years. In a previous study on the same sample (Beijers et al., 2010), we found that PNS was associated to more respiratory, general, and skin illnesses, and more antibiotic use during the first postnatal year. Therefore, we hypothesize that children from mothers with higher levels of PNS will suffer from more respiratory, general, and skin illnesses, and receive more antibiotic treatments, during the first six postnatal years.

METHODS

Participants and procedure

This study is part of an ongoing longitudinal study in which 192 mothers were followed from week 37 of pregnancy on. Inclusion criteria were an uncomplicated, singleton pregnancy, clear understanding of the Dutch language, no drug use and no current physical health problems. The 174 participants of the earlier health assessment study (first 12 months, Beijers et al., 2010) were followed again at 30 months (*N*=167), 48 months (*N*=159), 60 months (*N*=161), and 72 months (*N*=149). Reasons for drop out were lack of time or other personal circumstances. There were no differences in the maternal PNS variables (general anxiety, pregnancy-related

anxiety, daily hassles, pregnancy-related daily hassles, maternal evening cortisol, maternal cortisol decline) between participating mothers and drop outs (*N*=25, independent samples t-tests *p*'s>0.05). The Ethical Committee of the Faculty of Social Sciences (Radboud University) approved the study following the Helsinki Declaration (ECG 300107 and ECG 22111/130112) and mothers gave written informed consent.

Around week 37 of pregnancy, mothers filled out questionnaires about their experienced levels of stress and anxiety, and reported on their demographics. Furthermore, they collected saliva samples to determine circadian cortisol concentrations. Postnatally, the mothers also filled out questionnaires about anxiety and stress at 3, 6, 12, 30, 48 and 72 months.

In the first six years of life, maternal reports on children's illnesses and health complaints were obtained by semi-structured interviews as follows: at age 30 months (pencil and paper questionnaire), 48 months (pencil and paper questionnaire), 60 months (phone interview), and 72 months (online questionnaire). Furthermore, from the 174 mothers, 130 mothers gave written permission to request their children's medical records (prescribed medications) from their general practitioners. These records were used to assess antibiotic use. There were no differences in the maternal PNS variables between mothers who gave permission to request the medical records and mothers who did not give permission (N=44, independent samples t-test *p*'s>0.05).

Measurements

Infant health

Mothers reported on their child's health during the past year (30, 48, 60 and 72 months). They were asked how often the child had a specific illness or health complaint (e.g. having a cold) ending with an open question about possible illnesses that were not asked for. Basically, the same questionnaire was used at all time points. The items were classified following the International Classification of Primary Care (ICPC; Lamberts and Wood, 1987). The classes used in the current study were identical to those of Beijers et al. (2010), namely Respiratory, Digestive, Skin, and General. For each time point, a sum score per class was created by adding up all the illnesses and health complaints. See Table 1 for illnesses and complaints reported in our sample.

The number of antibiotic treatments from age one to six was obtained from medical records.. This time period was chosen because in a previous paper on this sample the relation between PNS and antibiotic use was examined between 0 and 12 months of age (Beijers et al. 2010).

General anxiety

Maternal prenatal anxiety symptoms were measured using a Dutch translation of the 20-item state anxiety subscale of the State-Trait Anxiety Inventory (STAI; α=0.93) (Spielberger, 1983; van der Ploeg et al., 1981). Items were scored on a 4-point scale, with a higher score indicating more anxiety.

Pregnancy-related anxiety

Pregnancy-related anxiety was measured using the Pregnancy-Related Anxieties Questionnaire-Revised (PRAQ-R; van den Bergh, 1990). Two subscales, scored on 5-point scales, were used, namely 'fear of giving birth' (3 items, α=0.70) and 'fear of bearing a handicapped child' (4 items, α=0.83). Higher scores indicated more anxiety.

Daily hassles

The occurrence and intensity of daily hassles was measured using a 49-item Dutch questionnaire (Alledaagse Problemen Lijst, APL, test-retest reliabilities between 0.76 and 0.87; Vingerhoets et al., 1989). Participants had to check if they encountered a daily hassle during the past 2 months and scored on a 4-point scale how much it bothered them. Example items are 'you had a conflict with your partner', and 'family or friends were involved in a traffic jam'. The score used in the analyses is a mean valence rating; the sum of the total (negative) valence was divided by the frequency of daily hassles. A higher score indicated more negativity.

Pregnancy-related daily hassles

Pregnancy-related daily hassles were measured using a Dutch translation of the Pregnancy Experience Scale (PES; DiPietro et al., 2004), consisting of 43 items (α=0.87). Example items are 'discussing baby names with your spouse', and 'preparing the nursery'. Participants had to rate the listed pregnancy-related experiences on two 5-point scales, indicating (1) the extent to which the experience was experienced as a hassle, and (2) the extent to which the experience was experienced as an uplift. A total score was calculated by dividing the sum of intensities of hassles by the sum of intensities of uplifts. A higher score indicated more negative emotional valence toward pregnancy.

Prenatal cortisol

Around week 37 of pregnancy (mean: 37 weeks, 0.8 days, SD: 9.4 days), mothers collected saliva samples by passive drooling into sterile containers with screw caps to determine cortisol concentrations. Five samples were collected at two consecutive days at awakening, 30 minutes after waking, and at 12:00, 16:00 and 21:00 h. Samples were stored in a freezer (-20 Celsius) until analysis. An in-house competitive radioimmunoassay was used to measure cortisol (Laboratory of Endocrinology, University Medical Center Utrecht, the Netherlands). Intra- and inter-assay variations were below 10%. Cortisol concentrations between days correlated

strongly, therefore mean cortisol concentrations over the two days were calculated (for more details see Tollenaar et al., 2011; Beijers et al. 2010). The evening cortisol concentrations and cortisol decline over the day (wakening minus evening) were not correlated (r=-0.11, p>0.05), but were highly correlated with other cortisol measures: area under the curve (decline: r=.18; evening: r=.85), morning (decline: r=.83; evening: r=.45), and cortisol awakening response (decline: r=-.33; evening: r=-.10). The evening cortisol and the cortisol decline over the day were used in the analyses because of this pattern of correlations, together with the fact that this study is a follow up on the Beijers et al. (2010) study, in which these variables were also used as independent variables. Additional reasons are that higher evening cortisol and flattened diurnal cortisol rhythms have been related to many psychopathologies (Goodyer et al. 2001), and a flatter diurnal cortisol curve is often found in adults and children under chronic stress (Cicchetti et al., 2010; Gunnar & Vazquez., 2001; Heim et al., 2008; Miller et al., 2007).

Statistical analyses

Multilevel modeling analyses using mixed models in SPSS 22.0 were conducted to test whether maternal reported PNS and maternal prenatal cortisol concentrations were related to children's health between 18 months and six years of life.

Next, scores higher than 3 standard deviations above the mean were defined as outliers and deleted (respiratory N=11, digestive N=10, general N=9). All analyses were performed with and without outliers. The PES variable needed square root transformation to achieve normality. The residuals of the analyses with skin illnesses and antibiotic treatments violated the assumption of normality, even after transformation. Therefore, we conducted multiple hierarchical regression analyses with skin problems (sum of assessments at 30 till 72 months, covering the period of 18 to 72 months) and antibiotic treatments (sum of prescriptions from one to six years of age) as dependent variables.

Three multilevel analyses were conducted with respiratory, digestive, and general illnesses as dependent variables. At level 1 the illnesses were introduced, which were nested within the children in level 2. First, for each dependent variable the intraclass correlation was calculated to test whether multilevel analyses were appropriate. For respiratory illnesses, the intraclass correlation was 43%, meaning that 43% of the variance was associated with differences between infants. This indicates that a multilevel analysis was appropriate. The intraclass correlation for digestive illnesses was 26%, and for general illnesses 38%, also indicating that multilevel analyses were appropriate. Second, a build-up strategy was used by adding the variables to the model one by one. After adding a variable to the model, the -2 log likelihood ratio scale after least square estimation was examined. If the added variable significantly contributed to the model, it remained in the model. The variables were added as follows: linear time (random), quadratic time, confounders, maternal prenatal anxiety

and stress (maternal reports and cortisol concentrations), and interactions between time and maternal prenatal anxiety and stress predictors (using centered scores). The best fitting models are presented in the results section.

Two multiple hierarchical regression analyses were performed, with skin problems and antibiotic use as dependent variables. Log10 transformations were used to fulfill the assumption of normality. No outliers were detected in these transformed variables. For each dependent variable two models were analyzed. In the first model all possible confounders and prenatal predictors were added. In the second model, to eliminate irrelevant variables and increase power, only variables that explained at least 1% of the variance in the first model were included (part correlation ≥ 0.10). The final models, with confounders in step 1, and prenatal predictors in step 2, are presented in the results (see also Beijers et al., 2010).

Confounders

The following confounders were included maternal educational level, prenatal smoking, prenatal alcohol use, birth weight, infant biological sex, attendance of center based childcare, and duration of breastfeeding. Attendance to center-based childcare at 12 months and 30 months (57.5% and 60.9% respectively) were strongly correlated (r=0.78, p<0.001). Therefore, only attendance to center-based childcare at 30 months was included. Number of siblings was measured at each time point and included as a repeated measure variable in the multilevel analyses. In the hierarchical regression analyses, birth order (first born or not) was included. To control for postnatal state anxiety, the STAI was included as a confounder at each time point in the multilevel analyses. In the hierarchical regression analyses a mean score of the postnatal STAI measures was included (significant correlations over time; r's>0.303, p<0.001) to avoid multicollinearity.

RESULTS

Descriptive analyses

Table 2 presents the descriptive statistics. Correlations between PNS, and child illnesses and antibiotic treatments are presented in Table 3. Maternal reports of PNS were related to respiratory, general, and digestive illnesses at different ages (r's ranging between 0.16 and 0.36), but not to skin illnesses or antibiotic treatments. Regarding cortisol measures, children of mothers with a steeper cortisol decline during the day had more digestive illnesses in the 30-month assessment.

Main analyses

The final multilevel models with respiratory, digestive, and general illnesses are summarized in Table 4. For all illnesses, a main effect of time shows that respiratory (c^2(406.54)=-5.46, p<.001), digestive (c^2(419.52)=-3.61, p<.001) and general illnesses (c^2(380.33)=-4.30, p<.001) decline over age. With respect to respiratory illnesses, the analyses showed that higher levels of fear of bearing a handicapped child (c^2(497.198)=3.69, p<0.05) and higher levels of prenatal state anxiety (c^2(497.91)=2.63, p<0.01) were significantly related to more child respiratory illnesses. These main effects are moderated by time. The interaction effect of fear of bearing a handicapped child with time shows that the higher the fear, the higher the number of respiratory illnesses in early life, with the association disappearing over time. As indicated by the region of significance the association is significant until 38.3 months (c^2(382.66)=-2.23, p<.05, see Figure 1). The interaction effect of general anxiety with time is similar; the higher the general anxiety during late pregnancy, the higher the number of respiratory illnesses in early life. This association remains significant for a longer period of time, namely till 45.2 months of age, as indicated by the region of significance (c^2(380.75)=-3.09, p<.01, see Figure 2).

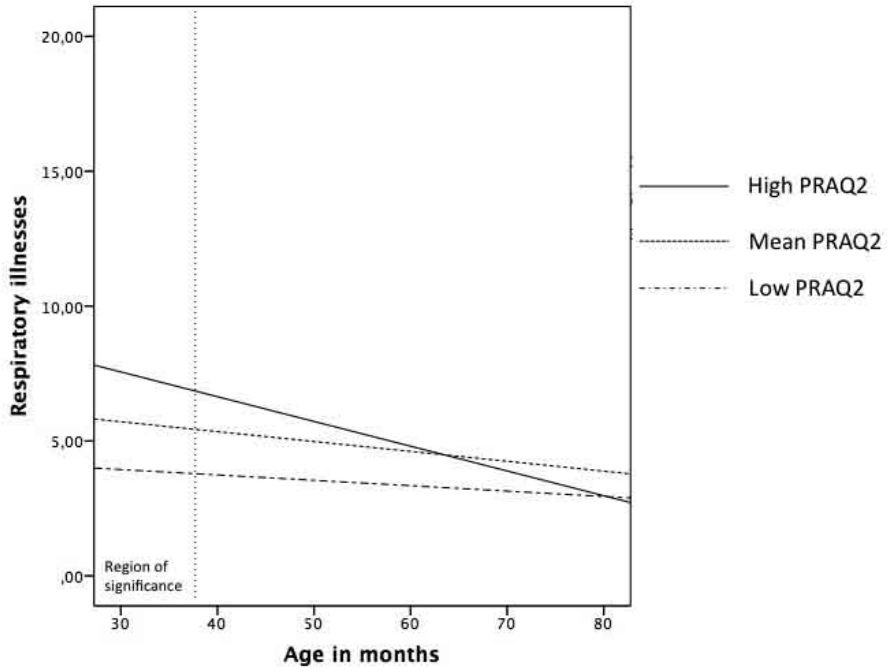

Figure 1. Interaction effect prenatal $PRAQ_{fear\ handicapped\ child}$ * time on children's respiratory illnesses.

Note: children's illnesses in the preceding year were assessed at 30, 48, 60, and 72 months, reflecting the period between 18 and 72 months. Slope for high STAI: p<0.001, slope for mean STAI p<.01 and low STAI p>0.05. Region of significance: <38.30 months.

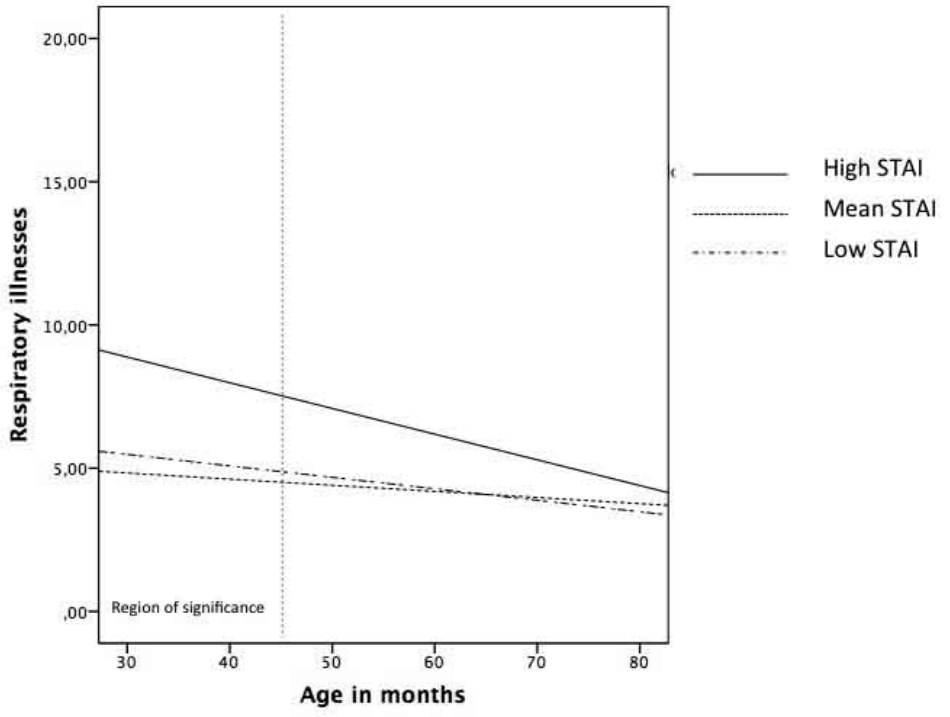

Figure 2. Interaction effect prenatal STAI * time on children's respiratory illnesses.

Note: children's illnesses in the preceding year were assessed at 30, 48, 60, and 72 months, reflecting the period between 18 and 72 months. Slope for high STAI: $p<0.01$, slope for mean STAI $p<0.05$ and low STAI $p>0.05$. Region of significance: <45.15 months

Furthermore, a larger maternal cortisol decline over the day was related to more child digestive illnesses ($c^2(477.94)=3.10$, $p<.01$). The interaction effect between cortisol decline and time indicates that the larger the maternal cortisol decline over the day, the higher the number of digestive illnesses in early life. This association is significant until the age of 37.3 months, as indicated by the region of significance ($c^2(391.51)=-2.87$, $p<.01$, see Fig. 3).

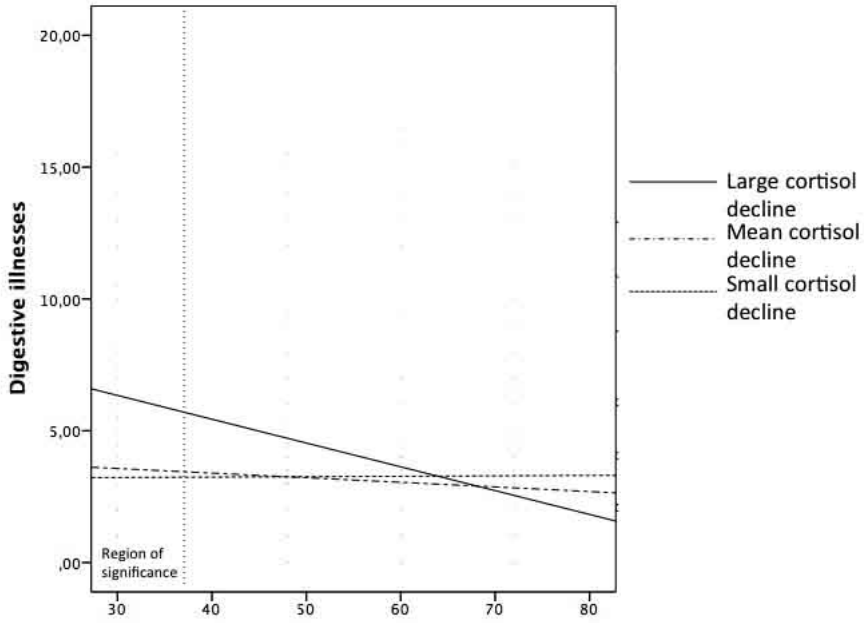

Figure 3. Interaction effect prenatal cortisol decline * time on children's digestive illnesses.

Note: children's illnesses in the preceding year were assessed at 30, 48, 60, and 72 months, reflecting the period between 18 and 72 months. Slope for large cortisol decline: $p<0.001$, slope for mean cortisol decline: $p<0.01$, slope for small cortisol decline $p>0.05$. Region of significance: <37.30 months

Lastly, the multilevel results for general illnesses show that a lower educational level ($c^2(135.75)=-3.31$, $p<.01$) and higher maternal postnatal anxiety ($c^2(135.92)=3.39$, $p<.01$) were related to more child general illnesses. PNS was not related to general illnesses.

The final multiple regression models with antibiotic use and skin problems as dependent variables are presented in Table 5. Regarding antibiotic use, model 2 (including the prenatal predictors), was significant ($F(8,113)=2.88$, $p<0.006$), and shows that higher prenatal maternal daily hassles were related to more child antibiotic prescriptions between one and six years of age ($b=0.24$, $p<.05$). Regarding skin problems, both models were non-significant ($p>0.05$) indicating that PNS was not related to skin problems.

DISCUSSION

The results of this study show a positive relation between maternal anxiety and stress (PNS) in late pregnancy and child respiratory and digestive illnesses until toddlerhood, and antibiotic use between age one and six. Hence, these results extend the previous results of the Beijers et al. (2010) study on the links between PNS and illnesses and health symptoms in the first year of life. More precisely, more maternal prenatal general and pregnancy-specific anxiety symptoms were related to more respiratory illnesses. Additionally, interaction effects with time show that the higher the general anxiety and the higher the fear of bearing a handicapped child during late pregnancy, the higher the initial number of illnesses during early life and until toddlerhood. Furthermore, a larger decline of maternal cortisol during the day was related to more digestive illnesses, until around three years of age. Lastly, higher levels of prenatal daily hassles were positively related to antibiotic use between one and six years of age. In contrast with the study of Beijers et al. (2010), PNS was not related to general and skin illnesses.

The positive relation between prenatal anxiety symptoms and the child's respiratory illnesses is in line with previous studies on maternal prenatal stress and child wheezing and respiratory illnesses, as described in a recent meta analysis of van de Loo et al. (2016). This relation may be explained by effects of the mother's anxiety on the development of the offspring's immune system. Immune development starts during early fetal life and is vulnerable to environmental factors in utero and after birth (Goenka and Kollmann, 2015; Marques et al. 2010). First, an anxious mother's own immune functioning may be affected, directly influencing the developing fetal immunity through a reduced transplacental transfer of passive immunity, a phenomenon occurring especially in late pregnancy (Merlot et al., 2008). Secondly, the functioning of the placenta might be affected by PNS. To protect the fetus from extremely high concentrations of maternal cortisol during pregnancy, the 11βHSD2 enzyme in the placenta has a protective barrier function, inactivating most of the maternal cortisol into cortisone. However, this barrier function appears to function less optimally in anxious mothers, resulting in the fetus being exposed to more cortisol during pregnancy (O'Donnell et al., 2012). As glucocorticoids can have an immune-suppressive effect on immune function parameters (Marques et al., 2010), this can result in a suppressed immune response in the infant. Finally, the infant intestinal microbiota, that plays an important role in the development of immunity, may be involved (Dimmitt et al., 2010). Maternal microbes, transferred from the mother to the infant during and after birth, are the first colonizers of the infant gut (Gosalbes et al., 2013; Tannock et al., 1990). Prenatal anxiety symptoms may negatively affect the composition of the mother's own microbiota (Sekirov et al., 2010), hence affecting the microbiota she passes on to her child. In support of this hypothesis, a study in Rhesus monkeys showed that infants from mothers who experienced stress during pregnancy had altered intestinal microbiota and more diarrheic symptoms than infants from mothers who were not stressed (Bailey et al., 2004). Furthermore, we recently reported links between maternal PNS and the composition of the

infant intestinal microbiota, and how children with this altered composition had more health problems in the first months of life (Zijlmans et al., 2015). For other potential mechanisms linking prenatal anxiety and infant health see the review by Beijers et al. (2014).

As mentioned before, the positive relation between prenatal anxiety symptoms and child respiratory illnesses continued until toddlerhood, and later disappeared. As can be seen in table 2, respiratory illnesses and symptoms are reported more frequently than digestive and general illnesses. The relatively frequent occurrence of respiratory complaints may make them an especially good marker for studying subtle differences in immune functioning in healthy children. Possibly, children from mothers with high levels of prenatal anxiety are born with an immature and less well-functioning immune system, making them especially vulnerable for pathogens in their environment during early years. The fact that the association between prenatal anxiety and respiratory complaints disappears after toddler age, may be a consequence of the general decline in respiratory illnesses over time. This decrease in respiratory symptoms and complaints may make it difficult to discern variance explained by PNS in later childhood years.

The finding that pregnant mothers with a steeper cortisol decline over the day during late pregnancy had children with more digestive illnesses is difficult to explain. Previous studies showed that a flatter diurnal cortisol curve is often related to chronic stress in children, adolescents and adults (Cicchetti et al., 2010; Gunnar & Vazquez., 2001; Heim et al., 2008; Miller et al., 2007). Therefore, we had hypothesized that a flatter cortisol decline would represent higher maternal stress and anxiety levels, and that this in turn would be related to more health problems in children. Previous studies on the relation between prenatal maternal cortisol decline and child outcomes support this hypothesis (Zijlmans et al. 2015). For example, one study found that mothers with a flatter daily cortisol decline in late pregnancy gave birth to infants with lower birth weight (D'Anna Hernandez et al. 2012). In the previous study on this population (Beijers et al., 2010), a relation was found between a flatter maternal late pregnancy slope and more infant respiratory illnesses in the first year, making the present results all the more intriguing. One possible explanation for our present findings is that in our sample the cortisol decline was positively correlated with the total cortisol concentrations during the day ($r=.18$, $p<.05$; Area Under Curve with respect to the ground). This could potentially explain our findings, as maternal cortisol concentrations can cross the placenta and in turn affect HPA axis development. The HPA axis acts in concert with the gut brain axis, and is in turn related to gut related diseases, such as diarrheic illnesses (Sekirov et al., 2010). However, given the paucity of earlier research and especially research reaching into middle childhood, it is not yet possible to draw firm conclusions. More extended longitudinal studies on the development of child health in the light of maternal prenatal cortisol physiology are needed to increase our understanding on the subject. Studies involving large birth cohorts would be especially helpful in this context.

No relation was found between PNS and skin illnesses between 18 months and six years of life, while Beijers et al. (2010) did find a relation between PNS and skin illnesses during the first postnatal year. In line with these findings, an epidemiological study that also focused on the first six years of life, showed a relation between stress-related maternal factors during pregnancy and an increased risk for childhood eczema up to the age of 2 years but not later (Sausenthaler et al., 2009). Additionally, the lack of associations in the present study may be explained by the general decrease of eczema, one of the most frequently reported skin illnesses in our sample, over time (Nivel, 2013).

A positive relation was found for postnatal anxiety and general illnesses. This secondary finding could be explained in at least three manners. First, anxious mothers may detect and report more general illnesses in their children because they are more focused on their child's well being and notice more subtle health changes. Second, anxious mothers may be less capable of buffering their children from environmental stressors and may even provoke stress in their children, which in turn may lead to immunosuppression and more general illnesses in the children. And third, children who are often ill may provoke anxiety in mothers. The general illnesses measured in our study consisted mainly of fever and ear infections. A study by Lagerlov et al. (2003) in preschool children showed that fever often results in anxious feelings in caregivers. Parents feel an increased responsibility for the child and the burden of parenting increases. Hence, general illnesses could provoke anxiety in mothers.

A strong point of this research is the longitudinal design, in which illnesses were measured every year, covering the period from 18 months until age six. Also, in addition to diagnosed illnesses (e.g. eczema, asthma) we recorded subtle health complaints (e.g. coughing, fever), which may be a better representation of the general functioning of the immune system. Such health complaints are also of societal relevance as they often result in parents staying at home from work to care for the child. This is particularly the case with fever, which is part of the category general illnesses in our study. Children suffering from fever are not allowed to go to daycare or school. Furthermore, mothers do not often delegate the care of a sick child to others and therefore mostly stay at home to care for their child during illness (Lagerlov et al. 2003). Finally, PNS and child's health were measured using different methods (questionnaires, cortisol) and different sources (mothers, general practitioners).

Our research also has some limitations. The assessment of subtle health complaints made us necessarily dependent on maternal reports, which may be subjective. However, we also found a positive relation between PNS and antibiotic use, supporting the relation between PNS and child health by medication prescribed by general practitioners. Furthermore, mothers from our sample are highly educated which could affect the generalizability of the study. For future studies we would recommend to shorten the reporting period of health complaints from one year to monthly or bimonthly reporting. Also, other potential confounders or mediators, such as (epi)genetics, immune parameters, and diet, that could influence the relation between prenatal stress and child health are important to include in future studies. Finally, to obtain

more insight in potentially sensitive gestational periods in which the fetus is more susceptible to maternal cortisol concentrations and lower psychological wellbeing, we recommend to study the impact of fetal exposure to maternal PNS during all trimesters of pregnancy.

To conclude, this research showed that prenatal anxiety symptoms and cortisol are related to children's respiratory and digestive illnesses in early life, until around the age of three years. Furthermore, higher prenatal anxiety was related to more child antibiotic use between one and six years of age. Programming effects of PNS may lie behind these apparently persistent relations. These findings point in the direction of possible effects of PNS persisting beyond the first year of life and into toddlerhood, but disappearing at older ages.

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Table 1. Illnesses and health complaints, divided per class, assessed at 30, 48, 60, and 72 months.

Respiratory	Digestive	General	Skin
R07 sneezing/nasal congestion	D10 vomiting	A03 fever	S74 dermatophytotis
R71 whooping cough	D11 diarrhea	A12 allergy	S84 impetigo
R72 strep throat	D12 constipation	A72 chickenpox	S86 dermatitis seborrhea
R74 upper resp infection acute	D13 jaundice	A74 rubella	S87 dermatitis/atopic eczema
R78 acute bronchitis/bronchiolitis	D99 disease digestive system, other	A76 viral exenthema other	S88 dermatitis contact/allergic
R81 pneumonia		H71 otitis media acute/myringitis	
R96 asthma			
R97 allergic rhinitis			

Table 2. Descriptive statistics for children and mothers included in the present study

Demographics (N=174)	
Maternal age (years), mean ± SD (range)	32.60 ± 3.80 (21.90 to 42.90)
Maternal educational level	
Primary education	3.60 (N=6)
Secondary education	20.50 (N=36)
College or university	75.90 (N=132)
Smoking during pregnancy, %	4.00 (N=7)
Alcohol ingestion during pregnancy, %	16.70 (N=29)
Birth weight, mean ± SD (range), gram	3630.32 ±464.32 (2645.00 to 4730.00)
Infant biological sex, %	
Girl	47.70 (N=83)
Birth order, %	
First	40.80 (N=71)
Second or more	58.20 (103)
Additional confounders	
Duration of breastfeeding, mean ± SD (range), months	5.42 ± 4.28 (0.00 to 12.00)
Attendance at center-based child care at age 30 months, %	60.90 (N=106)
Postnatal state anxiety (STAI) mean 3, 6, 12 months, mean ± SD (range)	28.52 ± 6.44 (20.00 to 60.70)
Postnatal state anxiety (STAI) 30 months, mean ± SD (range)	29.91 ± 8.12 (20.00 to 79.00)
Postnatal state anxiety (STAI) 48 months, mean ± SD (range)	28.07 ± 6.60 (20.00 to 49.00)
Postnatal state anxiety (STAI) 72 months, mean ± SD (range)	30.71 ± 8.50 (20.00 to 68.00)
Prenatal psychological anxiety and stress; mean ± SD, range	
Prenatal anxiety (STAI)	32.20 ± 8.88 (20.00 to 64.00)
Pregnancy related daily hassles (PES)	0.33 ± 0.23 (0.00 to 1.40)
Prenatal daily hassles (APL)	1.14 ± 0.46 (0.00 to 2.50)
Fear of giving birth (PRAQ-R)	5.36 ± 2.48 (3.00 to 15.00)
Fear handicapped child (PRAQ-R)	9.17 ± 3.38 (4.00 to 20.00)
Prenatal cortisol concentrations; mean ± SD, range, nmol/L	
Decline (awakening minus evening)	6.70 ± 4.46 (-2.80 to 24.00)
Evening	9.48 ± 2.72 (0.90 to 20.00)
Infant health[1]; mean ± SD, range	
Respiratory 30 months	6.78 ± 5.72 (0.00 to 30.00)
Respiratory 48 months	5.37 ± 4.83 (0.00 to 24.00)
Respiratory 60 months	3.70 ± 4.54 (0.00 to 27.00)
Respiratory 72 months	4.52 ± 3.88 (0.00 to 20.00)

Digestive 30 months	5.28 ± 5.71 (0.00 to 36.00)
Digestive 48 months	3.16 ± 4.11 (0.00 to 19.00)
Digestive 60 months	2.84 ± 4.07 (0.00 to 24.00)
Digestive 72 months	3.19 ± 3.06 (0.00 to 15.00)
General 30 months	3.98 ± 3.07 (0.00 to 17.00)
General 48 months	2.58 ± 2.60 (0.00 to 15.00)
General 60 months	2.30 ± 2.68 (0.00 to 20.00)
General 72 months	2.24 ± 2.14 (0.00 to 12.00)
Skin 30 months	1.71 ± 3.69 (0.00 to 13.00)
Skin 48 months	1.59 ± 3.51 (0.00 to 12.00)
Skin 60 months	1.24 ± 3.28 (0.00 to 12.00)
Skin 72 months	1.44 ± 3.19 (0.00 to 13.00)
Skin, total 6 years[2]	5.47 ± 9.90 (0.00 to 48.00)
Antibiotic use, mean ± SD, range	
Antibiotic treatments, total 6 years[2]	2.04 ± 2.60 (0.00 to 14.00)

[1]Number of illnesses/complaints during the past 12 months

[2]Total number of skin illnesses/complaints and total number of antibiotic treatments were calculated for hierarchical regression analyses

Table 3. Correlations between Maternal PNS Variables and Children's Health variables for the period between 18 - 72 months of age.

	STAI	**APL**	**PRAQ-R** birth	**PRAQ-R** handicapped	**PES**	**Cortisol** decline	**Cortisol** evening
PNS questionnaires (N=174)							
STAI							
APL	.30**						
PRAQ-R_{birth}	.30**	.09					
PRAQ-$R_{handicapped}$	.14	.14	.14				
PES	.35**	.25**	.28**	.20**			
Prenatal maternal cortisol							
Corstisol decline (N=148)	.02	.01	-.03	-.17*	.16		
Cortisol evening (N=154)	-.02	-.05	.08	-.07	-.21**	-.11	
Cortisol AUC_{ground} (N=140)	-.02	-.02	.07	-.03	-.09	.18*	.85**
CAR (N=147)	-.01	-.18*	-.02	-.02	-.12	-.33**	-.10
Child Health Variables							
Respiratory 30 months (N=159)	.20**	.15	.05	.16*	.14	-.06	-.04
Respiratory 48 months (N=160)	.36**	.24**	.14	.27**	.14	-.07	-.12
Respiratory 60 months (N=162)	.13	.02	.02	.01	.16*	.11	-.06
Respiratory 72 months (N=148)	.01	-.08	.05	.10	.06	.01	-.03
Digestive 30 months (N=160)	.13	-.00	.08	-.13	.20*	.27**	.03
Digestive 48 months (N=160)	.11	.09	.13	.09	.02	.05	.02
Digestive 60 months (N=163)	.17*	.17*	-.02	.07	.01	-.04	-.07
Digestive 72 months (N=149)	-.04	-.06	.01	-.04	-.02	-.05	-.03
General 30 months (N=160)	.14	.07	.02	.01	.08	.03	.04
General 48 months (N=160)	.27**	.16*	.13	.09	.14	.03	-.02
General 60 months (N=164)	.10	.15	.09	.04	.14	.11	-.05
General 72 months (N=147)	-.02	.08	-.01	.04	.07	.12	.12
Skin, total 6 years (N=126)	-.02	.03	.09	.01	-.01	-.04	-.12
Antibiotic use, total 6 years (N=130)	-.09	.14	-.19*	.07	-.08	-.09	-.06

**p<.01, *p<.05

STAI: state anxiety; APL: daily hassles; PRAQ-R_{birth}: fear of giving birth; PRAQ-$R_{handicapped}$: fear of bearing a handicapped child; PES: pregnancy specific daily hassles.

Note. Spearman correlations were used for all correlations with the PES, skin problems and antibiotic use (because of non-normality). All other correlations are Pearson correlations.

Table 4. Estimates for the best fitting multilevel models for respiratory, digestive, and general illnesses for the period between 18 – 72 months of age, predicted by PNS.

	Estimate	SE	P
Respiratory			
Intercept	-5.553	2.797	0.048*
Time linear	-0.054	0.010	<0.001**
Attendance child care center 30 months	0.012	0.570	0.983
Siblings	0.179	0.360	0.620
Postnatal state anxiety (STAI)	0.036	0.045	0.428
Prenatal state anxiety (STAI)	0.185	0.070	0.009*
Prenatal PRAQ-$R_{handicapped}$	0.624	0.169	<0.001**
Cortisol decline over the day	0.038	0.060	0.524
Prenatal STAI * time	-0.003	0.001	0.026*
Prenatal PRAQ-$R_{handicapped}$ * time	-0.009	0.003	0.002**
Deviance	2742.409		
Digestive			
Intercept	1.757	1.158	0.130
Time linear	-0.037	0.010	<0.001**
Attendance child care center 30 months	0.234	0.485	0.629
Number of siblings	0.609	0.326	0.063
Cortisol decline over the day	0.420	0.136	0.002**
Cortisol decline over the day * time	-0.007	0.002	0.004**
Deviance	2724.257		
General			
Intercept	6.711	1.252	<0.001**
Time linear	-0.160	0.037	<0.001**
Time quadratic	0.001	0.000	0.001**
Maternal educational level	-0.287	0.088	0.001**
Attendance child care center 30 months	0.116	0.261	0.658
Number of siblings	0.142	0.172	0.406
Postnatal state anxiety (STAI)	0.063	0.019	0.001**
Cortisol decline over the day	0.040	0.027	0.138
Deviance	2016.590		

Note. The presented results contain dependent variables without outliers. Similar results were found when outliers were included.
**p<.01, *p<.05

Table 5. Final multiple regression model with antibiotic use and skin problems as dependent variables, predicted by prenatal anxiety and stress.

	B	β	R^2_{model}	F_{change}	R^2_{change}	*p*
Antibiotic use[a]						
Step 1						
Maternal education	-.046	-.196*	.078	2.295	.060[c]	
Infant biological sex	-.115	-.183*				
Attendance day care	-.061	-.096*				
Postnatal STAI	-.000	-.008*				
Step 2						
Prenatal STAI	-.046	-.109*	.180	3.266	.102[c]	.006
Prenatal daily hassles	-.166	-.240*				
Fear of giving birth	-.022	-.178*				
Evening cortisol	-.011	-.099*				
Skin problems[b]						
Step 1						
Birth weight	-.000	-.070*	.020	0.807	.492	
Attendance to day care	-.079	-.072*				
Postnatal STAI	-.007	-.090*				
Step 2						
Prenatal STAI	-.049	-.072*	.024	0.502	.004	.574

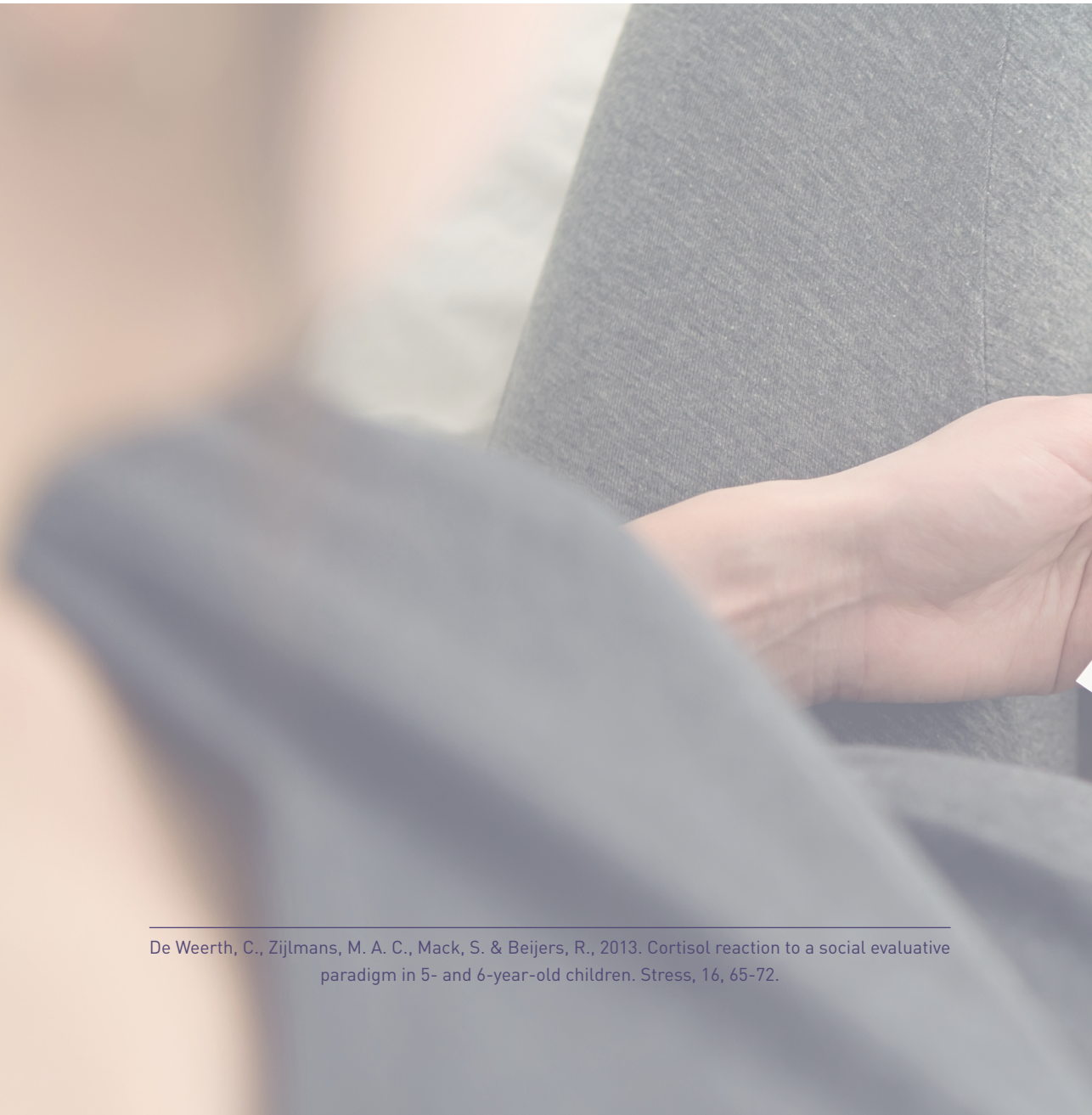
*p<.05

[a] Excluded variables: prenatal smoking, prenatal alcohol use, birth weight, first born, duration of breastfeeding, fear of handicapped child, cortisol decline

[b] Excluded variables: maternal education, prenatal smoking, prenatal alcohol use, infant biological sex, first born, duration of breastfeeding, prenatal state anxiety, fear of handicapped child, pregnancy specific daily hassles, cortisol decline, evening cortisol

[c] R2 change reflects the variance that is explained by adding the variables to the model, controlling for the confounding variables

Note. Antibiotic use covers the period between 12 – 72 months. Skin problems covers the period between 18 – 72 months



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Chapter 4

Cortisol Reactions to a Social Evaluative Paradigm in 5- and 6-year-old Children

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ABSTRACT

The goal of the present study was to develop a stress paradigm to elicit cortisol elevations in the group as a whole in 5- and 6-year-olds. To this end we tested a paradigm containing elements of social evaluative threat, unpredictability and uncontrollability, and with a duration of 20 minutes.

The Children's Reactions to Evaluation Stress Test (CREST) is composed of three short tasks which children have to perform in front of a judge. The tasks are rigged so as to provoke (partial) failure in the child's performance. Participants were 42 children (*M*=68.0 months, SD=4.3). Six saliva samples were taken during the testing session to obtain cortisol measurements of baseline concentrations, stress reactivity, and recovery.

Our findings showed that this paradigm was effective in provoking a significant cortisol increase in the group as a whole, with no effects of possible confounders (child's sex, child's age, parental educational level, school, time of testing, sex of experimenter, and sex of judge). The mean cortisol increase for the group was 127.5% (*SD*=190.9); 61% of the children could be classified as reactors (mean increase of 214%, *SD*=201.5), and 39% as non-reactors (mean decrease of 7.8%, *SD*=16.8).

To our knowledge, this is the first study that shows a significant cortisol response for the group as a whole in this age group to a standardized laboratory paradigm. As such, this paradigm is a promising tool to be used in future research on early life interactions between physiology and psychology.

INTRODUCTION

The hypothalamic pituitary adrenal (HPA) axis, with its end product, the glucocorticoid hormone cortisol, is an important regulator of our physiological reactions to stressors. Although humans are regularly faced with stress and need this physiological response to survive, abnormal reactivity of the HPA axis is related to mental disorders (Fairchild et al., 2008, Petrowski et al., 2010) and poorer physical health (Segerstrom & Miller, 2004). However, issues of causality in the relations between abnormal HPA axis reactivity and (psycho)pathology remain unclear. Since the HPA axis is sensitive to environmental factors and changes over time (Gunnar and Quevedo, 2007), it is important to examine HPA axis reactivity at several time points during development to notice gradual change. Only then can we begin to understand the complex interaction between physiology and psychology in early life that can lead to mental and physical disorders in adulthood.

The experience of stress is elicited by a physical or psychologically challenging situation (Gunnar and Quevedo, 2007). Different stimuli or situations are experienced as challenging at different ages. For example, babies react with an increase in cortisol to physical stressors such as vaccinations (Jansen et al., 2010), whereas children above age seven react to psychological stressors that contain social evaluation, such as a public speaking task (Gunnar et al., 2009). Apparently, different stressors are needed to examine HPA axis reactivity over time. Gunnar et al. (2009) showed in a review that for children between 5 and 6 years of age various paradigms have been used, including frustration tasks (Luby et al., 2003), rigged failure tasks (Lewis and Ramsay, 2002), rigged competition (Donzella et al., 2000), strange events (Quas et al., 2004), and separation tasks (Luby et al., 2003). However, none of these paradigms were able to elicit a significant cortisol increase in the group as a whole.

The goal of this study was to develop a paradigm that would elicit cortisol elevations in the group as a whole in 5- and 6-year-olds. Requirements were that the paradigm would be easy to carry out, and not overly stressful (i.e. not overly distress the children and followed by a quick recovery). To this end, we designed a paradigm (the Children's Reactions to Evaluation Stress Test; CREST) with characteristics of social evaluation, unpredictability and uncontrollability. These elements have been found to be stressful in children aged 8 or older and in adults (Dickerson and Kemeny, 2004, Gunnar et al., 2009). Another element that has been suggested to be stressful for young children is failing publicly on a task that they believe even younger children could complete successfully (Gunnar et al., 2009). This element of forced failure was therefore also included in the paradigm. In short, the CREST was composed of three tasks that children had to perform in front of a judge. The tasks were rigged so as to provoke (partial) failure in the child's performance. Our hypothesis was that this paradigm would produce significant cortisol increases in the group as a whole in 5- and 6-year-olds. The possible effects of the following confounders were explored: child's sex, child's age, parental educational level, school, time of testing, sex of experimenter, and sex of judge.

METHODS

Participants

Children were recruited from four regular primary schools: one in the city of Nijmegen and three in nearby villages (The Netherlands). In The Netherlands, children attend primary school from age four onwards, with the first two years consisting of kindergarten. Parents of children attending their second year of school were approached by letter (N=179). In this letter the study was described, and parents were invited to enroll their child if he/she wished to participate. Forty-six applications were received, from which three children were excluded due to a clinically referred diagnosis and/or daily use of medication affecting cortisol secretion. The data of a fourth child was excluded due to physical illness (earache) on the testing day. The final group therefore consisted of 42 children (20 boys and 22 girls), with ages ranging between 57.0 and 75.9 months (*M*=68.0 months, SD=4.3). Written informed consent was obtained from all parents, and the study was approved by the Ethical Committee of the Faculty of Social Sciences, Radboud University Nijmegen, that follows the Helsinki Declaration.

Procedure

Prior to the testing session, the parents were phoned and informed about the procedure of the experiment in detail. They were asked to tell their child that the experimenter would ask them to do a couple of tasks during the session, but not to tell their child the exact nature of the tasks. Furthermore, all parents were asked to fill out a short demographics questionnaire including maternal and paternal educational level, and parents' and child's country of origin. The paradigm was carried out in a mobile lab (research van) parked next to the child's school. All the children were working in their own classroom prior to the testing session. This has the advantage of creating a relatively standard pre-test situation. Furthermore, because school is a familiar environment where children are accustomed to performing tasks, being taken to the mobile lab for the testing session was expected to avoid the possible arousal and anticipation stress of coming to the university lab for a testing session.

The testing took place in the afternoon, starting between 13.15 and 15.30 hours, in order to avoid the circadian morning peaks of cortisol (Kudielka and Wüst, 2010). Furthermore, because food and physical activation can influence cortisol concentrations, the teacher was asked not to allow the child to eat or do physical activities 30 minutes prior to the start of the testing session. Finally, the teacher was also asked to tell the child that the experimenter would ask him/her to do a couple of tasks during the session, but not to reveal the exact nature of the tasks

Experimental protocol: CREST (Children's Reactions to Evaluation Stress Test)

The paradigm had a duration of 20 minutes, and consisted of three tasks (15 min), followed by a period of stress due to an anticipated evaluation (5 min). The timeline of the experimental paradigm is presented in Figure 2. The children were tested by two researchers: an experimenter and a judge. The experimenter had the role of explaining the tasks and guiding the child through the testing session. If the child showed signs of distress the experimenter gave extra support to the child during the tasks. The judge had the role of evaluating the child's performance on the tasks. The age of both the experimenter and the judge ranged between 20 and 26 years.

After collecting the child in the classroom, the experimenter brought the child to the research van. To check whether children were really naive about the CREST, the experimenter asked them what the parents, teacher, or other children had told them about the session. There were no indications that any of the children had prior knowledge about the procedure. Subsequently, the experimenter motivated the child by presenting four presents (tissue, used eraser, bubble blower, kaleidoscope) and letting the child decide which present he/she liked most and which present he/she liked least. The child was then told that he/she had to perform some tasks in front of a judge. It was emphasized that the judge would decide which present the child deserved (i.e. the most liked or the least liked), depending on his/her task performance. Next, the judge entered the van and took a seat in front of the child and the experimenter.

During the *first task*, the child was asked to stand still in front of the judge, so that the judge could evaluate how still the child was able to stand. The child was told that it was extremely important not to move; otherwise an alarm would go off. This alarm consisted of a timer clicked onto their pocket with two wires that were fastened with tape to their wrist and ankle. First, the experimenter demonstrated the task with a 30-second performance, and the alarm did not go off. Next, the child had to stand still in front of the judge for 60 seconds. However, irrespective of the child's movements, the alarm went off on two preprogrammed times (after 20 and 40 seconds). Each time the alarm went off, the child was reminded by the judge that it was very important not to move.

During the *second task*, the child listened to a story about animals (3 minutes) recorded on an mp3 player. Five seconds of silence were implemented after each animal name, with eight animal names in total. The child was asked to imitate the sound belonging to the animal, every time he/she heard the name of an animal. The judge would evaluate the child's performance by showing a green card each time the sound was perfect. Irrespective of the performance, the child was only shown a green card in three out of eight animal sounds (first, second, and sixth sound).

In the *third task*, the child was asked to make a tower of empty soft drink cans identical to the one shown by the experimenter. The experimenter then uncovered an example tower which was invisibly glued, and that consisted of a pyramid of cans on their side (four,

three, two, and one can(s) in each layer). The judge told the child that the task was very easy for children to perform, and should therefore work out fine. In reality, when the child tried to build the tower, the cans kept on rolling away making the task impossible. After three minutes, the judge instructed the child that he/she had to stop building the tower.

All three parts of the test hence contained elements of social evaluation, as the judge was observing and judging the child's behavior throughout. The three tasks were also uncontrollable, as the child's behavior was insufficient to control the outcome (i.e. alarm went off twice, five animal sounds were not judged as perfect, and the tower of cans was impossible to build). Finally, unpredictability was ensured by the child's prior lack of knowledge of the nature of the tasks, by the unpredictability of the judge's response, and by the alarm suddenly going off without the child moving.

After completion of the tasks, the child was told that the judge was going to decide if he/she deserved the most liked or the least liked present and the judge went away for five minutes. This was done to elicit stress due to an anticipated evaluation in the child. During these five minutes, the child waited and chose a drawing of a popular cartoon to color in on his/her own. After five minutes, the judge returned and informed the child that he/she had performed very well on the tasks and definitely deserved the nicest present. This was the end of the paradigm. Subsequently, the child was debriefed by telling him/her that the experimenters had been pulling his/her leg and by showing the child how the test was rigged. After the debriefing, the child stayed in the research van with the experimenter for an additional 35 minutes, during which the child colored and watched an educational TV program. This time was used to provide a calm and enjoyable experience after the stressfulness of the test, and for collecting the remaining saliva samples. The child received a letter for his/her parents, including a comprehensive debriefing and a short report on the child's reactions to the paradigm. Finally, the child was asked not to tell the content of the test to other children, and was returned to the classroom or picked up by parents if school was already out.

Coercion was not used to initiate or complete testing. One child showed some signs of distress during the paradigm, but these signs disappeared when the experimenter gave the child extra support. No other children showed behavioral signs of distress, and all were able to complete the session.

Cortisol measurements

Six saliva samples were taken to obtain cortisol measurements of baseline, stress reactivity and recovery concentrations. Two samples measured baseline concentrations: one was taken just before the stress test (C1; pre-stress) and one 15 minutes after starting the stress test, after completion of the three tasks but before the period of stress due to an anticipated evaluation (C2; pre-response; previously it has been shown that salivary cortisol increases are not seen

at this time point after the start of stressor exposure (Dickerson & Kemeny, 2004). To assess stress reactivity, two samples were obtained 25 and 35 minutes after the beginning of the stress test (C3 and C4, respectively). Lastly, two samples obtained at 45 and 60 minutes after the beginning of the stress test were used as recovery measurements (C5 and C6, respectively). Eye sponges (BD Visispear™, Waltham, MA) were used as saliva sampling devices (deWeerth et al., 2007). The participant had to put an eye sponge in his/her mouth for approximately one minute. After that, the eye sponge was transferred into a plastic tube. These samples were taken to the lab and centrifuged for 10 minutes with a G-force of 3948 g units, and then stored in a freezer (-25°C) before analyses. Samples were analyzed by the Psychobiology Laboratory at the University of Trier, Germany. The inter-assay coefficients of variation ranged between 7.1% and 9%; the lower detection limit was 0.173 nmol/L for a 50 µl saliva sample.

Confounders

The following confounders were included because they can possibly influence cortisol reactivity: child's sex, child's age, parental educational level, school, time of testing, sex of experimenter, and sex of judge. Child sex was not matched to the sex of the experimenter or the judge. Parental educational level was computed by averaging the mother's and father's highest completed educational level (secondary or intermediate vocational education, higher vocational education, and university).

Statistical analyses

Logarithm transformations were applied to skewed data and data was checked for outliers. All cortisol concentrations were skewed and log transformed. One outlier was detected in the variable 'percentage increase'. This outlier was replaced by the variable mean plus three times its standard deviation (Hasings et al., 1947)[1].

The percentage increase in cortisol was calculated as highest stress reactivity value (C3 or C4) minus lowest baseline value (C1 or C2) divided by lowest baseline value (C1 or C2), and multiplied by 100. To calculate how many children had reacted to the paradigm with relevant cortisol increases, we used the definition of reaction by Schuetze et al. (2008), namely of an increase in cortisol from baseline to peak of at least two times the rate of error in the assay and two times larger than the lower limit of assay sensitivity. Hence, children showing an increase from baseline (lowest value of sample 1 and 2) to peak (highest value of sample 3 and 4) of 18% and an absolute increase from baseline to peak of 0.35 nmol/L were considered to have reacted to the paradigm with an increase in cortisol.

1 *Note:* removal of the outlier rendered similar significant results.

To analyze the course of the cortisol concentrations (baseline – stress reactivity – recovery), a repeated measures of analyses of variance (ANOVA) was computed. Greenhouse-Geisser corrections were applied where appropriate. To analyze if this paradigm induced a significant increase between the baseline cortisol concentration (lowest of samples 1 and 2) and stress reactivity concentration (highest of samples 3 and 4), a paired samples T-test was computed. A paired-samples T-test was also computed between the stress reactivity concentration (highest of samples 3 and 4) and the recovery concentration (lowest of samples 5 and 6) to analyze if the children's cortisol significantly decreased after the stress period.

RESULTS

Preliminary analyses

Table 1 shows the demographic characteristics and potential confounders of the sample. Correlations between the absolute increase in cortisol (highest peak concentration minus lowest baseline concentration) and the possible confounders child's sex, child's age, parental educational level, time of testing, sex of experimenter, and sex of judge, were non-significant (see Table 2). The absolute increase in cortisol did not differ between the children of different schools either ($F_{3, 37}$=0.41, p>0.05). These confounders were therefore not taken into account in the main analyses.

Main analyses

To analyze the course of the cortisol concentrations during the testing session, a repeated measures ANOVA was computed. The analysis revealed a significant quadratic time effect for the repeated measurements of cortisol (F(1.96, 78.5)=7.54, p=0.001). Paired samples T-tests showed a significant difference between baseline and peak cortisol concentrations (t40=-5.05, p<0.001, η^2=0.39), indicating that the paradigm induced a significant increase in children's cortisol concentrations for the group as a whole (see Figure 1). Furthermore, there was a significant difference in cortisol between peak and recovery concentrations (t41=7.88, p<0.001, η^2=0.60), indicating that the children's cortisol concentrations recover after the paradigm.

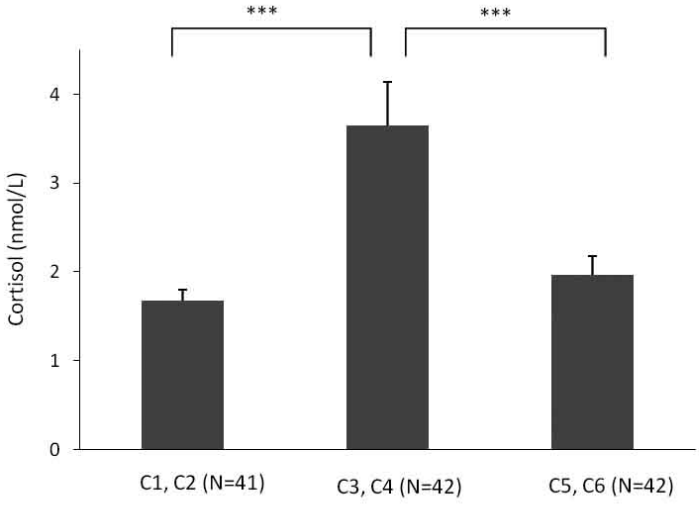

Figure 1. Salivary cortisol concentrations (mean ^ SEM) over the CREST paradigm: baseline concentration (lowest of samples C1 and C2), stress concentration (highest of samples C3 and C4), and recovery concentration (lowest of samples C5 and C6) for the group as a whole. N = number of children/ samples for each collection point. Paired samples t-tests showed a significant difference in cortisol concentrations between baseline and stress measurements, and a significant difference between stress and recovery measurements (***p < 0.001).

Although the group as a whole showed an increase in cortisol as a reaction to the paradigm, important inter-individual variability in reactivity was observed. The percentage increase in cortisol ranged between -52.0% and 747.8% (*M*=127.5, *SD*=190.9). Based on the definition of responders of Schuetze et al. (2008), of the total group 61% were found to have reacted significantly (t=-5.67, p<0.001, df=24), showing a mean increase in cortisol of 214%, whereas 39% were found not to have reacted, showing a non-significant 7.8% mean decrease in cortisol (t=1.75, p>0.05, df=15), see Figure 2.

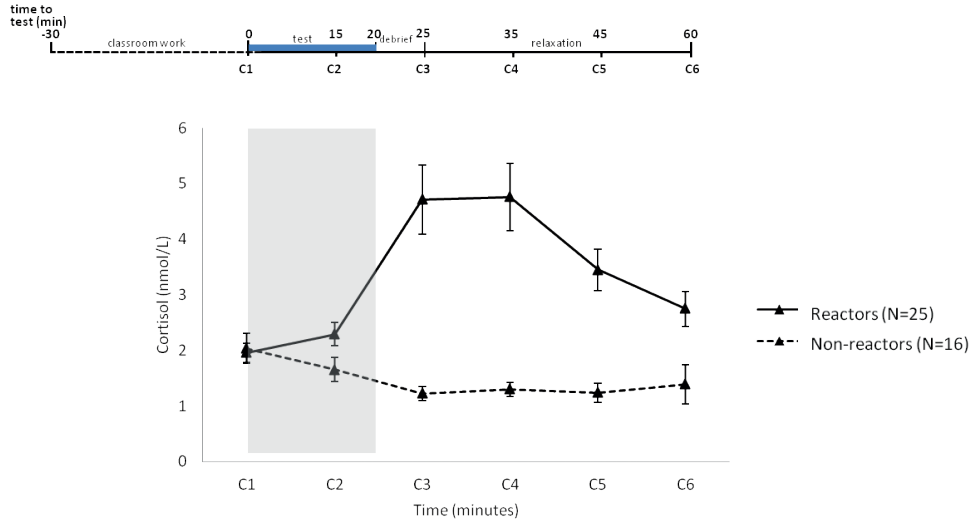

Figure 2. Top: detailed timeline of the CREST paradigm. Saliva sampling points for cortisol assessment are marked as C1, C2 (baseline), C3, C4 (stress reaction), C5, C6 (recovery). The thick bar indicates the stress phase (20 min: 15 min of performance stress plus 5 min of stress due to an anticipated evaluation). Bottom: salivary cortisol concentrations (mean ± SEM) over the CREST (gray box) for reactors, N=25 children; mean peak increase in salivary cortisol concentration [mean increase from lowest baseline sample (C1/C2) to highest stress sample (C3/C4)] = 214%; $p<0.001$, paired t-test; and non-reactors, N = 16 children; mean decrease in cortisol [mean change from lowest baseline sample (C1/C2) to highest stress sample (C3/C4)] = 7.8%; not significant, paired t-test.

DISCUSSION

In the present study we evaluated 5- and 6-year-olds' cortisol reactions to a social evaluative paradigm. Our findings showed that this paradigm was effective in provoking a significant cortisol increase in the group as a whole. The possible confounders children's sex, children's age, parental educational level, school, time of testing, sex of experimenter, and sex of judge showed no relation with children's cortisol reactivity. This implies that the CREST could be a suitable paradigm to elicit a cortisol response for both 5- and 6-year-old boys and girls from different SES-backgrounds and schools, and that the paradigm can be carried out by both male and female researchers.

These results show that a paradigm including elements of social evaluative threat, uncontrollability and unpredictability is adequate for inducing a cortisol response in 5- and 6-year-olds. The mean increase in cortisol concentrations from baseline to stress reactivity

was 127.5%. This mean cortisol increase is comparable to the mean increase obtained by stress tests for children above the age of 8, including the Trier Social Stress Test for Children (Buske-Kirschbaum et al., 1997, around 125% mean increase; Kudielka et al., around 120% mean increase; Yim et al., 2010, around 150% mean increase), and to the mean increase obtained in a recent study in which a new effective laboratory stressor was developed for 3-year-olds (Kryski et al., 2011; around 80% mean increase). It is important to note that, although effective, the CREST was not extremely stressful. The children showed no behavioral signs of being overly stressed by the paradigm, and displayed a relatively rapid post-stressor cortisol recovery (see Figure 1), as well as signs of being relaxed and in a good mood after the debriefing.

To our knowledge, this is the first paradigm showing that social evaluative threat, which is strengthened with elements of unpredictability and uncontrollability, results in a cortisol response in children of age 5 and 6. This is in line with research showing that this combination of elements instead of the separate elements produces cortisol increases in adults and older children (Dickerson and Kemeny, 2004, Gunnar et al., 2009). The strengths of the paradigm are first, that a 'judge' observed the child's performance during the three tasks. This researcher 'judge' was previously unknown to the child. This judging element most probably gave salience to the *social evaluative threat* factor of the procedure, producing a high ego involvement. Second, the content of the tasks was unknown beforehand, and the judging and alarm functioning were unrelated to the child's movements or quality of the noises, making the testing session *unpredictable*. And third, the forced failure on all three tasks made the procedure a highly *uncontrollable* situation for the children. Moreover, the tasks appeared to be relatively easy beforehand, and in the can tower task the children were also informed that the tower was very easy for other children to build.

Another, perhaps complementary, explanation for the cortisol response to this social evaluative paradigm could be that it combines several stressful tasks into one paradigm. While some of the earlier stress paradigms were based on a single task (e.g. Luby et al., 2003), the CREST combines three different tasks and a period of stress due to an anticipated evaluation into one single paradigm. It is possible that the use of these stressful tasks individually would not lead to significant increases in cortisol, and that only a combination of tasks leads to a situation that is stressful enough to provoke a cortisol reaction. It is also possible that children differ in which task(s) they experience as (more) stressful. For example, some children could become more stressed by not being able to stand still, while others become more stressed by not being correctly judged for producing perfect animal noises or building an apparently easy can tower. By combining different tasks, we increase the probability that all children are stressed by at least one of them. For example, in a study using the MacArthur Story Stem Battery (MSSB; von Klitzing et al., 2003), 5-year-olds had to complete a story about stressful everyday life events with the use of play figures. Although this stressor was chosen because it includes high ego involvement, it only led to a significant cortisol increase in girls (Hatzinger, 2007). In another study focusing on rigged failure, most of the children did not show a cortisol

response to the task. However, from the 15% of the children who did show a cortisol increase, all but one was male (Donzella et al., 2000). This suggests that a test that combines tasks with both high ego involvement and rigged failure (as well as other stressful elements), could be stressful for both girls and boys. In sum, the present study's succession of three short stressful tasks followed by a period of stress due to an anticipated evaluation could therefore at least partly explain the effectiveness of this paradigm for the group as a whole. The tasks could produce additive effects of stress and/or, due to the different nature of the tasks, they could ensure that most of the children were stressed by one of the tasks.

Finally, a further component of the paradigm that may have stimulated the children's cortisol responses could be that by increasing the children's motivation for participating by promising a preferred gift if they performed well and an unwanted gift if they did not perform well, children were more motivated to succeed in the test. Being motivated to succeed would in turn make the performance on the tasks more relevant and the forced failure therefore more stressful to the children.

A question is why a subgroup of participants (39%) failed to show a cortisol *increase* as a result of the paradigm. The initial cortisol values of this non-reacting group were not significantly different from those of the reacting group, so differences in pre-stress levels were not behind the differences in reaction. In this context is important to note that having 39% of participants not react to a stress test is not unusual. Although often not reported, most stress tests for children fail to produce a cortisol reaction in all individuals (Klimes-Dougan, 2001; Gunnar et al., 2009). Given the nature of our study group, i.e. young children, our paradigm was limited by ethical constraints and therefore designed not to be overly stressful. It is therefore very possible that some children were simply not stressed by the paradigm. Nonetheless, many other possible explanations exist for the large inter-individual differences in reactivity. Possible candidate factors that could play a role in individual cortisol reactions are temperament, experience with social evaluative situations, and adverse experiences in early development. Also, increases versus decreases in cortisol as reactions to stressors could be representing different biological profiles that are related to the emotional reactions to stressors. According to Moons et al. (2010), in a study with the TSST in adults, anger reactions to psychosocial stress would be related to increases in cortisol, while fear reactions would be related to decreases in cortisol. Measuring children's emotions as a reaction to the CREST paradigm, and relating them to their cortisol increases, decreases, or lack of change in cortisol, is a logical next step in this line of research. Future research will therefore hopefully shed more light on why some children 'fail' to react with a cortisol increase to this test while others show increases of more than 700%.

Limitations and future directions

This study found that a social evaluative paradigm, strengthened with elements of unpredictability and uncontrollability, elicits a cortisol response in young children. Therefore, the paradigm could be a promising tool for future research on cortisol reactivity and recovery in 5- and 6-year-olds. However, there are some limitations. First, although we asked the teacher not to allow the child to eat or do physical activities 30 minutes prior to the testing session and all children were working in the classroom before the test, we do not have measures of the teachers' compliance with our request. In future studies, researchers should either assess the teachers' compliance or include a short period of rest before starting the test. Another limitation is that we only used cortisol as a measurement of stress reactivity and recovery. Including behavioral measures or measures of the sympathetic nervous system, such as cardiovascular measures and measures of alpha amylase, could render a more complete picture of young children's stress reactions to this paradigm. Finally, future research is needed to replicate our findings in larger samples. In addition, it would be interesting to determine whether the CREST is effective for younger or older age-groups, and to investigate whether it can be used to study possible abnormalities in stress reactivity in clinically referred groups.

CONCLUSION

This study showed that our new laboratory paradigm - CREST - induced a significant cortisol increase in 5- and 6-year-olds. No effects of possible confounders (child's sex, child's age, parental educational level, school, time of testing, sex of experimenter, and sex of judge) were observed. To our knowledge, this is the first study that shows a general cortisol response in 5- and 6-year-olds to a standardized laboratory paradigm. As such, this paradigm is a promising tool to be used in the future and might help to unravel the complex early life interactions between physiology and psychology that can lead to (psycho)pathology in adulthood.

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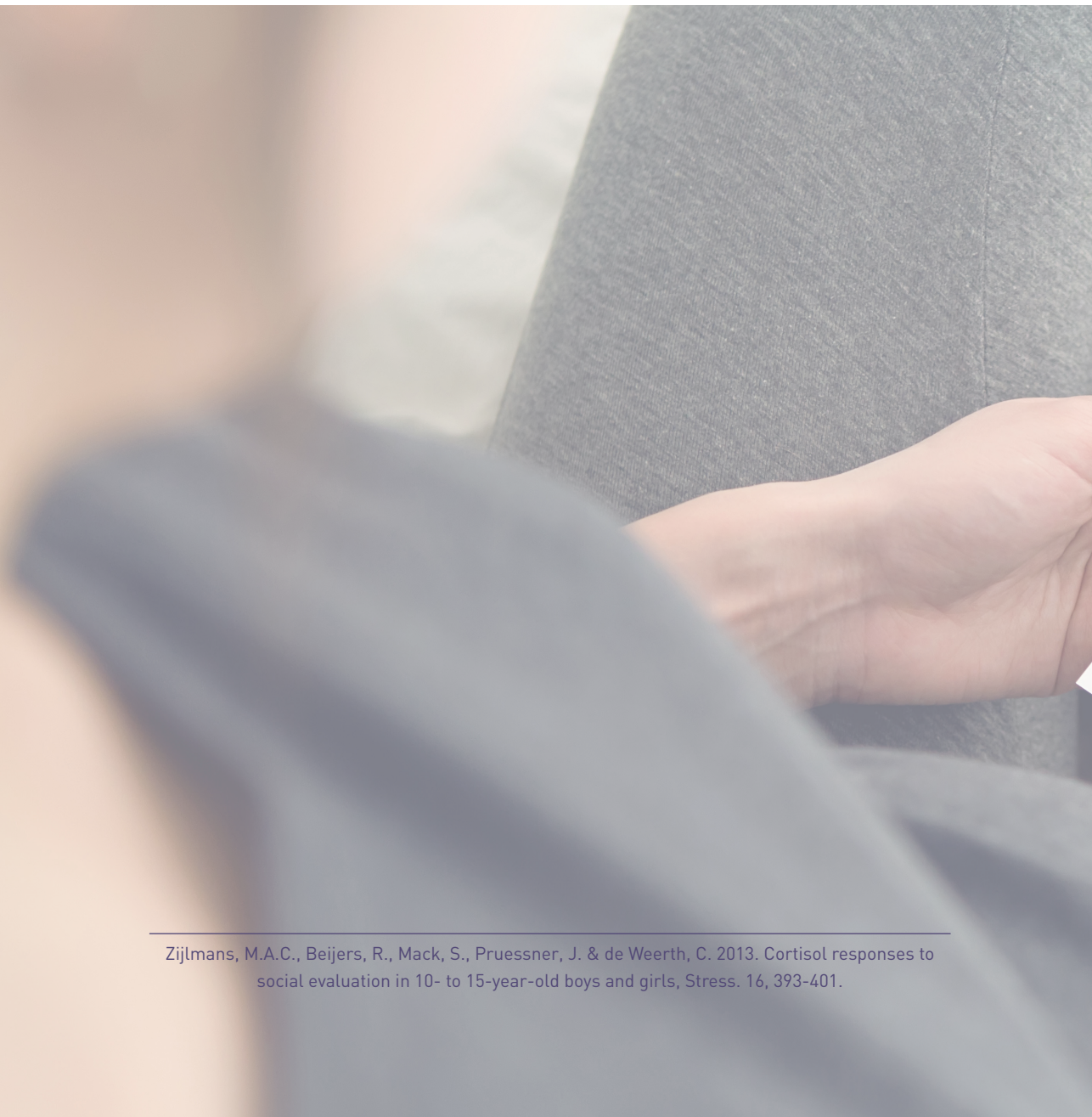
Table 1. Descriptive statistics of demographic characteristics and confounders (N=42)

Variables	Value
Maternal educational level (%)	
Secondary or intermediate vocational education	17.9
Higher vocational education	42.9
University	39.3
Paternal educational level (%)	
Secondary or intermediate vocational education	14.3
Higher vocational education	31.0
University	23.8
Child age in months, mean ± SD (range)	68.0 ± 4.3 (57.0-75.9)
Child sex (%)	
Boys	47.6%
Girls	52.4%
Time of testing, mean ± SD (range)	14.0 ± 35.4 (13.1-15.3)
School (%)	
School 1	26.2%
School 2	40.5%
School 3	21.4%
School 4	11.9%
Sex of experimenter (%)	
Male	14.3%
Female	85.7%
Sex of judge (%)	
Male	71.4%
Female	28.6%

Table 2. Correlations between the actual increase in salivary cortisol concentration (nmol/L) and possible confounders.

	child's sex	child's age[1]	parental educational level[1]	time of testing[1]	sex of experimenter	sex of judge
Δ cortisol	0.04	0.06	-0.12	0.13	0.09	-0.22

Note. [1]Pearson correlations; others are Spearman correlations. All non-significant.



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Chapter 5

Cortisol responses to social evaluation in 10- to 15-year-old boys and girls

Maartje A.C. Zijlmans, Roseriet Beijers, Simon Mack, Jens Pruessner & Carolina de Weerth

ABSTRACT

This study investigated cortisol responses and perceived stress of 10 to 15-year-olds to a computerized paradigm including elements of social evaluation, unpredictability and uncontrollability. Both age and sex differences were examined.

Participants were 52 children and adolescents (23 boys, *mean age* = 12.5 years). Over the course of a roughly two hour testing session where subjects were exposed to the computerized testing paradigm, seven saliva samples were obtained to measure pre-stress, stress reactivity and recovery concentrations of cortisol. In addition, subjective emotional stress experiences were recorded.

The results showed no effect of age on cortisol responses. Furthermore, although both sexes reported experiencing the paradigm as (equally) stressful, only boys reacted with significant cortisol increases (M = 162.93%).

To our knowledge, this is the first computerized stressor that induces cortisol responses in 10 to 15-year old boys. Whether girls' perceived stress results in the activation of other biological systems, such as the sympathetic nervous system as well as in differential activation of brain regions, remains to be determined. Future studies investigating sex differences in stress reactivity during adolescence should include neuroimaging, as well as psychophysiological measures, to unveil some of the mechanisms behind the current findings.

INTRODUCTION

In response to stressful challenges, the Hypothalamic Pituitary Adrenal axis (HPA axis) becomes activated, resulting in the release of a cascade of hormones that helps the body to mobilize energy resources to cope more efficiently with a stressful situation. The hormone cortisol, secreted by the adrenal cortex, is the end product of this axis (Sapolsky et al., 2000). In humans, the cortisol stress response is considered adaptive and necessary. However, repeated or chronic activation of the HPA axis especially early in life can result in atypical HPA axis reactivity, with blunted or exaggerated HPA axis responses to stress. This, in turn, has been linked to psychopathology later in life (e.g., Heim and Nemeroff, 2001; Heim et al., 2008; Petrowski et al., 2010).

Infants are born with a functional HPA axis, reacting with the secretion of cortisol when confronted with stressors (Jansen et al., 2010). During development and in response to experience, the HPA axis' regulation is further fine-tuned and adapted to each child's individual environment. An important period during development in which humans are faced with an increasing number of (psychological) challenges, is the transition from middle childhood to adolescence. There is some discussion in the literature whether this period is also accompanied by organizational effects on the regulation of the HPA axis, which might lead to blunted HPA axis reactivity in 11 to 13 year olds (Gunnar and Vazquez, 2006). However, some studies have also reported normal HPA axis reactivity during that period (Kudielka et al., 2004). Investigating developmental changes in HPA axis reactivity during adolescence is especially important as adolescence is known to be a vulnerable period for the development of a variety of psychological disorders, including depression, anxiety and substance dependence (Kessler et al., 2005). Moreover, different psychological challenges and physiological changes are faced by male and female adolescents which could be related to differences in HPA axis reactivity.

A few studies have examined cortisol responses in children during middle childhood and adolescence with the use of laboratory stressors. Social evaluation, combined with uncontrollability and unpredictability, have proven to be effective in eliciting a physiological stress response with laboratory stressors in both adults and children (Dickerson and Kemeny, 2004; Gunnar et al., 2009). Stroud et al. (2009) examined the stress responses of 7- to 17-year-olds, and showed that both children and adolescents reacted with significant increases in cortisol levels during a social performance task. In this study, the cortisol response was larger for the adolescents as compared to the younger children. Another study in children and adolescents aged 11 to 16 found a significant cortisol increase in reaction to a social performance paradigm only in older boys (13-15 year olds) (Klimes-Dougan et al., 2001). These boys showed increases in cortisol of 40% above baseline. In similarly aged 12- to 15-year-old children, Westenberg et al. (2009) also found a significant cortisol increase in reaction to a public speaking task, with no significant main effects of age and sex. Furthermore, a study by Gunnar et al. (2009) examined stress responses in children of four different ages. Nine-year-

old children and 15-year-old adolescents reacted with a cortisol increase to the test. Of the 13-year-olds, only the girls showed a cortisol increase, while the 11-year-olds, and 13-year-old boys showed no increase of cortisol in response to the stressor. Finally, Kudielka et al. (2004) showed significant cortisol increases in 9 to 15-year olds in response to the Trier Social Stress Test (TSST; Kirschbaum et al., 2003).

To summarize, previous research suggested that there might be developmental changes in stress reactivity during the period of middle childhood to adolescence in the physiology of stress reactivity to stressors. However, only a few studies are available, and the findings are inconclusive. In addition, the role played by additional factors including sex in the developmental changes in stress reactivity also remains to be elucidated.

Thus, the aim of the present study was to examine the effects of age and sex in physiological stress reactivity (cortisol) in 10 to 15-year-old children. To this end we used a newly developed laboratory stress paradigm that includes elements of social evaluation, unpredictability and uncontrollability. Two important factors led to the decision to develop a new stress paradigm. First, we wished to develop a paradigm that was specifically aimed at adolescents. To achieve this, the evaluation was more focused on personal characteristics than on academic achievement. We hypothesized that this would be stressful for teenagers, because their personal identity and social self are developing and important for acceptance by others (Harter 1990). Also, comparisons with peers were included in the paradigm because of the importance of peer relations in middle childhood and adolescence. The second reason was of a pragmatic nature. Extant effective laboratory stressors for children mostly include public speaking, requiring several experimenters to carry them out (test leader, two or more panel members). The paradigm designed for the present study is computer-based and therefore more efficient than public speaking tasks.

Based on previous research and the age range of the present study, our hypothesis was that both younger and older children would react with significant increases in cortisol to the paradigm. Furthermore, and because of the contradictory findings in the literature, we examined sex differences in stress reactivity, but had no specific hypotheses on the direction of possible differences.

METHODS

Participants

Children were recruited from 5 primary and 3 secondary schools in Nijmegen (The Netherlands) by distributing 1075 advertisements and information letters for both children and their parents. This recruitment resulted in 76 applications. Participants who used medication, had a diagnosis for neuropsychiatric or mental disorders, and children with recent exposure to traumatic

events were excluded from the study (N=7). Furthermore, seventeen children were not included in the study because of scheduling difficulties and illness on the testing day. The final group consisted of 52 children (23 boys) with ages ranging from 10.4 to 15.5 years (*M* = 12.52, SD = 1.21). Written informed consent was obtained from all participants and their parents. The study was approved by the Faculty of Social Sciences' Ethical Committee of the Radboud University Nijmegen, which follows the international Helsinki Declaration.

Procedure

The testing session took place in the Behavioural Science Institute laboratory of the Radboud University Nijmegen. Sessions took place between 1:00 PM and 8:00 PM and had a total duration of 130 minutes. Participants were asked to abstain from eating solids and performing extensive physical activities 45 minutes before arriving in the lab. After arrival, participants were photographed with a neutral facial expression against a neutral background in a black T-shirt. This photo of their head and shoulders was for later use in the computer task. Afterwards, participants relaxed for 40 minutes in a testing room which was equipped like a living-room, including carpet, sofas and stereo. Participants were then taken to a cubicle where the stress-provoking computer paradigm took place. In the cubicle participants were verbally instructed by the test leader about the computer paradigm (10 minutes). After that, the participant completed the computer paradigm (3 minutes practice, 32 minutes actual paradigm), followed by one assessment of perceived stress and debriefing (5 minutes). The cubicle part had a total duration of 50 minutes. In the final 40 minutes, participants again relaxed in the 'living-room' while reading magazines (first 20 minutes) and filling out extra questionnaires (last 20 minutes). Stress (i.e., cortisol in saliva) was measured -45, 5, 30, 40, 50, 60, and 80 minutes in reference to the onset of the computerized paradigm. At the end of the testing session each child received a 10 Euro voucher as a gift for participation. See Figure 1 for a timeline of the laboratory visit.

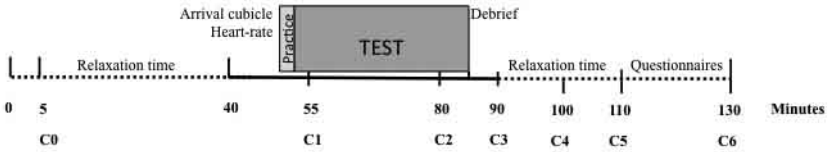

Figure 1. Timeline of the testing session. The cortisol measurements are marked as C0 to C6.

Computer paradigm: Social Evaluative Stress Test (SEST)

Upon entering the cubicle participants were connected with two fingers of their non-dominant hand to a "heart-rate device" connected to the computer. This device was actually a dummy, but the participant was told that it registered heart rate and that even small movements of the hand could negatively affect its working. As movements could ruin the data, the child was asked to keep this hand as still as possible. However, and irrespective of the child's actual movements, the alarm light on the screen turned on at two predetermined time points during the answering of the 36 questions (see below) to indicate inappropriate movements.
The investigator then told the child that multiple-choice questions would appear on the computer screen. These could be answered with the dominant hand by means of a button box. A panel would judge the answers as well as the participant's facial expression and posture by means of a camera next to the computer. After that the children completed a practice session with a total of 28 easy, non-personal questions (e.g. what is the color of this flower?). Thereafter, the following instructions were displayed on the screen in Dutch:

> "The Dutch Film Association (DFA) has produced a brand new and thrilling 3D film. They want it to be a success! They asked our research institute to find out whether kids of your age like the film. We are looking for kids who *represent the youth of today*. Their opinion about the film is important to us. They will be the first kids to watch the film and give their opinion about it! From research we know that *nice* kids represent the youth of today best. To find out whether you are nice and represent the youth of today, we ask you to fill in personal questions on the computer. A strict panel will compare your answers with those of four other children and select the three who represent today's youth the best. Those three kids may watch the film and give their opinion. However, if you belong to the remaining two you will have to give a presentation in front of the panel telling them why you think you are nice and represent the youth. If you are able to convince the panel, you may watch the film as well."

After these instructions, cortisol sample 1 (C1) was taken. To increase the credibility of the story, the photo of the participant together with photo's of the four other "participating children", as well as a photo of the "panel" (i.e., 4 adults sitting in lab coats behind a table, with neutral expressions on their faces) were displayed on the screen. Besides that, one of the lab-coated panel members from the photo entered the room before the test began to resolve a "program error that was delaying the start" and introduced him/herself to the participant.

Figure 2 shows the set-up of the computer screen during the test session. The photos on the right showed the position of each child: children in the green zone would watch the movie, while children in the red zone would have to give a presentation to the jury.

Figure 2. Set up computer screen. The photo is derived from the Radboud Faces Database (Langner et al. 2010) and is not from a participant.

After the panel member had left, a total of 36 multiple choice questions were displayed with a fixed time window of 20 seconds per question (the seconds left were shown on the screen). The questions included personal questions (i.e., addressing personal traits, ideas, hobbies, and position in life), and knowledge questions (i.e., addressing knowledge of facts with high levels of difficulty). Personal and knowledge questions were shown in a fixed design; in each block three personal questions were followed by three knowledge questions. Prior to testing, twenty children had evaluated the personal questions on age appropriateness and meaningfulness. Based on their feedback, two test versions were designed: for ages 10-12 and 13-15. In total,

participants were presented six blocks of questions. After each block intermediate judgments of the panel changed the participant's position from initially high to low. After answering all the questions, participants looked at a neutral screen for 5 minutes before being told that their final ranking was the fourth position and that they now had to prepare a presentation for the panel. After another 5 minutes, perceived stress was assessed and the stress test ended with the debriefing of the participant. In the debriefing all participants were told that they did not actually have to present in front of the jury. Additionally, they were informed that the SEST was preprogrammed in such a way that it was impossible to win the competition.

The SEST was programmed using E-prime version 1.0 (Schneider et al., 2002), and was designed to exclude speech and movement in the participant. In this way it is compatible for use during future neuroimaging sessions. Moreover, the experimental design will enable explicit allocation of neural activation to specific test elements: the control condition (i.e., training session without social evaluation and including only easy, non-personal, knowledge questions), the test session with personal questions, and the test session with knowledge questions.

In sum, the SEST contains elements of social evaluation (i.e. picture of jury, comparison with other children, several judging moments, camera), unpredictability (i.e. unknown paradigm) and of uncontrollability (i.e. 'heart-rate device' going off twice irrespective of movements, time pressure for answering questions, falling in the ranking, too difficult questions). In this way the paradigm capitalized on the elements that are known to induce stress in children and adults in laboratory settings.

Cortisol

Seven saliva samples were taken to obtain cortisol measurements of pre-stress levels, stress levels, and recovery levels. The saliva samples were obtained by having the participants spit through a short straw into a 2.5 ml tube at -45, 5, 30, 40, 50, 60, and 80 minutes in reference to the onset of the computerized paradigm. All participants provided samples of sufficient volume and quality for assay. The samples were frozen at -20°C before they were analyzed at the Psychobiology Laboratory of the University of Trier, Germany. The inter-assay coefficients of variation ranged between 7.1% - 9.0%; the sensitivity of the assay was 0.173 nmol/L.

Confounders

Perceived stress

To measure perceived stress, participants rated two items on a 7-point scale (1 = *not at all stressful/exciting* to 7 = *very stressful/exciting*). These items were: 1. How stressful/exciting did you find the experiment; and 2. How stressful/exciting did you find the expectation of giving

a presentation in front of the panel? There was a significant correlation between the items: $r(49)=0.62$, $p<0.001$. Therefore, the mean score of the two items was used in the analyses.

Credibility

Participants rated four items on a 7-point scale to assess the credibility of the social evaluative paradigm. These items were: 1. To what extent did you believe the cover story?, 2. To what extent did you believe in the presence of other participants?, 3. To what extent did you believe in the existence of the jury?, and 4. To what extent did you believe the heart-rate measures? The scores on the four items were all positively correlated, with r's ranging between 0.22 and 0.53. To obtain one measure of credibility, the mean score of the four items was used in the analyses.

Additional confounders

To control for potential confounders that can influence cortisol reactivity, the following variables were included in the analyses: means of transportation (arrival by public transport or car versus arrival by bike), school type (primary versus secondary school), and time of day.

Statistical analyses

The first cortisol measurement C0 (t=-45min) showed an excessive variation between subjects, likely indicating a confounding effect from activities taking place prior to the arrival at the laboratory. It was thus excluded from all analyses. The six remaining cortisol samples (C1-C6) were used to study reactivity and recovery to the stressor. Absolute increase (delta) in cortisol was calculated by abstracting the lowest of samples 1 and 2 from the highest of samples 3, 4, and 5.

Skewed data was log-transformed, and all measures were checked for outliers. Each cortisol measurement (C1 - C6) contained one positive outlier (six in total). Furthermore, one positive outlier in the absolute increase in cortisol was detected. All outliers were replaced by the group mean plus 3 standard deviations (Hasings et al., 1947).

To analyze if this paradigm induced a significant increase between the baseline cortisol level (lowest of samples 1 and 2) and stress reactivity level (highest of samples 3, 4 and 5) for the group as a whole, a paired samples T-test was computed. A paired samples T-test was also computed between the stress reactivity level (highest of samples 3, 4 and 5) and the recovery level (sample 6) to analyze if the participants' cortisol significantly decreased after the stress period.

To examine differences between middle childhood and adolescence, two age groups were created (age 10-12, age 13-15). To analyze the main effect of the paradigm on the cortisol levels, a three factor (sex by age group by time) mixed design analyses of variance (RM-ANCOVA) was computed. Greenhouse-Geisser corrections were applied where appropriate. As between subject factors child's sex and age (age group 10-12, age group 13-15) were included in the

main analyses. Dependent variable were the cortisol levels (with time as the within subject factor -5, 30, 40, 50, 60, and 80 minutes in reference to the onset of the stressor). Because of the relatively small sample size and in order to avoid possible spurious results (Simmons et al. 2011), we only included confounders that significantly correlated with the absolute increase in cortisol as covariates in the main analyses.

Independent samples T-tests for all separate cortisol measurements were conducted to analyze differences between boys and girls, and differences between middle childhood (age 10-12) and adolescence (13-15).

RESULTS

Table 1 shows the demographic characteristics and potential confounders of the sample for the total group as well as separated by sex and age groups. Independent sample t-tests showed that the time of testing occurred significantly later for girls than boys ($t(50)=-2.48$, $p<0.05$). Also, the older children were tested significantly later compared to the younger age group ($t(50)=-2.20$, $p<0.05$). Boys showed significantly higher absolute cortisol increases (delta) than girls ($t(50)=-3.49$, $p<0.001$), and younger children showed marginally significant higher cortisol increases than the older children ($t(50)=1.89$, $p=0.07$). Furthermore, the children's mean scores on perceived stressfulness and credibility of the test indicated that on average they found the test stressful and believable. Girls rated the credibility of the test higher than boys ($t(49)=-2.03$, $p<0.05$), while there were no significant differences in perceived stressfulness between boys and girls and between younger and older children.

Paired samples T-tests showed a significant difference between pre-stress cortisol levels and peak levels ($t(51)=-4.04$, $p<0.001$, $d=0.66$), indicating that the paradigm induced a significant increase in children's cortisol levels for the group as a whole (see Figure 3). Furthermore, there was a significant difference in cortisol between peak levels and recovery levels ($t(51)=5.37$, $p<0.001$, $d=0.62$), indicating that the children's cortisol levels returned back to baseline after the end of the paradigm. Regarding sex differences, for boys, paired samples T-test showed a significant increase in cortisol ($t(22)=-4.04$, $p<0.001$, $d=1.22$), followed by a significant decrease in cortisol ($t(22)=4.37$, $p<0.001$, $d=0.82$). For girls the increase in cortisol showed only a trend for significancy ($t(28)=-1.88$, $p=0.07$, $d=0.27$) while the ensuing decrease in cortisol was significant ($t(28)=3.54$, $p<0.001$, $d=0.56$).

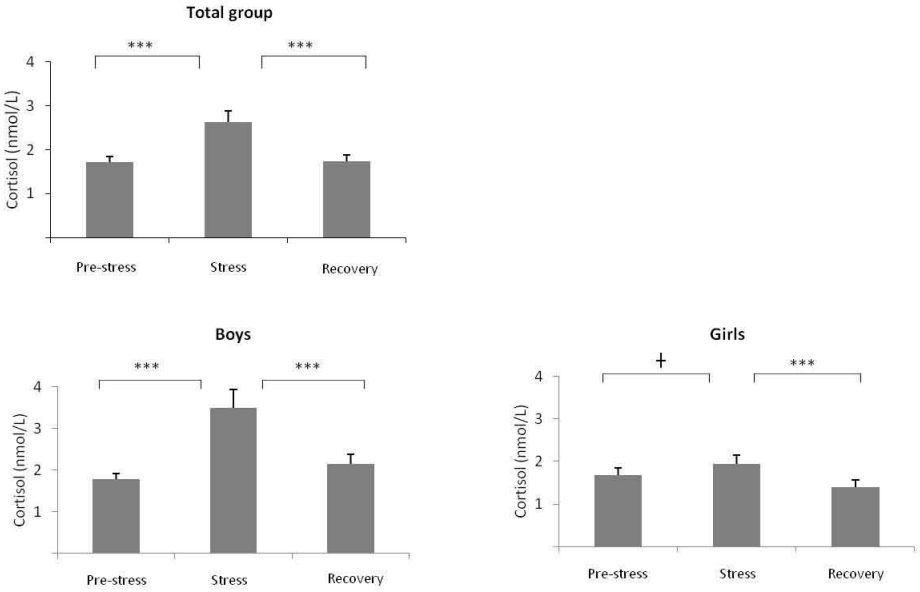

Figure 3. Salivary cortisol levels (means ± SEM) over the stress paradigm: pre-stress level (lowest of samples 1 and 2), stress level (highest of samples 3, 4, 5), and recovery level (sample 6) for the group as a whole (N=52), for boys (N=23) and for girls (N=29). Paired samples T-tests were used to examine the difference between pre-stress level, stress level and recovery level. ***p<.001, + p=0.07

Figure 4 shows the course of the cortisol levels separately for girls and boys. Independent samples T-tests showed that boys have significantly higher cortisol values than girls at C3 ($t(33.43)=3.06$, $p<0.05$, $d=0.93$), C4 ($t(30.04)=2.70$, $p<0.05$, $d=0.83$), C5 ($t(34.30)=2.82$, $p<0.05$, $d=0.85$), and C6 ($t(50)=2.80$, $p<0.05$, $d=0.80$).

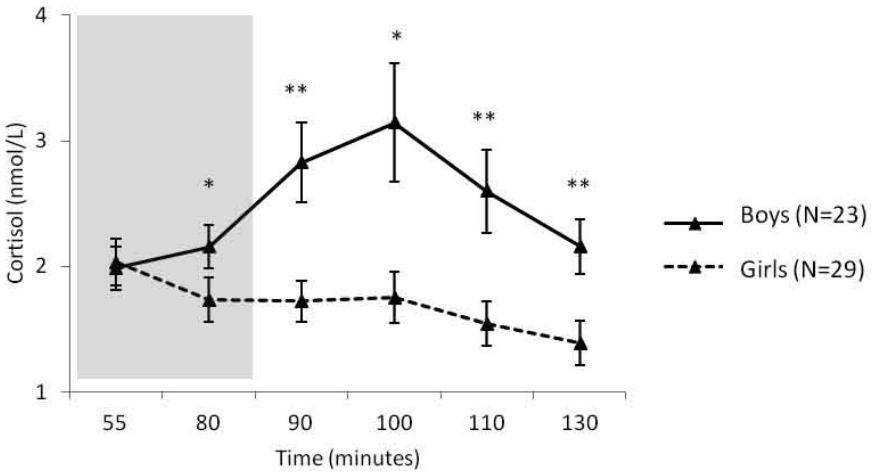

Figure 4. Cortisol levels (means ± SEM) over the social evaluative stress test (SEST) for boys (N=23; mean increase in cortisol = 162.93%), and girls (N=29; mean increase in cortisol = 44.41%). The gray box indicates the stress phase. Independent samples T-tests were used to examine the difference in the six cortisol measurements between boys and girls. **p<0.001, *p<0.05

Figure 5 shows the course of cortisol levels for the two age groups (10-12 and 13-15). While younger children seem to react with higher cortisol levels as compared to older children, independent samples T-tests showed that, apart from the two trends for significance, C4 ($t(48.63)=1.89$, $p=0.06$, $d=0.48$) and C5 ($t(49.94)=1.78$, $p=0.08$, $d=0.46$), there were no significant differences between the six cortisol measurements of the younger and older children.

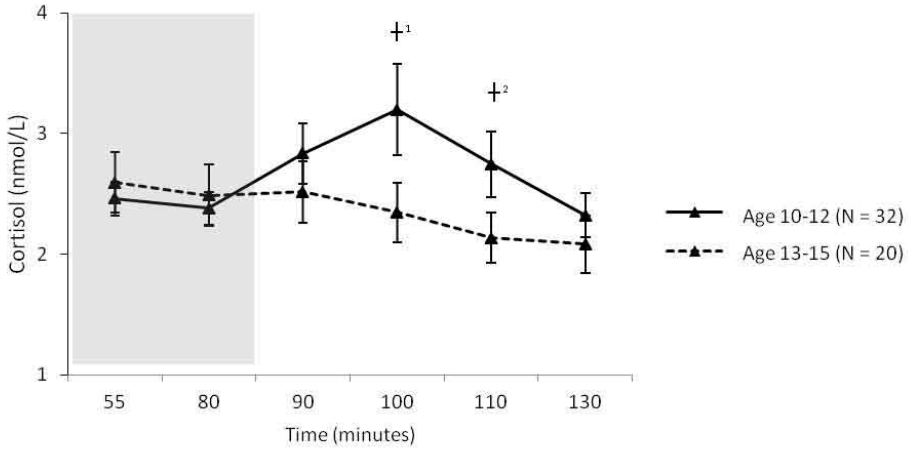

Figure 5. Cortisol levels (means ± SEM) over the social evaluative stress test (SEST) for 10-12 year olds (N=32; mean increase in cortisol = 79.85%), and 13-15 year olds (N=20; mean increase in cortisol = 30.17%). The gray box indicates the stress phase. Independent samples T-tests were used to examine the difference in the six cortisol measurements between age groups.
† [1] p=0.06, † [2] p=0.08

The correlations table (see Table 2) showed that the absolute increase in cortisol (delta) was not correlated with child age and was significantly correlated with child sex; boys reacted with significantly higher increases in cortisol than girls ($r(50)=-0.39$, $p<0.01$). Of the confounders, means of transportation was significantly correlated with the delta cortisol ($r(47)=-0.32$, $p<0.05$). Children who had cycled to the lab showed lower deltas than children who had come by car/bus. Therefore, means of transportation was included as a confounder in the main analyses. Furthermore, the delta cortisol was significant correlated with perceived stress, with children reporting higher levels of perceived stress showing higher absolute increases in cortisol ($r(49)=0.29$, $p<0.05$). The delta cortisol was not correlated with the other confounders (i.e. time of testing, school type, and credibility of the paradigm), and these were therefore excluded from further analyses. Furthermore, the correlations table shows that when the group was split by sex, only means of transportation continued to be significantly positively correlated with the delta cortisol in girls ($r(25)=-0.48$, $p<0.05$).

ANOVA analyses

To analyze the dynamic of the cortisol levels across the testing session, a repeated measures ANCOVA was computed, with child sex and age group (10-12 and 13-15) as a between subject factor, and means of transportation and perceived stress as confounders. The analysis revealed no significant main effect of time for the repeated measurements of cortisol (F(2.64, 113.66)=0.20, p>0.05). However, and in line with the reported T-tests, the cortisol measurements significantly interacted with child's sex (F(2.64,113.66)=5.41, p<0.01), with boys showing higher cortisol reactivity to the paradigm than girls. Child's age did not significantly interact with the cortisol measurements (F(2.64,113.66)=0.32, p>0.05). Furthermore, the confounders means of transportation (F(2.64,113.66)=5.35, p<0.01), and perceived stress (F(2.64,113.66)=3.72, p<0.05) significantly interacted with the cortisol measurements as described above.

DISCUSSION

This study examined cortisol responses of 10 to 15-year-olds to a computerized paradigm including elements of social evaluation, unpredictability, and uncontrollability. Both age and sex differences were examined. The results showed no effects of age on cortisol responses. In contrast, it appeared that the cortisol response was significantly affected by sex. Although both sexes reported the paradigm as (equally) stressful, only boys reacted to the paradigm with significant cortisol increases. To our knowledge, this is the first computerized stressor that induces cortisol responses in 10 to 15-year old boys. The question remains why boys, and not girls, responded with increased cortisol levels to the paradigm.

From a psychological point of view, an explanation for finding substantial cortisol reactions in boys can be found in the elements of the paradigm we used. Previous research showed that men are physiologically more affected by achievement type stressors when compared to women (Stroud et al., 2002). Our paradigm included achievement (i.e. knowledge questions in competition with peers) which could have led to stronger cortisol increases in boys. On the other hand, previous research has also shown that women react more physiologically to interpersonal challenges and social rejection (Stroud et al. 2002). Our paradigm contained an interpersonal challenge (i.e. competition with peers, personal questions) and social rejection (i.e. ranking based on personal questions), but these elements were perhaps not powerful enough in their present form to trigger stress in the girls. This could be due to the use of an indirect, computerized interpersonal challenge and rejection, instead of more direct, face-to-face one. However, if the nature of the paradigm were responsible for the sex differences in cortisol responses, one would expect boys to not only show higher cortisol responses, but also to report the task as more stressful. This was not the case: both boys and girls reported the

paradigm to be equally stressful. This makes it probable that not only psychological factors are behind the sex differences found in physiological reactivity.

Physiological differences between boys and girls could also (partly) explain why only boys reacted with cortisol reactions to the paradigm. In adults, there is a difference between males and females in their physiological reactivity to acute stressors. Females show greater interoceptive corticolimbic reactivity to acute stress, whereas males show stronger peripheral reactivity, including the HPA axis (Ordaz and Luna, 2012). This sex difference emerges in mid- to late- adolescence and is probably caused by gonadal hormones. During puberty, estrogen levels increase in girls with high levels of estrogen receptor density especially in the corticolimbic circuitry (ter Horst et al., 2009). Estrogens block the receptor binding of cortisol by facilitating the production of corticosteroid binding globulin, a molecule that binds to cortisol (McEwen, 2002). The increased levels of estrogens during puberty are related to the age of menarche (DiVall and Radovick, 2008). Moreover, estrogens fluctuate during the menstrual cycle, with the highest concentrations in the follicular phase. Although in adult females the menstrual phase is related to cortisol responses (Kajantie and Philips, 2006), there is only one study that looked at this during adolescence. Bouma et al. (2009) found that adolescent free cycling girls (age 15-17) reacted with lower cortisol responses to a stressor than boys. However, there were no effects of menstrual phase, possibly because girls of this age had not developed a stable menstrual cycle yet. Because our sample was fairly young, it is likely that not all the girls were already menstruating. Unfortunately, neither the onset of the menstrual cycle, nor the menstrual cycle phase was assessed in the current study. Thus, we can only speculate that the observed sex differences in cortisol responses in the current study might have been caused in part by girls with increased levels of estrogens.

Another factor influencing the concentration of estrogens is the use of oral contraceptives (OC). Previous research in adults showed that OC users show lower cortisol responses to a laboratory stressor than non OC users, with cortisol levels of OC users similar to women who were in the follicular phase (Kajantie and Philips, 2006). A study including adolescents found that OC users showed no response to the stress test (Bouma et al., 2009). Given that the mean age of OC users in the Netherlands is 16.1 years (Stichting Farmaceutische Kengetallen, 2009), and that the mean age of our sample was 12.5 years with the oldest participant not reaching the age of 16 years yet, we think it is unlikely that OC use played an important role in this study. However, we did not directly assess the use of oral contraceptives in our female participants, thus we can't exclude with certainty the possibility that some of our girls might have used oral contraceptives.

Despite the fact that the different ages in our sample perceived the paradigm as equally stressful, our results showed an interesting trend for reactivity differences. The youngest age group (age 10-12) had marginally higher cortisol levels in reaction to the paradigm than the older age group (13-15). Here again, both psychological and physiological factors could explain these differences. For example, younger children, due to lack of experience, might

be more stressed by the anticipation of a presentation in front of a jury. Also, pubertal stage and hormones could be behind the differences. However, and on a cautionary note, these differences were only marginally significant and our sample size was small, making these interpretations more speculative. Moreover, the fact that the oldest age group contained more girls than boys (65% girls) could also explain these marginal differences. Additional research with larger samples and with broader age ranges is needed in order to detect developmental changes in stress reactivity to specific stressors. Nonetheless, the current results do add support for the idea that cortisol reactivity can be observed at all ages, including the transition from childhood to adolescence, as we clearly saw cortisol increases in boys. The current results thus support the idea of a consistent HPA axis reactivity across the lifespan (Kudielka et al., 2004), and shed further doubt on the suggestion of a hypoactive HPA reactivity period during the transition from childhood to adolescence (Gunnar et al., 2009). Here, it might perhaps be useful to take cultural differences as a possible explanatory factor for the different results into account, as studies that have found continuity in HPA axis reactivity are based on European samples (Kudielka et al., 2004; Westenberg et al., 2009, present study), while studies showing a lack of cortisol reactivity during the transition from childhood to adolescence are based on North American samples (Stroud et al., 2009; Klimes-Dougan et al., 2001; Gunnar et al., 2009).

Strengths of this study are the inclusion of seven cortisol measures, as well as a pre-stressor resting period of 40 minutes in order to obtain a reliable baseline measure of cortisol. Nonetheless, some limitations should also be noted. The most important limitation is the lack of information on pubertal stage, age of menarche, menstrual cycle and OC use. This limits our capacity for understanding the physiological mechanisms behind our results (e.g. the menstrual cycle phase could explain the lack of a cortisol reaction in some of the girls). Another limitation is that our group was relatively small. This could have, for example, limited our power to detect age differences in cortisol reactivity.

Future research is also needed to examine the difference in physiological stress reactions in adolescent boys and girls. Because girls might react to stress in ways other than cortisol increases, additional measures of stress reactivity would be useful, such as measures of the sympathetic nervous system and brain reactivity. The present paradigm can be easily adapted to use during functional neuroimaging and could therefore be used to measure both cortisol and brain reactivity to the same acute stressor.

CONCLUSIONS

This study found that a computerized paradigm including social evaluation, unpredictability and uncontrollability was efficient for increasing cortisol levels in 10 to 15-year-old boys. Although girls of the same age did not show increases in cortisol as a reaction to the protocol, both sexes reported experiencing the paradigm as (equally) stressful. These differences in the relations

between reported stress and cortisol reactivity are intriguing and an interesting subject for future research on the development of psychophysiological mechanisms during adolescence. Because the paradigm can be easily adapted for use in neuroimaging studies, future mapping of male and female adolescent brain reactivity during the stressor could help shed more light on the subject.

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Table 1. Descriptive statistics and possible confounders of the sample (N=52)

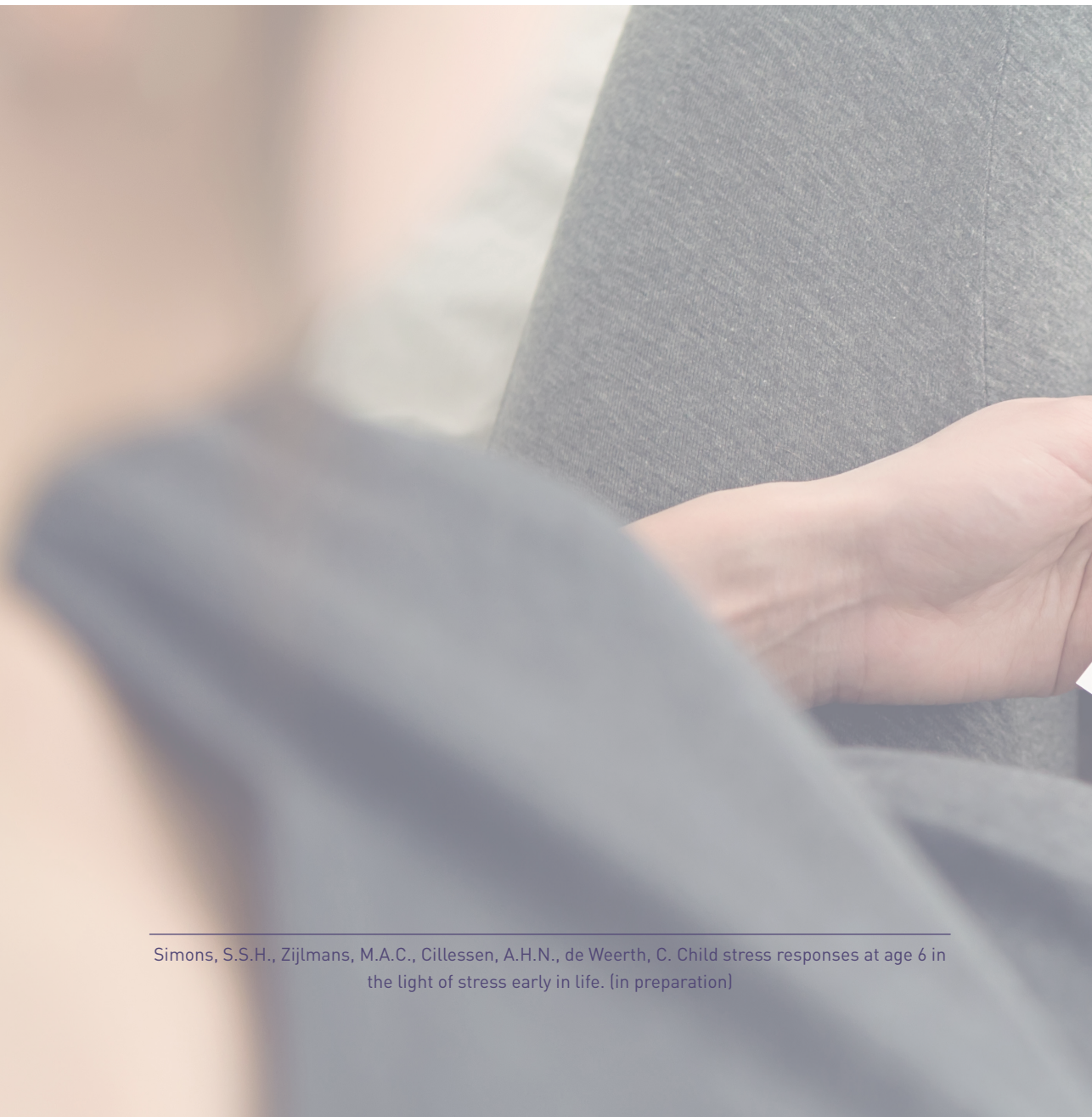
Variables	Total group	Boys	Girls	p	Age 10-12	Age 13-15	p
	N=52	N=23	N=29		N=32	N=20	
School type (%)							
Primary school	38.46	47.80	31.00		62.50	5	
Secondary school	59.62	52.20	65.50		37.50	95	
Missing	1.92	0	3.50		0	0	
Child's age in years, mean ± SD (range)	12.52 ± 1.21 (10.35 – 15.48)	12.30 ± 1.13	12.69 ± 1.2				
Child's sex (%)							
Boys	44.23	100	0		50	35	
Girls	55.77	0	100		50	65	
Time of testing, mean ± SD (range)	15.30h ± 75 min (12.00 – 19.00)	15.10h ± 69 min	16.00h ± 73.5 min	*	16.06h ± 77 min	15.20h ± 70 min	*
Means of transportation (%)							
Public transport or car	46.15	47.80	48.1		53.10	35	
Bicycle	48.08	47.80	51.9		40.60	60	
Missing	5.77	4.40	0		6.30	5	
Δ cortisol (baseline – peak, nmol/L)	0.91 (1.62)	1.70 (2.02)	0.28 (0.79)	**	1.23 (1.86)	0.39 (0.94)	†
Perceived stress, mean ± SD (range)	4.22 ± 1.32 (1-6.50)	4.25 (1.45)	4.19 (1.24)		4.42 (1.38)	3.90 (1.20)	
Credibility, mean ± SD (range)	4.49 ± 1.15 (2-6.75)	4.13 (1.05)	4.77 (1.16)	*	4.52 (1.03)	4.44 (1.35)	

Note: Independent samples T-tests showed no differences between the groups unless otherwise indicated; **p<0.001, *p<0.05, †p=0.07

Table 2. Correlations between the actual increase in cortisol level and potential confounders for the total group (N=52), boys (N=23), and girls (N=29).

	Sex	Age group	Transport	Time of day	School type	Perceived stress	Credibility
Δ cortisol	-.39**	-.26	-.32*	-.05	.00	.29*	.07
Δ cortisol boys		-.13	-.27	.40[†1]	.21	.42[†2]	.25
Δ cortisol girls		.07	-.48*	-.12	.75	.14	.29

Note: Spearman correlations were used for categorical variables (age group, sex, transport, time of day, school type). Pearson correlations were applied for continuous variables (perceived stress, credibility). [†1]p=0.06, [†2]p=0.05, *p<.05, **p<.01



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Chapter 6

Child Stress Responses at age 6 in the Light of Stress Early in Life

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ABSTRACT

Individuals differ in their physiological and behavioral stress responses and alterations in these responses have been associated with (mental) health. Therefore, it is important to understand the development and correlates of such stress responses. This study investigated potential predictors of physiological and behavioral stress responses of 6-year-old children to a stressful social evaluative situation (performance in front of a judge). Specifically, we investigated whether physiological (cortisol) and behavioral (gazing) stress responses were associated with stress early in the child's life, in the form of maternal prenatal and early postnatal (first 6 months) distress. In addition, associations between the two stress responses were studied.

To this end, 149 children (M_{age} = 6.09 years; 70 girls) participated in a social evaluative stress test (CREST) during which 6 cortisol saliva samples were collected. Physiological stress responses were operationalized as cortisol stress reactivity and total stress cortisol. To operationalize behavioral stress responses, gazing at the judge during the stress test was observed. Maternal prenatal distress was measured with questionnaires and physiological (cortisol) measures, and early postnatal distress was measured using questionnaires.

Results indicated that less maternal fear of giving birth, higher maternal prenatal evening cortisol concentrations, and more maternal feelings of anxiety in the first 6 postnatal months all uniquely predicted higher total stress cortisol concentrations in children at age 6. Additionally, children with higher cortisol stress reactivity gazed less in the direction of the judge.

These results suggest that maternal distress early in the child's life may program children's later HPA-axis functioning and that in 6-year-olds confronted with a stressful social situation, gazing may be used to deal with the stressor.

INTRODUCTION

In stressful and/or threatening social situations individuals react physiologically and behaviorally. One physiological system that becomes activated is the hypothalamic-pituitary-adrenal (HPA) axis, producing the hormone cortisol, which mobilizes energy to respond (e.g., Nicolson, 2007). Behaviorally individuals may react, for example, by adjusting their attention (Wilson and MacLeod, 2003) and gazing at a threatening stimulus. Efficient stress responses are needed to deal with day-to-day stressors. However, there are individual differences in physiological and behavioral responses (e.g., Wilson and MacLeod, 2003; Kudielka et al., 2009) and alterations in these responses have been associated with (mental) health (e.g., Wilson and MacLeod, 2003; Susman et al., 2010; Phillips et al., 2013). Therefore, it is important to understand the development and correlates of such stress responses. The present study investigated the early life environment as a potential predictor of later stress responses. Both the uterine and early postnatal environments are thought to play an important role in shaping the development of the offspring's stress system (Heim et al., 2004; Elzinga et al., 2008; Gunnar et al., 2009). These effects may potentially affect development over a longer period of time or even permanently, and are called programming effects (Seckl and Meaney et al., 2004; van den Bergh et al., 2005). In the current paper, we specifically studied associations between stress early in the child's life, in the form of maternal prenatal and early postnatal (in the first 6 postnatal months) distress (i.e., stress and anxiety), and children's physiological (cortisol) and behavioral (gazing) responses to a social evaluative stressor at age 6.

Previous studies on the associations between maternal prenatal distress and child cortisol stress responses have rendered complex results. In one study, more prenatal reported distress was associated with higher cortisol responses to a bathing session in 5-week-olds, and lower cortisol responses in 2- and 12-month-olds towards a vaccination and maternal separation, respectively (Tollenaar et al., 2011). In another study, more perceived maternal prenatal stress was associated with higher cortisol reactivity to a heel-stick in newborns and to a toy removal task in 10-month-olds (Leung et al., 2010). Findings for the associations between maternal prenatal cortisol and child cortisol responses have also been mixed (for a review, see Zijlmans et al., 2015). For example, higher maternal prenatal cortisol was associated with larger cortisol responses to a heel-stick in newborns and to a vaccination in 4- to 6-year-olds (Gutteling et al., 2004; Davis et al., 2011), while Tollenaar et al. (2011) found no associations between maternal prenatal cortisol and child cortisol stress responses during children's first year.

Less is known about associations between prenatal distress and children's gazing behavior in stressful and threatening situations. However, prenatal programming mechanisms may generally affect children's behavioral vigilance, and thereby their gazing behavior. In line with this, infants of mothers who were more stressed during pregnancy looked away from the mother or objects used for a longer time period during a peek-a-boo task (Lin et al., 2014). Prenatal distress has been associated with other child behavioral responses as well. For

example, maternal prenatal life events were positively associated with observed fearfulness during a lab task in 14- to 19-month-olds (Bergman et al., 2007). Further, positive associations have been found between anxiety and attention/gazing to threatening stimuli (Wilson and MacLeod, 2003), and between prenatal maternal anxiety and children's self-reported anxiety (8- and 9-year-olds; van den Bergh and Marcoen, 2004), suggesting that there may be associations between maternal prenatal distress and child gazing behavior in stressful and threatening situations.

Early maternal postnatal distress may also affect children's stress responses. Associations have been found between parenting stress and self-reported parenting, suggesting lower parenting quality, in parents of 2- to 5-year-olds (Anthony et al., 2005; Guajardo et al., 2009). Lower parenting quality in turn may be stressful for young children (Loman and Gunnar, 2010). In line with this, mothers with an anxiety disorder were less sensitive and more intrusive, and had 9-month-olds with higher cortisol responses to a fearful situation than healthy controls (Feldman et al., 2009).

To our knowledge, research on associations between early postnatal maternal distress and child gazing behavior in stressful or threatening situations is lacking. Maternal distress early in children's lives may also affect their behavioral stress responses via caregiving. Braungart-Rieker et al. (1998) found that in 4-month-olds, mothers' sensitivity was positively associated with children's visual attention towards them during a stressful still face paradigm. Also 5- and 6-month-old children of responsive parents looked more at their parent during a procedure including free play, still faces, and reunions, than children of less responsive parents (Haley and Stansbury, 2003). Receiving lower quality care, which in itself can be considered a stressor, may affect the child's threat and stress response system as well as its behavior (Loman and Gunnar, 2010).

An additional goal of the present study was to investigate possible associations between cortisol responses and gazing behavior in response to the stressor. The few previous studies on these types of associations have yielded diverse findings. In university students, larger increments of cortisol during a stressful interview were associated with more eye contact and engagement with the interviewers (Sgoifo et al., 2003). In contrast, in 6- to 17-year-olds, higher cortisol reactivity during a social challenge (i.e., interview, singing, silent and oral reading) was associated with less gazing towards the interaction partner (Hessl et al., 2006). Finally, in 10-year-olds no direct link between cortisol reactivity and gazing towards an evaluating judge was found (de Veld et al., 2014).

In sum, the goals of the present study were to investigate whether maternal distress in late pregnancy and the first 6 postnatal months predicted children's cortisol and gazing responses to a social evaluative stressor. Associations between children's cortisol responses and their gazing behavior during the stressor were also examined.

METHODS

Participants

The data for this study were collected in an ongoing longitudinal project on the psychobiology of child development (BIBO project; Radboud University). Healthy mothers and their children are followed since late pregnancy. All mothers gave written informed consent; the study was approved by the Institutional Ethical Committee following the Helsinki Declaration (ECG 300107 and ECG 22111/130112). The project began with 220 mother-child dyads, 193 were still in the project when the child was 3 months old (see for details, Beijers et al., 2011). At child age 6, 188 mother-child dyads were still in the project and were invited to participate in the 6-year data collection. Of this group, 149 children participated in a school visit that included a social evaluative stress test for which parents gave written informed consent (M_{age} = 6.09; *SD* = 0.14; Min = 5.87, Max = 6.85; 70 girls). Reasons for non-participation were: a preference of the school or child not to participate (*n* = 4), relocation of the family abroad (*n* = 3), or other reasons (e.g., parents saw the procedure as too challenging for their child, considered the project as too demanding, or had personal motives, *n* = 32). Of the invited 188 children, the 39 who did not participate did not differ significantly from the participating 149 children in maternal age at delivery, maternal educational level during pregnancy, child gender, or temperament (Children's Behavior Questionnaire short form; Putnam and Rothbart, 2006), all *p's* > .05. The current study used prenatal data and data collected when the children were 3 months, 6 months, and 6 years old.

Procedure

Maternal prenatal distress.

Around the 37th week of pregnancy mothers were asked to complete questionnaires regarding their feelings of general and pregnancy-specific stress and anxiety. They also were asked to collect diurnal saliva samples to determine their cortisol circadian rhythm.

Maternal early postnatal distress.

To determine maternal postnatal feelings of stress and anxiety early in children's lives (first 6 months) mothers were asked to complete questionnaires about their feelings of general stress and anxiety when their child was 3 and 6 months old.

Child stress responses at age 6.

When children were 6 years old families were invited to let their child participate in a social evaluative stress test during a school visit. If parents agreed, the researchers visited the child's school with a mobile laboratory. In 8 of the 149 cases, the mobile lab visited the child at home.

All visits took place in the afternoon of a regular school day and started between 12:15 and 15:15 hours. The visits began with the Children's Reactions to Evaluation Stress Test (CREST; de Weerth et al., 2013; Simons et al., 2016) during which the child carried out three forced-failure tasks containing elements of unpredictability and uncontrollability before a judge. In the first task (restrained movement task; 1 minute) children were asked to stand as still as possible, with an alarm clicked on their clothes. They were told that the alarm would beep if they moved. During the task the alarm went off twice on preprogrammed times independently of children's actual movement. In the second task (animal story task; 3 minutes) children listened to a recorded story about animals and were asked to fill in eight gaps in the story by making the sound of the animal just mentioned in the story. In this task children were told they would receive visual feedback from the judge (a green card) if they performed the sound perfectly. However, the judge showed the green card in only three of the eight sounds, irrespective of the child's performance. In the third task (tower of cans task; 3 minutes) children were asked to build a tower of horizontally lying cans which was almost impossible to do (see de Weerth et al., 2013). After the three tasks, the judge left the room for 5 minutes to 'evaluate' the child's performance. The total stress test procedure took 20 minutes (15 minutes for the three tasks, 5 minutes anticipation of the judge's evaluation). The CREST has been shown to effectively trigger an increase in cortisol concentrations in 5- and 6-year-olds (in an independent sample: de Weerth et al., 2013; in this sample: Simons et al., 2016). After the stress test, the judge told the children that they performed perfectly well, rewarded them with a present, and a thorough debriefing took place. This was followed by a 25-minute recovery phase and another 25 minutes of tasks not described in this paper. The entire procedure was guided by a trained researcher who helped with saliva sampling, other tasks, and supported the child when necessary during the CREST.

Measures

Maternal prenatal and early postnatal distress.

Pregnancy-specific anxiety.

Two subscales of the Pregnancy-specific Anxiety Questionnaire-Revised (PRAQ-R; Buitelaar et al., 2003; Huizink et al., 2003; 2004a; 2004b) were used to measure pregnancy-specifix anxiety. The subscales measured fear of giving birth (3 items) and fear of bearing a handicapped child (4 items) using a 5-point scale. Cronbach's α was 0.73 and 0.82, respectively. Higher scores represented higher levels of maternal pregnancy-specific anxiety.

Pregnancy-specific stress.

The Pregnancy Experience Scale (PES; DiPietro et al., 2004) was used to measure pregnancy-specific stress. Each of 43-items in this questionnaire described a pregnancy-specific experience. Mothers rated on a 4-point scale to what degree each item resulted in a positive and a negative experience. Cronbach's α was 0.87 for positive raitings and 0.87 for negative raitings. To derive a score for the experienced negative emotional valence towards pregnancy, the sum of the negative ratings was divided by sum of the positive ratings. Hence, higher scores represented a more negative emotional valence towards pregnancy that is, more pregnancy-specific daily hassles or stress.

General anxiety.

Prenatally, and when children were 3 and 6 months old, maternal feelings of general anxiety were assessed using the State subscale of the State-Trait Anxiety Inventory (STAI; van der Ploeg et al., 1981; Spielberger, 1983). This scale consists of 20-items answered on a 4-point scale. Higher scores represented higher levels of maternal state anxiety. Cronbach's α ranged from 0.90 to 0.93. To operationalize maternal postnatal anxiety in the first 6 postnatal months the average of the 3 and 6 month scores (Spearman's *rho* = .53, $p < .01$) was computed. This questionnaire was also used when the child was 6 years old (see section 2.3.4).

General stress.

Prenatally, and when children were 3 and 6 months old a Dutch questionnaire (Alledaagse Problemen Lijst; APL; Vingerhoets et al., 1989; test-retest reliabilities 0.76 - 0.87) was used to measure maternal stress. This questionnaire has 49 items describing daily hassles. Mothers indicated whether each item had occurred in the last two months, and, if so, rated how much it had bothered them using a 4-point scale. To derive a mean intensity rating the sum of the ratings given was divided by the number of occurred events. Higher sores thereby represented higher levels of experienced negativity due to daily hassles, indicating more stress. Early maternal postnatal stress (first 6 months) was operationalized as the average of the 3 and 6 month scores (Spearman's *rho* = .57, $p < .01$).

Cortisol.

During pregnancy, mothers were asked to collect diurnal saliva samples on two consecutive days at awakening (T1), 30 minutes after awakening (T2), at 12:00 hours (T3), 16:00 hours (T4), and 21:00 hours (T5). Samples were collected by passive drooling and stored in a freezer (-25 ºC). Subsequent cortisol analyses were carried out at the Laboratory of Endocrinology of the University Medical Center Utrecht (see, for details, Simons et al., 2015; 2016). Fluctuations in cortisol concentrations were diminished by excluding samples (5.7%) that deviated too much from the required sampling times as well as samples collected during or after the day of delivery (Beijers et al., 2010). Mean concentrations over the two days were calculated (Beijers et

al., 2010). To operationalize maternal physiological distress, diurnal cortisol decline (T1 minus T5) and evening cortisol (T5) were calculated. These measures were consistent with earlier research (Beijers et al., 2010) in which they were found to correlate highly with other prenatal maternal cortisol measures (total amount of diurnal cortisol, morning cortisol concentrations, and cortisol awakening response). Higher scores on diurnal cortisol decline represented a steeper diurnal decline in cortisol concentrations.

Child stress responses.

Physiological stress responses: cortisol responses.

At child age 6, six saliva samples (C1 - C6) were collected from the child during the stress test procedure to determine baseline, response, and recovery cortisol concentrations. As in the original CREST, baseline cortisol concentrations (C1 and C2) were collected immediately before the CREST started (C1) and 15 minutes after test onset (C2; de Weerth et al., 2013; Simons at al., 2016). Because physical activity and eating can affect cortisol concentrations (e.g., Dickerson and Kemeny, 2004; Nicolson, 2007), children were asked not to eat, drink, or be physically active 30 minutes prior to the test. Samples representing cortisol concentrations in response to the stressor (C3 and C4) were obtained 25 and 35 minutes after test onset. Samples representing cortisol concentrations during a recovery period (C5 and C6) were obtained 50 and 58 minutes after test onset. All saliva samples were collected using eye sponges (BD Visispeare, Waltham, MA; de Weerth et al., 2007). Centrifuged samples were stored at -25 ºC and analyzed in the Laboratory of Endocrinology of the University Medical Center Utrecht (see, for details, Simons et al., 2015; 2016).

Cortisol concentrations of 4 of the 149 children were excluded from all analyses because the children used medication that could affect their cortisol concentrations (n = 3) or because the timing of the saliva samples differed largely from the standard sampling times in the protocol (n = 1). A paired samples t-test indicated that the stress test induced a significant increase in cortisol from the lowest baseline concentrations (M = 6.06 nmol/L, SD = 2.70) to the highest peak response concentrations (M = 7.12 nmol/L, SD = 3.79), $t(141) = -4.41$, $p < .01$ (see Simons et al., 2016, for a figure representing the mean scores of the six sampling moments). Two indices of the cortisol stress response were calculated. Total stress cortisol was calculated as the area under the curve across all six samples: AUC = (C2 + C1) x 15/2 + (C3 + C2) x 10/2 + (C4 + C3) x 10/2 + (C5 + C4) x 15/2 + (C6 + C5) x 8/2. Cortisol stress reactivity was calculated by saving the standardized residual scores of a regression predicting the highest peak response concentrations from the lowest baseline concentrations (Schuetze et al., 2008; de Veld et al., 2012; Simons et al., 2016). Total stress cortisol and cortisol stress reactivity scores were log transformed to increase the normal distribution of the residuals of the regression analyses.

Behavioral stress responses: gazing behavior.

Gazing behavior was determined during the second subtest of the stress test, the animal story task. This subtest was chosen for the gazing observations because it is most similar to the Trier Social Stress Task (Kirschbaum et al., 1993), in that the child could move freely (as opposed to the restrained movement task), and was not focused on a manual task (as opposed to the tower of cans task). To observe gazing behavior during this test children were videotaped while performing, using a camera positioned above the judge. The videotapes were coded using Noldus Observer XT (version 11) and an ethogram developed to observe behavior during the CREST. Two independent observers coded the video's using interval coding, scoring the child's gaze direction every two seconds (see Table 1 for the ethogram for gazing behavior). Categories of the gazing behavior ethogram were mutually exclusive and hierarchically ordered. Scores for gazing behavior were calculated by counting the number of intervals in which the child looked at the judge (sum of 'looks at judge and experimenter' and 'looks at judge') divided by the total number of intervals (sum of all categories minus intervals in which looking behavior could not be scored: 'not visible'). Higher scores indicated more gazing towards the judge. Of the 142 videos, 7 could not be scored due to technical problems at recording. Of the 135 videos that were coded, inter-rater reliability between the two observers was calculated over 10 randomly selected videos and was excellent, Cohen's $k = 0.83$.

Potential confounders.

Child gender (girls = 0, boys = 1), maternal educational level at child age 6, and maternal anxiety at child age 6 were considered potential confounders. Maternal educational level was mothers' highest educational level ranging from "primary" (1) to "university" (8), followed by the option "other". "Other" answers ($n = 5$) were recoded into the closest matching option. Maternal anxiety at child age 6 was derived from the State subscale of the Sate-Trait Anxiety Inventory (see section 2.3.1.3). Cronbach's α was 0.93 for this measurement at child age 6.

Statistical analyses

To investigate associations between maternal distress (prenatal and during the first six postnatal months) and child stress responses, three hierarchical regressions were run with total stress cortisol, cortisol stress reactivity, and gazing behavior as dependent variables. In each regression, confounders that were significantly correlated with the outcome variable were included first, in Step 1 (Tabachnick and Fidell, 2007). Subsequently, the prenatal and early postnatal measures of maternal distress were added in the following Step. To investigate how physiological and behavioral stress responses were associated, Spearman correlations were calculated.

RESULTS

Descriptive statistics of the untransformed study variables are presented in Table 2. Spearman correlations are presented in Table 3. Higher cortisol stress reactivity scores were associated with less gazing at the judge (Spearman's *rho* = -.17, $p < .05$). Because girls' gazing was more directed towards the judge than boys' gazing (Spearman's *rho* = -.21, $p = .01$), child gender was included as a confounder in the prediction of gazing behavior. Because maternal anxiety at age 6 was associated with higher total stress cortisol concentrations at age 6 (Spearman's *rho* = .17, $p < .05$), maternal anxiety at age 6 was included as a confounder in the prediction of children's total stress cortisol concentrations. The correlations also showed that higher levels of maternal prenatal evening cortisol were associated with higher child total stress cortisol concentrations (Spearman's *rho* = .25, $p < .01$) and higher cortisol stress reactivity scores at age 6 (Spearman's *rho* = .25, $p < .01$).

Due to missing data, 30 of the 149 children were dropped out of all three regression analyses. These 30 children did not differ significantly from the other 119 children in gender, temperament, or maternal age or educational level, all *p's* > .05.

In the regression predicting total stress cortisol, Step 1 was not significant, $p > .05$ (see Table 4). Step 2 had significant additive value, $F_{change}(9, 95) = 2.04$, $p < .05$, $R^2_{change} = .16$, and the total model at Step 2 also was significant, $F(10, 95) = 1.92$, $p = .05$, $R2 = .17$ (see Table 4). Maternal fear of giving birth, maternal prenatal evening cortisol concentrations, and maternal feelings of anxiety in the first 6 postnatal months of the child's life all were uniquely associated with children's total stress cortisol concentrations (all *p's* < .05; see Table 4). Higher maternal fear of giving birth was associated with lower total stress cortisol at child age 6. Higher prenatal maternal evening cortisol concentrations and higher maternal feelings of anxiety in the first 6 postnatal months were both uniquely associated with higher total stress cortisol concentrations at age 6 (see Table 4).

The regression predicting cortisol stress reactivity was not significant, $p > .05$ (see Table 4).

In the regression predicting gazing behavior, Step 1 was significant, $F(1, 114) = 5.84$, $p < .05$, $R2 = .05$: girls looked more in the direction of the judge than boys (see Table 4). Step 2 did not have significant additive value and the model at Step 2 was not significant, both *p's* > .05 (see Table 4).

DISCUSSION

In this study, predictors of physiological and behavioral stress responses to a social evaluative stress paradigm were investigated in 6-year-old children. Specifically, we examined whether physiological stress responses (total stress cortisol and cortisol stress reactivity) and

behavioral stress responses (gazing) were predicted by stress early in the child's life, as indicated by maternal prenatal and early postnatal (first 6 months) distress. The associations between the two stress responses (physiological and behavioral) were also examined. Results indicated that less maternal fear of giving birth, higher maternal prenatal evening cortisol, and more maternal early postnatal (first 6 months) anxiety were all uniquely associated with higher child total stress cortisol concentrations. Additionally, higher cortisol stress reactivity was associated with less gazing at the judge during the social evaluative stress test.

Stress responses and stress early in life

The finding that prenatal maternal evening cortisol, fear of giving birth, and maternal early postnatal (first 6 months) anxiety were associated with total stress cortisol of 6-year-olds may suggest that prenatal and early postnatal maternal distress affect the development of the offspring's HPA-axis. This is in line with programming hypotheses and the idea that stress early in life is associated with later HPA-axis functioning (e.g., Lucas, 1991; Loman and Gunnar, 2010; Chaby, 2016).

The positive association between maternal evening cortisol and child total stress cortisol may be explained by a programming effect of higher maternal cortisol concentrations on children's HPA-axis development. Indeed, in rats maternal prenatal stress is linked to the offspring's number of hippocampal corticosteroid receptors and diurnal activity of the HPA-axis (Koehl et al., 1999). In humans, maternal cortisol may affect the fetus' developing brain and HPA-axis in various manners. For example, maternal cortisol may stimulate placental production of corticotrophin releasing hormone (CRH) which in turn may stimulate fetal production of (excessive) cortisol, affecting the developing fetal brain (Meaney et al., 1996). It is also possible that maternal cortisol reduces the placental blood flow which in turn may stress the fetus, thereby affecting the developing HPA-axis (e.g., Huizink et al., 2004a; Beijers et al., 2014; Zijlmans et al., 2015). Alternatively, high maternal evening cortisol concentrations may affect the fetus more indirectly, through maternal behaviour. For example, maternal sleep may be disturbed, in turn affecting maternal metabolic and inflammatory systems (see Beijers et al., 2014), thereby potentially affecting fetal development.

The positive association between maternal anxiety in the first 6 postnatal months and total stress cortisol of 6-year-olds may be explained by lower quality caregiving. This is in line with research indicating that mothers with an anxiety disorder are less sensitive and more intrusive and have 9-month-olds with higher cortisol stress responses (Feldman et al., 2009). An anxious mother may herself be a source of stress for the child if her anxiety leads to lower quality care (Loman and Gunnar, 2010), or an anxious mother may be less able to buffer her child from environmental stress. In both cases, children's HPA-axis functioning may be

affected. Alternatively, heightened stressfulness in the shared mother-child environment may partly explain this association.

The finding that children of mothers with more fear of giving birth had lower total cortisol concentrations seems contradictory to our other findings described above, and is difficult to explain. Not much is known about the effects of specific types of pregnancy anxieties on women's physiological states and behavior. It could be hypothesized that fear of giving birth is transferred to the child by increasing maternal cortisol concentrations. However, in our sample the association between maternal evening cortisol and child total stress cortisol was positive, whereas the link with maternal fear of giving birth was negative. There was also no evidence for associations between maternal fear of giving birth and maternal evening cortisol or diurnal cortisol decline. This suggests that mechanisms other than cortisol may underlie the association between fear of giving birth and child total cortisol concentrations. For example, perhaps fear of giving birth affects maternal lifestyle, such as eating or exercise (Beijers et al., 2014), in turn affecting the child's prenatal environmental conditions and potentially HPA-axis development. However, how this would result in lower instead of higher total stress cortisol in the child 6 years later remains unknown. Moreover, other research on the associations between maternal fear of giving birth and children's HPA-axis functioning found no association between both (Gutteling et al., 2004; Tollenaar et al., 2011; Simons et al., 2015). Future in-depth research on maternal pregnancy stress, lifestyle, and physiology is needed to shed more light on this subject.

The statistical analyses did not support associations between prenatal and early postnatal maternal distress and gazing or cortisol stress reactivity. These results may suggest that children's gazing and cortisol reactivity during stress are independent of maternal prenatal and early postnatal distress. However, study characteristics may also explain these findings. For example, in our protocol the judge gave the participants visual feedback on their performance (showed a green card); this may have constrained spontaneous gazing behavior and affected the stressfulness of the situation. Another type of stressor may have produced different patterns of reactions in the child, as well as different links with stress early in the child's life. Additionally, although previous research suggests that the early life environment is linked to later HPA-axis functioning (e.g., Kudielka et al., 2009; Loman and Gunnar, 2010), these links may be particularly salient in children experiencing more severe environmental stress than the children in our middle class sample.

The fact that stress early in life predicted children's total cortisol during the stress protocol, but not their cortisol stress reactivity may be related to the nature of these measures. While cortisol reactivity reflects the dynamics of the response to the stressor, total stress cortisol reflects cortisol concentrations during the entire procedure. As such, this last measure may more generally represent cortisol production during a school day or any day in general (circadian cortisol) more than in reaction to the stressor per se. The results may be suggesting that stress early in life is more predictive of basal functioning than physiological (and even

behavioral) reactivity in response to a specific acute stressor. Acute stress responses may be more variable, depending on the specific situation and stressor, and hence more difficult to link to stress early in life. In line with this are the earlier links found by Simons et al. (2015) between stress early in life and markers of cortisol circadian rhythm in early childhood.

Associations between physiological and behavioral stress responses

The negative association between gazing at the judge and cortisol reactivity scores is in line with a negative association between cortisol reactivity and gazing towards an interaction partner during a social challenge in 6- to 17-year-olds (Hessl et al., 2006). However, it is not in line with associations between larger increments of cortisol and more eye contact in adolescents during a social challenge (Sgoifo et al., 2003) and research in 10-year-olds showing no direct link between gaze aversion and cortisol reactivity during a social challenge (de Veld et al., 2014). The differences may be explained by differences in the stressors that were used. Sgoifo et al. (2003) used an interview in which students talked about their distinctive personality features, not resulting in a significant increase in cortisol in the group as a whole. De Veld et al. (2014) used a paradigm comparable to ours (performance in front of a judge), that lead to significant increases in cortisol in the group as a whole. Our protocol triggered looking at the judge for feedback; this was not the case in de Veld et al. (2014) where (verbal) feedback was provided only if mistakes were made or the child fell silent. These features may have affected gazing and/or cortisol responses, as well as their associations.

The negative association between gazing and cortisol stress reactivity scores in our study may suggest that keeping an eye on the judge is associated with lower cortisol stress reactivity. Children may have used gazing towards the information-providing judge in combination with cognitive strategies to reduce physiological arousal. This combination may have increased feelings of control, hence reducing uncontrollability and physiological responses (Dickerson and Kemeny, 2004). In line with this, it has been found that between children aged between 6 and 10 year, start to shift from predominately behavioral strategies (such as gaze aversion) to more cognitive emotion regulation strategies (Terwogt and Stegge, 1995). However, since false feedback was provided, uncontrollability probably kept playing a role. And given the correlational design, directions may be reversed: experiencing the situation physiologically as less stressful may result in more (relaxed) gazing at the judge. Other factors, such as shyness, also may have played a role. More shyness may result in less looking at the judge and higher physiological responses. Indeed, shyness predicted higher cortisol reactivity in response to meeting a stranger in 3-year-olds (Zimmerman and Stansbury, 2004). To date, few studies on stress responses include measures of behavior. More observational research in combination with experimental designs is needed to elucidate if children use behavior to deal with physiological stress. Experimental studies of behavior under stress, for example by

training children to look more or less at the judge, may clarify the regulatory functions of behavior which in turn may benefit future interventions.

That no association between gazing and total stress cortisol was found may be because gazing behavior, just as cortisol reactivity, reflects the dynamics of the responses to the stressor, while the total stress cortisol represents cortisol secretion during the whole protocol (i.e. including cortisol concentrations before and after the stressor).

Strengths and limitations

Strengths of this study are the relatively large longitudinal sample, the combination of physiological, behavioral, and psychological measures, and the use of an effective stress test for 6-year-olds. However, the study is limited in generalizability because the sample was primarily middle class. The use of a specific (social evaluative) stressor limits generalizability to other stressful situations. Because this study was not genetically informed, genetic or epigenetic (Meaney, 2010) transference cannot be ruled out. Finally, the correlational design precludes causal conclusions.

Future research

To increase generalizability, links between stress early in life and children's stress responses and between physiological and behavioral stress responses should be studied in various contexts and environments. Since gazing may modify physiological responses to acute stress it is interesting for future (intervention) research to study the causality of the links between physiological and behavioral stress responses. Moreover, gazing may moderate associations between stress early in life and children's HPA-axis functioning, possibly explaining the mixed findings of previous research. In this study, power issues prevented testing this but future research with larger sample sizes could test moderation by stressor type or behavioral strategies. Finally, since stress responses continue to develop after age 6 (Boyce and Ellis, 2005), it is interesting to examine how stress responses at older ages relate to stress early in life and what role cognitive development plays in these associations.

CONCLUSIONS

Maternal distress early in the child's life was associated with 6-year-old children's total cortisol secretion in a stressful social evaluative situation. Additionally, higher cortisol reactivity was related to less gazing at the social evaluative threat (the judge). The results suggest that stress

early in life may program children's later HPA-axis functioning and that 6-year-olds confronted with a stressful social situation may use gazing strategies to deal with the stressor.

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Table 1. Ethogram for Gazing Behavior during the Stress Test

Gazing behavior	Definition
Looks at judge and experimenter	Child looks at judge and experimenter at any moment within two second interval
Looks at experimenter	Child looks at experimenter at any moment within two second interval
Looks at judge	Child looks at judge at any moment within two second interval
Looks elsewhere	Child does not look at judge or experimenter at any moment during the two second interval, but looks upwards, downwards, or to the side
Eyes closed	Child has his/her eyes closed during whole two second interval
Not visible[b]	Eyes are not visible during whole two second interval

Note. Proportion scores were corrected for the intervals that were 'not visible'.
For the statistical analyses, categories 'looks at judge and experimenter' and 'looks at judge' were added up to represent the total number of intervals in which the child looked at the judge.

Table 2. Descriptive Statistics of All Study Variables

	N	M	SD	Min	Max
Confounders					
Child gender (% girls)	149	46.98			
Maternal educational level	144	6.76	1.39	3.00	8.00
Maternal anxiety, age 6	143	30.95	8.43	20.00	68.00
Predictors					
Maternal Prenatal					
Daily hassles	136	1.14	0.44	0.00	2.54
Anxiety	136	32.02	8.93	20.00	64.00
Pregnancy-specific hassles	136	0.32	0.22	0.00	1.43
Fear of giving birth	136	5.45	2.58	3.00	15.00
Fear of bearing a handicapped child	136	9.13	3.37	4.00	20.00
Diurnal cortisol decline (nmol/L)	119	6.75	4.50	-2.80	24.00
Evening cortisol (nmol/L)	125	9.72	3.73	0.85	37.00
Maternal Postnatal					
Anxiety, mean 3 and 6 months	149	28.70	6.64	19.50	59.50
Daily hassles, mean 3 and 6 months	149	1.15	0.38	0.00	2.28
Outcomes (child)					
Total stress cortisol (AUC)	134	375.80	169.48	73.80	1474.50
Cortisol stress reactivity[a]	142	0.00	1.00	-1.77	5.15
Gazing behavior	142	0.28	0.12	0.01	0.50

Note. AUC = area under the curve (total stress cortisol concentration).

[a]Due to the operationalization of reactivity as standardized residuals the mean of this variable is 0.00 and the SD is 1.00.

Table 3. Spearman Correlations between All Study Variables

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13[a].	14[a].
Confounders														
1. Child gender														
2. Maternal educational level	-.02													
3. Maternal anxiety, age 6	.13	-.01												
Predictors														
Maternal prenatal														
4. Daily hassles	-.20*	-.03	.11											
5. Anxiety	.08	.03	.46**	.23**										
6. Pregnancy-specific hassles	-.09	.01	.34**	.22*	.40**									
7. Fear of giving birth	-.11	.09	.25**	.08	.40**	.32**								
8. Fear of bearing a handicapped child	-.10	-.04	.07	.13	.11	.20*	.19*							
9. Cortisol decline (nmol/L)	-.02	.02	-.01	-.09	-.10	.16+	-.09	-.19*						
10. Evening cortisol (nmol/L)	-.05	-.01	.13	-.04	.03	-.20*	.05	-.02	-.24**					
Maternal postnatal														
11. Anxiety, mean 3 and 6 months	-.01	.11	.61**	.17*	.55**	.41**	.39**	.16+	-.03	.01				
12. Daily hassles, mean 3 and 6 months	-.15+	.04	.25**	.46**	.23**	.41**	.17*	.15+	.03	-.10	.36**			
Outcomes (child)														
13.Total stress cortisol (AUC)[a]	-.04	-.05	.17*	-.08	.02	.04	-.10	-.13	.01	.25**	.11	.03		
14. Cortisol stress reactivity[a]	.10	-.12	-.03	.07	-.12	-.09	-.10	-.15+	-.15	.25**	-.06	-.12	.46**	
15. Gazing behavior	.21**	.02	-.01	-.08	-.13	-.02	-.09	.02	-.01	-.03	-.00	-.01	-.14	-.17*

Note. AUC = area under the curve (total stress cortisol concentration). [a]log transformed. + $p \leq .10$, * $p \leq .05$, ** $p \leq .01$.

Table 4. Results from Regressions Predicting Total Stress Cortisol, Cortisol Stress Reactivity, and Gazing Behavior from Prenatal and Early Postnatal (first 6 months) Maternal Distress

	Model 1		Model 2	
	B	**ß**	***B***	**ß**
Total Stress Cortisol[a]				
Step 1				
Anxiety, 6 years	<.01	.08	<-.01	-.18
Step 2				
Daily hassles, prenatal			.01	.02
Anxiety, prenatal			<-.00	-.03
Pregnancy-specific hassles, prenatal			.06	.07
Fear of giving birth, prenatal			-.02	-.26*
Fear of bearing a handicapped child, prenatal			<-.01	-.07
Cortisol decline (nmol/L), prenatal			<.01	.01
Evening cortisol (nmol/L), prenatal			.01	.23*
Anxiety, mean 3 and 6 months			.01	.44**
Daily hassles, mean 3 and 6 months			.02	.05
R^2_{change}	.01		.16*	
R^2_{model}	.01		.17*	
Cortisol Stress Reactivity[a]				
Daily hassles, prenatal	.09	.21+		
Anxiety, prenatal	<-.01	-.07		
Pregnancy-specific hassles, prenatal	.04	.04		
Fear of giving birth, prenatal	-.02	-.23*		
Fear of bearing a handicapped child, prenatal	<-.01	-.08		
Cortisol decline (nmol/L), prenatal	<-.01	-.07		
Evening cortisol (nmol/L), prenatal	.01	.17+		
Anxiety, mean 3 and 6 months	<.01	.16		
Daily hassles, mean 3 and 6 months	-.07	-.17		
R^2_{change}	.12			
R^2_{model}	.12			

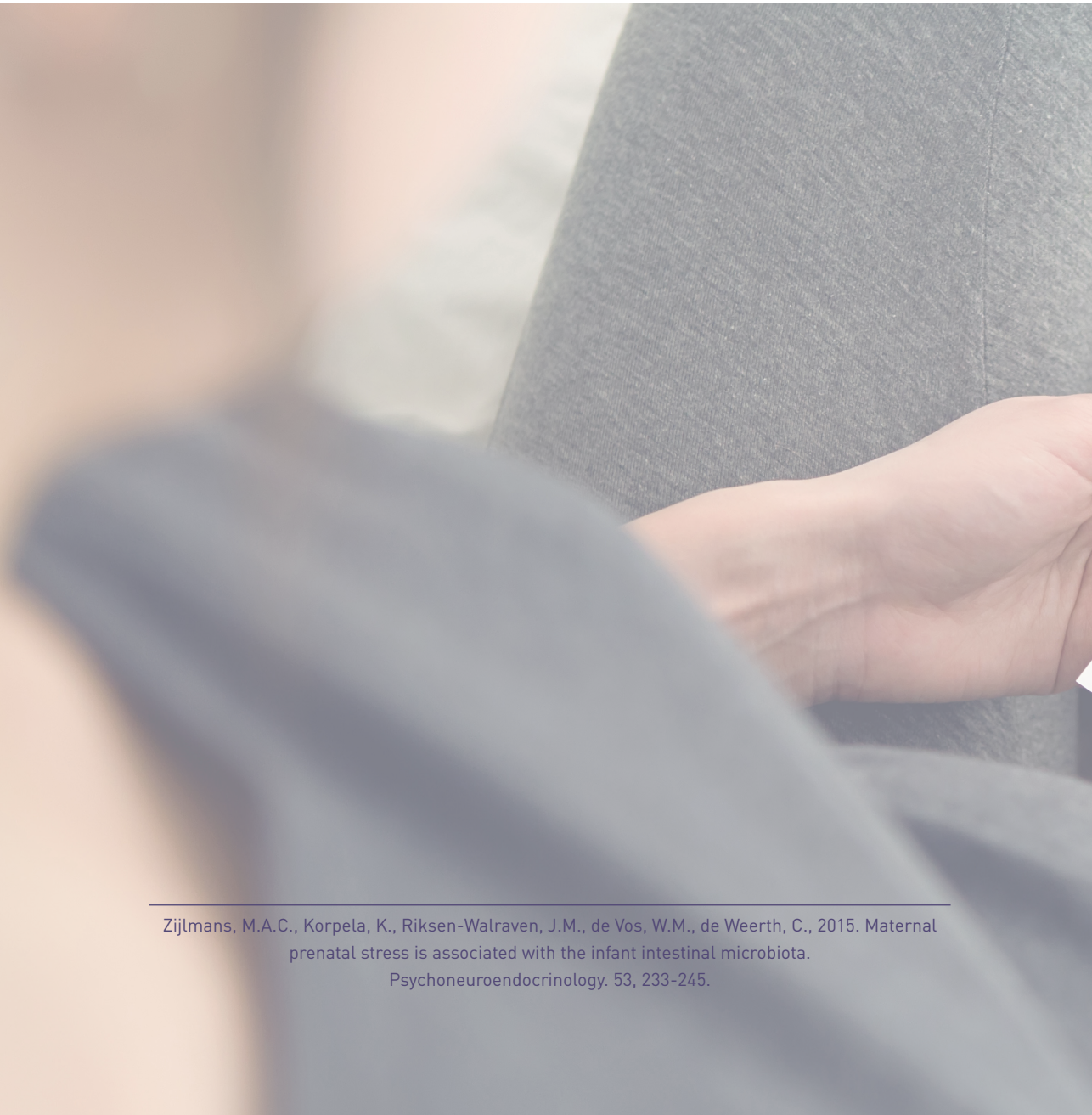
Note. [a]log transformed. No outliers were removed because Cook's distances indicated no potentially influential data points.

+ $p \leq .10$, * $p \leq .05$, ** $p \leq .01$.

Table 4. (continued)

	Model 1		**Model 2**	
	B	ß	*B*	ß
Gazing Behavior				
Step 1				
Child gender	-.05	-.22*	-.05	-.21*
Step 2				
Daily hassles, prenatal			<-.01	<-.01
Anxiety, prenatal			<-.01	-.22^{+}
Pregnancy-specific hassles, prenatal			.04	.06
Fear of giving birth, prenatal			<.01	.03
Fear of bearing a handicapped child, prenatal			<.01	<.01
Cortisol decline (nmol/L), prenatal			<.01	<.01
Evening cortisol (nmol/L), prenatal			<.01	.01
Anxiety, mean 3 and 6 months			<.01	.16
Daily hassles, mean 3 and 6 months			-.01	-.05
R^2_{change}	.05*		.04	
R^2_{model}	.05*		.09	

Note. No outliers were removed because Cook's distances indicated no potentially influential data points.
$^{+}$ $p \leq .10$, * $p \leq .05$, ** $p \leq .01$.



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Chapter 7

Maternal Prenatal Stress is associated with the Infant Intestinal Microbiota

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ABSTRACT

Maternal prenatal stress has been often associated with infant physical development and health, as well as psychological functioning and behavior. However, the mechanisms underlying these relations remain elusive. The goal of the present study was to prospectively investigate the development of the intestinal microbiota as a potential pathway linking maternal prenatal stress and infant health.

The development of the infant intestinal microbiota was followed over the first 110 days after birth in a healthy cohort of 56 vaginally born Dutch infants. Additionally, the relation between infant intestinal microbiota and gastrointestinal and allergic symptoms was examined.

Results showed that maternal prenatal stress, i.e., either reported stress or elevated basal maternal salivary cortisol concentrations or both, was strongly and persistently associated with the infants' microbiota composition as determined by a phylogenetic microarray. Infants of mothers with high cumulative stress (i.e., high reported stress and high cortisol concentrations) during pregnancy had significantly higher relative abundances of Proteobacterial groups known to contain pathogens (related to *Escherichia, Serratia,* and *Enterobacter)*, and lower relative abundances of lactic acid bacteria (i.e., *Lactobacillus, Lactoccus, Aerococcus*) and Bifidobacteria, altogether characteristics of a potentially increased level of inflammation. Furthermore, this aberrant colonization pattern was related to more maternally reported infant gastrointestinal symptoms and allergic reactions.

In conclusion, clear links were found between maternal prenatal stress and the infant intestinal microbiota and health. Although causality cannot be concluded, the results suggest a possible mechanism by which maternal prenatal stress influences the offspring development. These results suggest a potential for bacterial interventions to enhance offspring health and development in pregnant women with stress.

INTRODUCTION

Although the underlying mechanisms remain unclear, an increasing number of studies link maternal prenatal stress to infant physical development and health, and psychological functioning and behavior. Stress during pregnancy predisposes to premature birth and low birth weight (Mulder et al., 2002; Beydoun and Saftlas, 2008), eczema (Sausenthaler et al., 2009), asthma (Cookson et al., 2009), and respiratory, general and skin illnesses (Beijers et al., 2010). Regarding psychological functioning and behavior, children of prenatally stressed mothers often show more impulsivity, anxiety problems, ADHD symptoms, and worse cognitive and psychomotor development (Beydoun and Saftlas, 2008). Recently, the development of the infant gut microbiota has been put forward as a possible factor underlying the links between maternal prenatal stress and infant development (Beijers et al., 2014). Rhesus monkey infants whose mothers had experienced stress during late pregnancy, in the form of repeated exposure to an acoustic startle, had lower levels of Bifidobacteria and Lactobacilli and more diarrheic symptoms than the infants of non-stressed mothers (Bailey et al., 2004). Also, in adult mice a social stressor (i.e., social disruption) provoked a decrease in the relative abundance of the genus *Bacteroides* together with an increase in the relative abundance of the genus *Clostridium* (Bailey et al., 2009). The goal of the present study is to investigate the relation between maternal prenatal stress (i.e., reported stress and cortisol concentrations) and the development of infant intestinal microbiota and health in the first 110 days of life in humans.

The intestinal microbiota are known to play an important role in the maturation of an infant's gastro-intestinal tract, immunity, metabolism, as well as the hypothalamic-pituitary-adrenal system (Sudo et al., 2004; Dimmitt et al., 2010; Bäckhed et al., 2011). An aberrant acquisition of intestinal bacteria or a reduced complexity of the microbiota may delay immune maturation or alter the development of the immune system and stress responses (Sudo et al., 2004; Adlerberth and Wold, 2009; Sekirov et al., 2010). Bacterial colonization of the infant gut is thought to begin *in utero* (Gosalbes et al., 2013), and to accelerate dramatically during and after delivery, and during the first months of life (Palmer et al., 2007; Fallani et al., 2010). Microbes from the mother and, to a lesser extent, of the environment are thought to be the first colonizers of the infant's gut (Tannock et al., 1990; Gosalbes et al., 2013). After the initial establishment of the intestinal microbiota during the first year of life, the microbiota begins to stabilize to a unique individual composition, continuing to develop gradually throughout childhood and adolescence. To what extent the early colonization dictates later development and finally the stable adult composition, is currently unknown. Due to the intimate interaction between the developing intestinal microbiota and the immune system, the early-life development of the intestinal microbiota may have long-lasting consequences (Bäckhed et al., 2011).

Distortions in the intestinal microbiota are associated with a wide range of diseases, including the risk of diarrheal illness, food allergy, inflammatory diseases (atopic diseases and inflammatory bowel disease), irritable bowel syndrome, obesity, and diabetes (Sekirov et al.,

2010). Furthermore, as is the case with irritable bowel syndrome, gut-related diseases can develop or worsen during stressful periods (O'Mahony et al., 2009; de Palma et al., 2014). This may be due to the bidirectional communication between the central nervous system (CNS) and the gut (brain-gut axis; Dinan and Cryan, 2012), where both the autonomic nervous system (ANS) and the Hypothalamic Pituitary Adrenal (HPA) axis play important roles (Rhee et al., 2009). When the HPA axis is activated in reaction to stress, cortisol is produced as an end product. In rats, experimentally increased cortisone levels in pregnant females resulted in lower levels of total bacteria and gram negatives in the intestine of the pups (Schiffrin et al., 1993). This suggests that cortisone may influence the maternal microbiota, and thereby the transmission of bacteria to offspring. In humans it is as yet unknown if maternal prenatal psychological stress and cortisol concentrations are related to the infant gut microbiota.

The goal of the present human study is to prospectively investigate the relation between maternal prenatal stress and the development of infant intestinal microbiota and health in the first 110 days of life. A limitation of the Rhesus monkey study of Bailey et al. (2004) is that the intestinal microbiota analyses were carried out with traditional culturing approaches and were not able to show the more complex microbiota signatures. The present study avoids this limitation by using a high-throughput phylogenetic microarray (Rajilic-Stojanovic et al., 2009).

METHODS

Participants and procedure

This project is part of an ongoing longitudinal study in which 192 children are followed from the third trimester of pregnancy on. Infants were healthy, born at full term (≥ 37 weeks) and had a 5-min APGAR score ≥ 7. Inclusion criteria were an uncomplicated, singleton pregnancy, clear understanding of the Dutch language, no drug use and no current physical health problems (see Beijers et al., 2010 or Tollenaar et al., 2011 for more details). All mothers gave written informed consent, and the study was approved by the Ethical Committee of the Faculty of Social Sciences, Radboud University Nijmegen (ECG/AvdK/07.563).

Fecal samples at 9 time points were available for investigating the development of the infant intestinal microbiota from birth until ± 110 days of life (see de Weerth et al., 2013 for more details). For the current study, due to financial constraints, we made a selection of participants and fecal samples. Five out of the nine fecal sampling time points were selected. The mean (SD) of the postnatal collection days of these samples were: 6.7 (0.7), 12.5 (4.0), 24.8 (8.9), 83.8 (19.4), and 112.3 (15.4). Participants were selected based on reported prenatal maternal stress and anxiety. For five variables that measured maternal reported prenatal stress and anxiety, a median score was computed. Participants who scored above the median on 4 or 5 variables

were selected and categorized as the 'high maternal prenatal stress group' (N=48, 25% of the total sample). Participants who scored above the median on a maximum of 2 variables were selected and categorized as the 'low maternal prenatal stress group' (N=39, 20.3% of the total sample). Next, participants were excluded if the infant was missing three or more out of the five fecal samples (in both groups 10 participants were excluded for this reason). Children born with cesarean delivery (N=4, all in high maternal prenatal stress group) were also excluded. Seven participants were excluded because the microarray did not pass the quality check of high reproducibility (> 97 % Pearson's coefficient between two HITChip analyses) (Rajilic-Stojanovic et al., 2009). This resulted in a final group of 56 participants (low prenatal stress N=28, high prenatal stress N=28). The groups were significantly different on all of the five prenatal stress and anxiety and three postnatal stress and anxiety questionnaires, but did not differ on the maternal prenatal cortisol variables or the demographic characteristics (gender, birth weight, 5 minute APGAR score, maternal age, prenatal smoking, prenatal alcohol consumption, gestational age, infant use of antibiotics, duration of breastfeeding).

Measurements

Mothers filled out questionnaires on demographics, lifestyle (i.e., alcohol and smoking), and stress and anxiety in the third trimester of pregnancy (M=35.29 weeks, SD=1.22). The five stress and anxiety variables that were used in the present study are described below.

General anxiety

A Dutch translation of the state anxiety subscale of the State-Trait Anxiety Inventory (STAI; van der Ploeg et al., 1981; Spielberger, 1983, α between 0.90 and 0.96) was used to measure maternal prenatal anxiety. This subscale consists of 20 items scored on a 4-point scale. An example item is: 'I feel calm'. Higher scores indicated more anxiety.

Pregnancy-related anxiety

The pregnancy-related anxieties questionnaire-revised (PRAQ-R; van den Bergh, 1990) was used to measure pregnancy-related anxiety. Two subscales of this questionnaire scored on a 5-point scale, were used for analysis: PRAQ1. Fear of giving birth (three items; α between 0.79 and 0.83), PRAQ2. Fear of bearing a handicapped child (four items; α between 0.87 and 0.88). A higher score indicated more experienced anxiety.

Daily hassles

A Dutch questionnaire (Alledaagse Problemen Lijst, APL, Vingerhoets et al., 1989, with test-retest reliabilities between 0.76 and 0.87) was used to measure the occurrence and intensity of daily hassles. The questionnaire consists of 49 items for which participants have to indicate

whether or not the daily hassle has occurred in the past 2 months and how much it bothered them on a 4-point scale. A mean valence rating of the 49 items was computed by dividing the sum of total (negative) valence divided by the frequency of daily hassles. A higher value indicated more negativity.

Pregnancy-related daily hassles

To measure pregnancy-related daily hassles, a Dutch translation of the Pregnancy Experience Scale (PES) was used (DiPietro et al., 2004, α between 0.91 and 0.95). This questionnaire consists of 43 items and measures maternal appraisal of pregnancy related daily hassles. Participants have to rate pregnancy-related experiences (e.g., body changes during pregnancy) on two 5 point scales: one indicates the extent to which the experience was felt as a hassle, and the other indicates the extent to which the experience was felt as an uplift. The ratio of these ratings was computed by the sum of intensities of hassles divided by the sum of intensities of uplifts. A higher value indicated a larger negative emotional valence toward pregnancy.

Maternal prenatal cortisol

Around week 37 of pregnancy (M=37.37 weeks, SD=1.68) mothers collected 5 saliva samples on 2 consecutive days to determine cortisol concentrations. Samples were taken at awakening (day 1=07:48, SD=00:56, day 2=07:56, SD=01:02), 30 min after awakening, at 12:00, 16:00 and 21:00h. The cortisol concentrations at each sampling time strongly correlated between the days. To obtain one cortisol concentration per sampling time, the mean raw cortisol concentrations over the two days were calculated (for more details see Tollenaar et al., 2011). To determine the total cortisol secretion during the day, the Area Under the Curve with respect to the Ground (AUCg) was calculated over these means (Pruessner et al., 2003).

Infant gastrointestinal and allergic symptoms

During the first three months, mothers were asked to report on infant gastrointestinal symptoms and allergic reactions on a monthly basis, by means of a semi-structured interview (for more details see: Beijers et al., 2010). Mothers were asked if their infant had shown health symptoms during the past month, using yes-or-no items. Note that mothers reported about allergic reactions and not on diagnoses of allergies by physicians. Thereafter, the reported symptoms were coded using the International Classification of Primary Care (ICPC; Lamberts and Wood, 1987). Following the ICPC, the gastrointestinal symptoms reported by the mothers included: vomiting (D10), diarrhea (D11), constipation (D12), rectal bleeding (D16), gastroenteritis presumed infection (D73), disease digestive system, other (D99).

Confounders

Maternal age and educational level, parity, and infant birth weight were not associated with prenatal reported stress or cortisol concentrations and were therefore not included in the analyses. Potential confounders that were included in the analyses were breastfeeding during the study period, and postnatal maternal stress and anxiety when the infant was 3 months old. A significant proportion of the infants were not breastfed at each time point: 20% at age 7 days, 21% at 14 days, 30% at 28 days, 39% at 80 days, and 44% at 110 days. Therefore, breastfeeding was included as an explanatory variable in all models. Postnatal maternal stress and anxiety was assessed by means of the Perceived Stress Scale (Cohen et al., 1983), the state anxiety subscale of the STAI (van der Ploeg et al., 1981; Spielberger, 1983) and the daily hassles questionnaire (Dutch questionnaire Alledaagse Problemen Lijst, APL; Vingerhoets et al., 1989). Antibiotic use was very rare; at each time point of fecal sampling, maximally one infant had been given antibiotics, and this was found not to confound the analyses, so antibiotic use was not controlled for.

Collection of fecal samples and microbiota analysis

Parents collected the fecal samples of their infant at home and stored them at -20°C. The samples were transported in coolers with freezing cartridges or dry ice and stored at -20°C, and later at -80°C, until further processing at the Microbiology Laboratory of Wageningen University. DNA was extracted with the repeated bead-beating method as described by Salonen et al. (2010). The microbiota composition was analyzed twice with the phylogenetic microarray, the Human Intestinal Tract Chip (HITChip) (Rajilic-Stojanovic et al., 2009), which detects and quantifies over 1000 species-level phylotypes, with a specific focus on bacteria residing in the human intestine. The microarray consists of oligonucleotide probes for two hyper-variable regions (V1 and V6) on the 16S rRNA gene, allowing the identification, quantification and phylogenetic positioning of cultured and uncultured bacterial phylotypes. The DNA was amplified with PCR using the universal bacterial primers T7prom-Bact-27-for and Uni-1492-rev, and transcribed to RNA, which was labelled and hybridized on the microarray. The signal intensities of the oligonucleotide probes were translated into abundances of 1038 species-level phylotypes, 130 genus level-taxa, and 23 phylum-level taxa and clostridium clusters using the fRPA pre-processing algorithm (Lahti et al. 2013). The microarray has been shown to correspond to ca. 200 000 sequencing reads (Claesson et al. 2009). The microbiota data were transformed into relative abundances by dividing the signal intensities of each taxon by the total intensity of the sample.

Statistical analyses

All statistical analyses were conducted with R (R Development Core Team 2012), using the libraries nlme (Pinheiro et al., 2011) and vegan (Oksanen et al., 2013). To identify the most important maternal stress indicators to be utilized in further analyses, we first modeled the (log-transformed) relative abundance of each genus-level bacterial group, with each of the stress indicators separately as an explanatory variable, and controlling for breastfeeding and postnatal maternal stress. The stress indicators used were: the 5 separate questionnaire variables (raw scores), the sum of the 0-1 questionnaire scores (i.e., below or above the median) for the 5 prenatal stress variables (values ranging from 0 to 5), and the 5 separate cortisol concentrations (raw values) and the AUCg. This was done separately for each sampling age, and for all sampling ages together (using linear mixed effects models, with the individual as the random factor, with the function lme in the package nlme in R). Based on these models, the number of genus-level groups significantly associated at any sampling age with each stress indicator was calculated (Fig. 1). For further analyses, we selected the two stress indicators with the strongest association with the microbiota: the sum of the 0-1 questionnaire scores, and the 12:00 cortisol concentration.

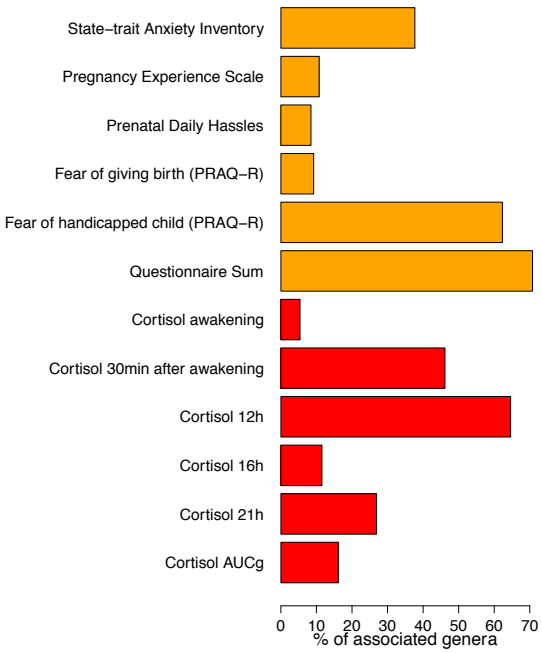

Figure 1. Percentage of genus-level bacterial groups significantly associated with the different maternal prenatal stress indicators at any sampling age (see Methods).

We estimated the amount of inter-individual variation in the total genus-level microbiota attributable to prenatal stress using permutational multivariate analysis of variance (Oksanen et al., 2013) (Fig. 2), controlling for breastfeeding and postnatal maternal stress by including these variables in the model. Including the relative abundances of all bacterial groups significantly associated with either stress indicator at any time point (101 genera, 78% of the microbiota), we performed a principal coordinates analysis (PCoA), using Manhattan distances (a measure of beta diversity). Based on the species-level data, we calculated the microbial alpha diversity as the inverse Simpson diversity index, over all species and within phyla.

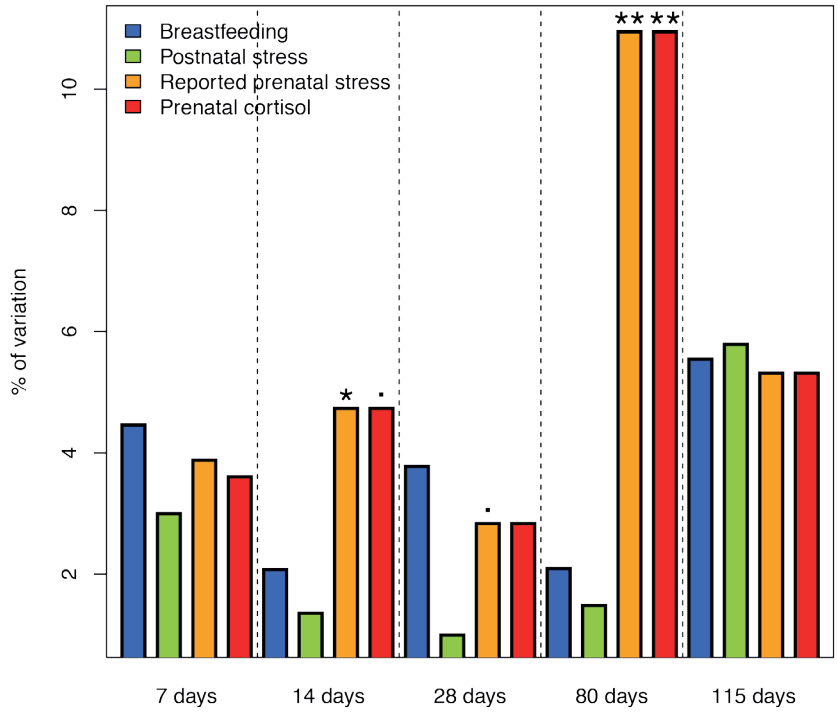

Figure 2. Percentage of inter-individual variation in the composition of the total microbiota attributable to differences in breastfeeding, maternal postnatal stress, and maternal prenatal reported stress and cortisol (based on Permutational multivariate analysis of variance). Asterisks indicate a significant contribution to the inter-individual variation: . = p< 0.1; * = p < 0.05; ** = p < 0.01.

RESULTS

Correlations between prenatal stress and anxiety variables

None of the reported stress and anxiety variables was significantly correlated with the cortisol variables, indicating that cortisol and reported stress represent independent measures of stress. The questionnaire-based stress variables were weakly to moderately correlated (r ranging from 0.22 to 0.74), and the cortisol concentrations measured at different times of day were weakly to strongly correlated with each other (r ranging from 0.15 to 0.87).

Comparison of the different stress indicators

Based on the number of significantly associated bacteria, the sum of the stress questionnaire scores and the cortisol concentration measured at noon were most strongly associated with the infant microbiota (Fig. 1). Of the individual questionnaire scores, the fear of a handicapped child (PRAQ2) had the strongest association with the infant microbiota, but not as strong as the sum of the questionnaire scores. We therefore selected the sum of questionnaire scores and the 12:00 cortisol concentration as measures of reported stress and cortisol concentration for further analyses. There was a weak positive correlation between the reported stress and the cortisol concentration (r = 0.25, $p < 0.001$). Both indicators were significantly associated with the relative abundances of over 60% of the bacterial genus-level groups at one or more time points during the first four months of the infants' life. A major part (78 %) of the microbial groups were associated with either reported stress or cortisol concentration.

The magnitude of the associations between the total infant microbiota and the prenatal stress (sum of questionnaires) and noon cortisol concentration were similar (Fig. 2). The effects of both prenatal stress indicators appeared comparable to or higher than those of breastfeeding, and were usually higher than the effects of postnatal maternal stress. The effects of prenatal stress on the infant microbiota were modest over the first month, peaked at 80 days, and were still clearly evident at 110 days.

As both stress indicators had similar associations with the microbiota, we combined the reported stress and the cortisol measure, forming a 'prenatal cumulative stress index': low reported stress + low cortisol concentration = low cumulative stress; low reported stress + high cortisol concentration, or high reported stress + low cortisol concentration = moderate cumulative stress; high reported stress + high cortisol concentration = high cumulative stress. See Table 1 for the descriptive statistics of these three groups.

Based on the temporal dynamics and the associations with the two chosen stress indicators (i.e., sum of the questionnaire scores and noon cortisol), we grouped the stress-associated bacterial genera into clusters within which the bacteria behaved uniformly (Table 2).

Prenatal cumulative stress is a major driver of inter-individual variation in infant microbiota

The major source of inter-individual variation in the infant microbiota was the ratio between a group of proteobacteria (*Escherichia, Enterobacter, Serratia;* referred to as bacterial group PRO1), and a group of lactic acid bacteria (*Lactobacillus, Lactococcus, Aerococcus;* group LAB) and Actinobacteria (*Bifidobacterium, Collinsella, Eggerthella;* group ACT1) (Fig. 3). There was a negative correlation between the relative abundances of PRO1 and LAB (r = -0.23, $p < 0.001$), which corresponded to the first principal coordinate (PC 1), and between PRO1 and ACT1(r = -0.40, $p < 0.0001$), which corresponded to the second PC (PC2) (Fig. 3). The infants in the low cumulative stress group were characterized by high relative abundance of LAB (69%, 377%, 305%, and 39% higher in the low cumulative stress, as compared to the high cumulative stress group at ages 7, 14, 28, and 110 days, respectively) and ACT1 (23%, 29%, 66%, 53% higher in the low cumulative stress group at ages 7, 14, 80, and 110 days). The infants in the high cumulative stress group tended to localize in the *Escherichia-Enterobacter*-end of the microbiota gradient (Fig. 3), with 853%, 256%, 1244%, and 699% higher abundances in the high cumulative stress group at ages 14, 28, 80, and 110 days, respectively. The sum of the first two principal coordinates (indicating a high relative abundance of PRO1 and low relative abundance of LAB and ACT1) was strongly associated with prenatal cumulative stress (Fig. 3). Infants in the high cumulative stress group, with the combined effect of high maternal prenatal reported stress and cortisol concentration, had the highest summed PC scores, and those in the low cumulative stress group had the lowest scores. Infants with only one prenatal cumulative stress factor, either high cortisol concentration or high reported stress, had an intermediate microbiota, suggesting a relatively linear association between cumulative prenatal stress and infant microbiota.

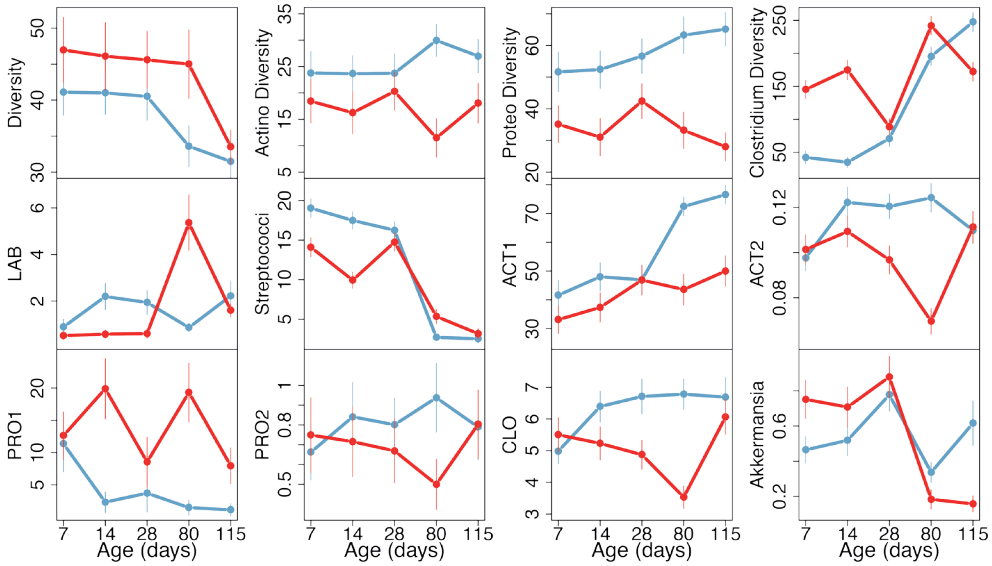

Figure 3. Associations between microbiota principal coordinates (beta diversity) and prenatal stress. Samples collected at different ages are shown in different panels for clarity. Light blue = low prenatal cumulative stress; light red = moderate cumulative stress; dark red = high cumulative stress (based on both questionnaires and cortisol, see text). The bacterial groups shown are *Enterobacter* (Ente), *Serratia* (Serr), *Escherichia* (Esch), *Aerococcus* (Aero), *Lactococcus* (Lact), *Eggerthella* (Egge), *Collinsella* (Coll), *Bifidobacterium* (Bifi) and those related to *Lactobacillus gasseri* (Lgas). a, b) Samples taken at 7 days of age; c, d) 14 days; e, f) 28 days; g, h) 80 days; i, j) 110 days. Asterisks indicate a significant difference from the low cumulative stress group: * = $p < 0.05$; ** = $p < 0.01$.

Temporal dynamics of the infant microbiota

We selected the low and the high cumulative stress groups and the bacterial groups most strongly associated with prenatal stress to illustrate the differences in the temporal dynamics (Fig. 4). In the low prenatal cumulative stress group, the overall diversity of the infants' microbiota decreased during the first four months of life, and was associated with the gradual establishment of dominant bacteria (mainly *Bifidobacterium*) (Fig. 4, blue lines; Fig. 5). However, the diversities within Actinobacteria, Proteobacteria, and Clostridia, the most abundant groups in the infant microbiota, increased with time (Fig. 4 blue lines). During the first month, the intestinal microbiota of the infants with low prenatal cumulative stress was characterized by a high abundance of streptococci and PRO1, which were gradually replaced by ACT1, LAB, *Clostridium* spp. (CLO), and Proteobacteria Group 2 (PRO2) (including low-abundance Proteobacteria, such as *Sutterella*; see Table 2). Similar patterns were evident at the phylum level (Fig. 5A).

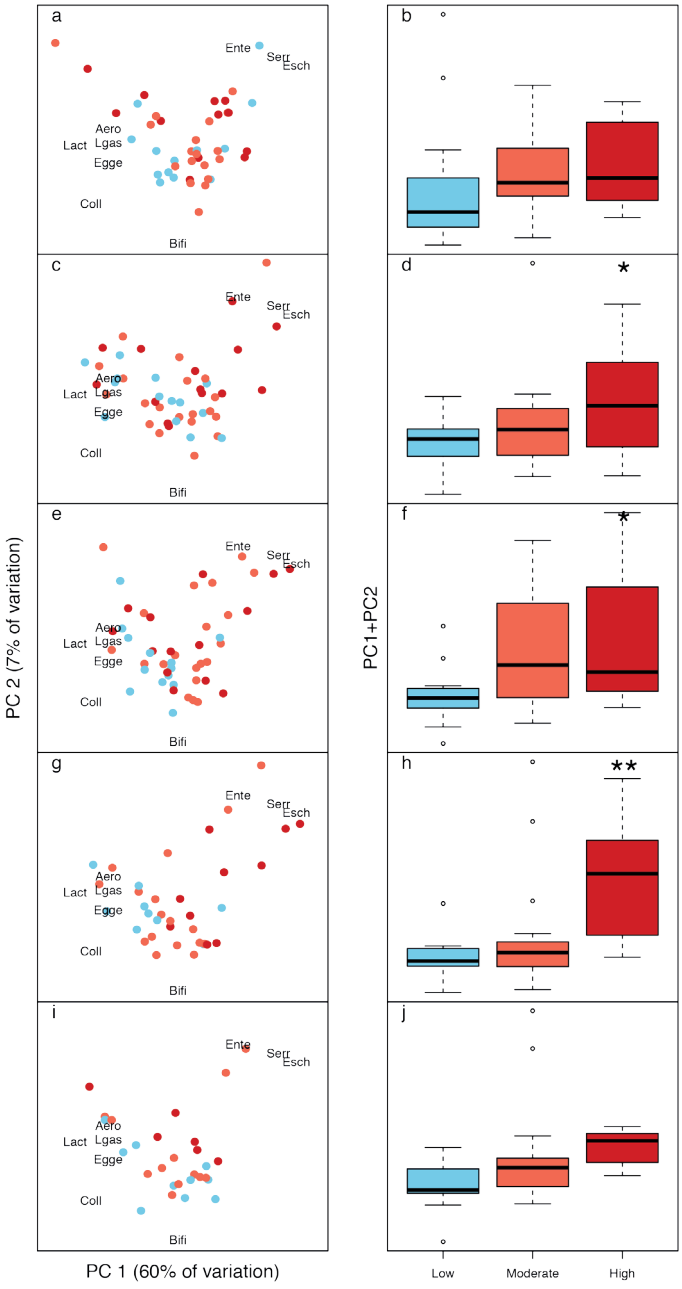

Figure 4. Development of the microbiota alpha diversity (inverse Simpson index, top row graphs) and the relative abundances (mean ± SE) of the significantly associated microbial groups (remaining graphs, see Table 2) in infants born to mothers with high (red) and low (blue) cumulative stress during pregnancy.

In the high cumulative stress group, the temporal development was different (Fig. 4, red lines). The overall diversity was consistently higher, due to more even relative abundances of different bacterial groups (Fig. 5), but the diversities within Actinobacteria and Proteobacteria were lower than in the low stress group. Furthermore, the relative abundance of PRO1 (and the total abundance of Proteobacteria, Fig. 5) was higher, and correspondingly the abundances of ACT1, ACT2, LAB, CLO and PRO2 were lower. The abundance of *Akkermansia* also differed significantly between the groups, as it declined dramatically in the high cumulative stress group after the first month and remained low thereafter (Fig. 4 red lines). The total abundance of Bacilli remained relatively high throughout the study period, whereas it declined considerably in the low cumulative stress group (Fig. 5).

Although we did not have sufficient power to properly investigate the potential influence of breastfeeding interacting with prenatal stress, we checked the results also separately for the breastfed and non-breastfed infants (data not shown). Compared to the observed association of prenatal stress and the infant microbiota, breastfeeding had a minor influence on the microbiota. Also, the microbiota association with stress was comparable between breastfed and non-breastfed infants. Therefore, we conclude that in this cohort, breastfeeding did not confound the interpretation of the results.

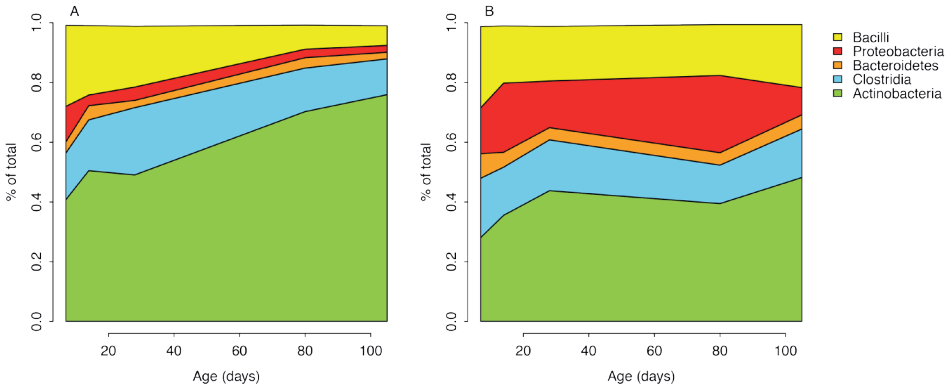

Figure 5. Average relative abundances of phylum-level bacterial groups (Firmicutes divided into Bacilli and Clostridia) during the study period in the low cumulative stress infants (A) and high cumulative stress infants (B). Bacilli includes the bacterial groups LAB and streptococci, Proteobacteria includes the groups PRO1 and PRO2, and Actinobacteria includes the groups ACT1 and ACT2. Members of the phylum Bacteroidetes were not strongly associated with prenatal stress and were therefore not further analysed. The phylum Verrucomicrobia (including only the genus *Akkermansia*) is not shown as its relative abundance of the total microbiota is very small (see Fig. 4).

Prenatal cumulative stress predisposes to gastrointestinal and allergic symptoms

Gastrointestinal symptoms (e.g., diarrhea, gastroenteritis, presumed infection and constipation) were more prevalent during the first three months of life in the high cumulative stress group (38%), as compared to the low cumulative stress group (22%). By the age of three months, 43% of the infants in the high cumulative stress group, and none in the low cumulative stress group, had shown allergic reactions. Note that due to the small sample sizes, these differences in symptom frequency were non-significant based on c^2-test ($p > 0.05$). The health differences between the groups appeared attributable to the differences in the intestinal microbiota. The infants with gastrointestinal symptoms had lower (albeit non-significantly) relative abundances of LAB (on average 0.5% of total microbiota) and *Akkermansia* (0.1%) than the infants without gastrointestinal symptoms (2% and 0.5%, respectively). Infants with allergic reactions by the age of three months had consistently lower abundances of LAB (0.5% vs. 2%, non-significant difference) and ACT1 (15% vs. 60%, $p < 0.001$), lower abundance of *Akkermansia* (only during the first month of life: 0.7% vs. 2%, $p < 0.05$), and higher abundances of PRO1 (30-50% vs. <10%, $p < 0.001$) than the infants without allergic reactions.

DISCUSSION

Maternal prenatal stress is associated with altered infant microbiota

We characterized the temporal dynamics of the human infant microbiota during the early stage of succession, using a phylogenetic microarray covering the majority of intestinal bacteria, in a cohort of 56 healthy, vaginally born infants, with varying exposure to maternal prenatal stress. Both maternal reported stress and salivary cortisol concentrations during late pregnancy were associated with dramatic shifts in the infant microbiota, which persisted until the end of the follow-up period at 16 weeks of age, even after correcting for breastfeeding and maternal postnatal stress. However, maternal reported stress and cortisol concentrations during pregnancy were only moderately correlated, suggesting that they tap into two different aspects of prenatal stress. Our secondary analyses showed that a combination of high reported stress and high cortisol concentrations (cumulative stress) was related to increased abundance of Proteobacteria such as *Escherichia* and *Enterobacter*, and decreased abundance of lactic acid bacteria and Actinobacteria. Most strikingly, such a colonization pattern was associated with maternally reported gastrointestinal symptoms and allergic reactions in the infant. The changes in the microbiota appear surprisingly universal, considering that the same pattern, a reduced number of Bifidobacteria and Lactobacilli, was found in rhesus monkeys prenatally exposed to a very different type of stressor (acoustic stress; Bailey et al., 2004).

We envisage three main mechanisms through which maternal prenatal cortisol concentrations may influence the infant intestinal microbiota. *First*, cortisol is known to control bile acid production in the liver, and regulate cholesterol and bile acid homeostasis (Rose and Herzig, 2013), plausibly thus directly influencing the maternal microbiota (Islam et al., 2011; Salonen and de Vos, 2014). High maternal cortisol may result in increased bile acid production, which could interfere with the natural development of the maternal intestinal microbiota during pregnancy (Koren et al., 2012). This could influence the transmission of microbiota from mother to infant (e.g., transfer of Bifidobacteria: Tannock et al., 1990). *Second*, maternal cortisol can cross the placenta and increase the fetal concentrations of cortisol (Duthie and Reynolds, 2013). This in turn could affect the development of the fetal HPA axis, resulting in higher infant basal cortisol concentrations and cortisol reactivity after birth (Tollenaar et al., 2011). Cortisol can affect the immune cells in the gut, change the permeability of the gut, disrupt the barrier function, and potentially influence the gut microbiota (Cryan and Dinan, 2012). *Third,* glucocorticoids in breast milk are correlated with salivary glucocorticoids in rhesus monkeys (Sullivan et al., 2011). Therefore, breast milk could be an additional potential vehicle by which mothers with higher prenatal concentrations of cortisol, and that continue to have high cortisol concentrations postnatally, transfer cortisol to the infant, affecting in turn the infant HPA axis and/or infant gut and finally, the infant intestinal microbiota. Although as yet hypothetical, the described pathways may explain how the maternal prenatal cortisol concentrations are related to the infant microbiota, and as such may constitute a basis for future research.

Reported prenatal stress, in the absence of elevated cortisol, had a similar impact on the infant microbiota as elevated cortisol in the absence of reported stress. One possible explanation is that, despite their normal levels of cortisol, mothers experiencing stress/anxiety transferred an altered microbiota to their infants, or exposed their infants to elevated cortisol concentrations via increased placental transfer due to reduced functioning of the11β-HSD2 enzyme (O'Donnell et al., 2012). Furthermore, maternal psychological stress could affect her physiology without direct involvement of the HPA axis, e.g., through maternal lifestyle (e.g., diet, sleep) or the immune system (Beijers et al., 2014). Hypothetically, mothers experiencing stress during pregnancy may have problems sleeping. Disturbed sleep produces inflammatory activation which may affect placental trophoblast invasion, affecting in turn embryo implantation and development. This may lead to abnormalities in the development of the fetal intestines, possibly finally affecting postnatal colonization by bacteria. Although this chain of events has yet to be determined, evidence for several of the steps has already been found (Beijers et al., 2014). Furthermore, stress influences the immune system that is in constant interaction with the intestinal microbiota and may directly affect the intestinal environment, especially with respect to inflammation. This pathway may lead to a selection of inflammation-tolerant bacteria, such as the enterobacteria (Lupp et al., 2007), which includes the genera *Escherichia, Enterobacter*, and *Serratia*.

We found only a weak correlation between maternal reported stress and cortisol, which suggests that they measure different kinds of prenatal stress. Summing these variables led to the strongest associations with the infant microbiota, indicating that the accumulation of feelings of stress/anxiety *and* high cortisol concentrations has a stronger relation with the infant microbiota than these stress variables independently. This is in line with the cumulative risk hypothesis (Rutter, 1979). Although the sum of the reported stress/anxiety variables included in our study showed the strongest relation with the infant intestinal microbiota, a stress/anxiety variable that seems to be especially important is the subscale 'fear of having a handicapped child', representing pregnancy-specific anxiety. Accordingly, pregnancy-specific anxiety was a strong predictor of infant cortisol reactivity in a larger cohort from this study (Tollenaar et al., 2011), as well as of infant baseline cortisol in other studies (Gutteling et al., 2005).

Health consequences of the aberrant microbiota

In early infancy, there appears to be a competitive balance between two groups of facultative anaerobic bacteria, the lactic acid bacteria and the Proteobacteria, the latter including the genera *Escherichia, Enterobacter* and *Serratia*. All known species belonging to the genus-level groups *Escherichia, Serratia,* and *Enterobacter* in the HITChip phylogeny can cause infections, particularly in susceptible individuals such as infants. Moreover, these are Gram-negative organisms, which produce lipopolysaccharide (LPS), an inflammatory endotoxin that has been implied in inflammation in a variety of metabolic diseases (Cani et al. 2014). Moreover, it has been suggested that LPS play a role in stress responses (Black 2002). A dominance of *Escherichia* and related organisms has been observed in infants treated with antibiotics (Fallani et al., 2010; Fouhy et al., 2012), in preterm infants later developing necrotizing enterocolitis (Normann et al., 2013), and in infants later developing colic (de Weerth et al., 2013). A dominance of enterobacteria in early infancy has been linked with allergy and eczema risk (Gosalbes et al., 2013), while lactic acid bacteria or Bifidobacteria appear to protect from allergies (Kuitunen et al., 2012). Furthermore, *Bifidobacterium infantis* has been shown to attenuate stress responses, and enteropathogenic *E. coli* to exacerbate them in infant mice (Sudo et al., 2004), suggesting that maternal prenatal stress may influence the HPA activity of the infant via the intestinal microbiota.

A low abundance of Lactobacilli and Bifidobacteria has been associated with increased crying in infants (de Weerth et al., 2013). Bifidobacteria, dominant members of the healthy infant microbiota (Fallani et al., 2010), are often not detected in prematurely born infants (Normann et al., 2013), in infants treated with antibiotics (Fouhy et al., 2012), or in infants born via caesarean section (Biasucci et al., 2010), suggesting that bacteria belonging to this genus are highly sensitive to environmental disturbances and are habitat specialists. In a

recent paper (Subramanian et al., 2014) several species of *Bifidobacterium* and *Lactobacillus* were found to be associated with healthy microbiota development in children.

To conclude, *Bifidobacterium* may be a reliable indicator of a healthy infant microbiota. The colonization pattern observed in the infants whose mothers had experienced stress during pregnancy resembles the patterns previously reported in infants with compromised health. Indeed, infants in the high cumulative stress group had a higher risk of gastrointestinal symptoms throughout the study period, and most strikingly, a higher risk of developing allergic reactions during the first three months of life. Health symptoms were also associated with a reduced level of *Akkermansia*. *Akkermansia muciniphila* is a mucus-degrading intestinal symbiont, residing in the mucus layer in close contact with the host (Belzer & de Vos 2012). It is known to improve intestinal barrier function and reduce LPS levels in mice fed a high-fat diet (Everard et al., 2013) and thought to have a protective and anti-inflammatory influence (Belzer & de Vos 2012).

CONCLUSION

In conclusion, maternal prenatal stress, based on questionnaires or on elevated basal cortisol concentrations, or on both, was strongly and persistently associated with the infants' microbiota composition and colonization pattern. Furthermore, the altered colonization pattern appeared to predispose the infants to gastrointestinal symptoms and allergic reactions. This is the first study to show a link between maternal prenatal stress, and the infant intestinal microbiota in humans. These results suggest that offspring health may be improved by modifying the intestinal microbiota during pregnancy, especially in women with stress.

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Table 1. Descriptive statistics for infants and mothers included in the present study

	Low adversity (N=18)	Moderate adversity (N=24)	High adversity (N=14)	p
Demographics				
Gender of infant				
Male	11	15	8	
Female	7	9	6	
Birth weight (gram; mean ± SD, range)	3563.39 ± 601.72 (2810-4600)	3567.38 ± 512.77 (2645-4730)	3731.86 ± 287.47 (3280-4150)	
5 minute APGAR score (mean ± SD, range)	9.65 ± 0.61 (8-10)	9.55 ± 0.74 (7-10)	9.57 ± 0.85 (7-10)	
Maternal age (years; mean ± SD, range)	32.42 ± 3.10 (26.30-36.90)	32.38 ± 3.36 (24.90-40.10)	32.46 ± 3.70 (26.30-38.40)	
Prenatal smoking				
Yes	0	1	0	
No	18	23	14	
Prenatal alcohol consumption				
Yes	2	3	2	
No	16	21	12	
Infant use of antibiotics[a]	1	1	0	
Breastfeeding month 0-4 (weeks; mean ± SD, range)[b]	9.28 ± 6.66, 0-16	9.52 ± 6.37, 0-16	10.77 ± 6.69, 0-16	
Gestational age (days; mean ± SD, range)	281.33 ± 8.97, 260-293	281.21 ± 7.36, 267-296	283.14 ± 7.52, 271-295	
Postnatal experienced stress[c] (mean ± SD, range)	23.39 ± 3.79, 17-30	26.54 ± 6.57, 17-45	28.15 ± 5.74, 20-40	<.10
Postnatal anxiety[c] (STAI; mean ± SD, range)	25.72 ± 4.86, 20-36	29.67 ± 10.18, 20-58	32.23 ± 10.18, 20-58	
Postnatal daily hassles[c] (mean ± SD, range)	0.77 ± 0.39, 0-1.4	1.16 ± 0.48, 0.2-2.7	1.15 ± 0.43, 0-1.8	<.05
Maternal stress variables; mean ± SD, range				
Prenatal anxiety (STAI)	25.28 ± 4.98, 20-41	31.08 ± 8.38, 20-51	35.21 ± 6.33, 23-46	
Pregnancy related daily hassles (PES)	0.20 ± 0.10, 0-0.40	0.39 ± 0.23, 0.20-1	0.52 ± 0.32, 0.10-1.20	
Prenatal daily hassles (APL)	0.76 ± 0.35, 0-1.30	1.18 ± 0.50, 0.30-2.40	1.27 ± 0.32, 0.60-1.80	

Fear of giving birth (PRAQ-R)	4.14 ± 1.53, 3-8	6.17 ± 3.50, 3-15	7.43 ± 3.27, 3-15	
Fear handicapped child (PRAQ-R)	6.11 ± 1.32, 4-9	8.42 ± 2.95, 4-15	10.21 ± 3.31, 5-15	
Cortisol awakening (nmol/L)	16.30 ± 4.38, 9.50-24	15.37 ± 3.73, 8.90-22.2	18.11 ± 4.78, 12.60-27	
Cortisol awakening + 30 min (nmol/L)	20.74 ± 5.21, 9.8-31.5	20.95 ± 5.09, 11.3-31	24.29 ± 6.90, 13.3-38	
Cortisol 12:00h (nmol/L)	13.13 ± 2.03, 9.6-16.1	14.78 ± 3.28, 7.6-19.7	19.78 ± 2.57, 17.1-25	<.001
Cortisol 16:00h (nmol/L)	11.02 ± 1.87, 6.9-14.9	12.14 ± 2.27, 7.8-16.1	15.06 ± 2.62, 11.6-20.9	<.001
Cortisol evening (nmol/L)	8.50 ± 1.51, 5.50-10.80	9.01 ± 2.44, 4-14.80	11.55 ± 3.24, 6-18.2	<.01
Cortisol AUCg[d] (nmol/L)	45.87 ± 7.09, 30-58.10	49.73 ± 9.01, 31-66.70	63.08 ± 10.18, 50.5-84.10	<.001

Note: One way ANOVA's showed no significant differences between the groups unless otherwise indicated.

[a]the infant in the low prenatal adversity group received antibiotics during month 1, the infant in the moderate prenatal adversity group received antibiotics during month 2.

[b]1 infant from the low prenatal adversity group, 5 infants from the moderate prenatal adversity group, and 2 infants from the high prenatal adversity did not receive any breastfeeding at all.

[c]postnatal stress and anxiety measured at 3 months

dArea Under the Curve with respect to the Ground

Table 2. Grouping of the stress-associated genera, based on similar temporal dynamics and associations with the stress indicators.

Group (CODE)	**Genera**
Lactic acid bacteria (LAB)	*Lactobacillus*, *Lactoccus*, *Aerococcus*
Actinobacteria 1 (ACT1)	*Actinomycetaceae*, *Bifidobacterium*, *Collinsella*, *Eggerthella*
Actinobacteria 2 (ACT2)	*Atopobium*, *Corynebacterium*, *Micrococcaceae*
Proteobacteria 1 (PRO1)	*Escherichia*, *Serratia*, *Haemophilus*, *Proteus*, *Enterobacter*
Proteobacteria 2 (PRO2)	*Aeromonas*, *Alcaligenes*, *Anaerobiospirillum*, *Aquabacterium*, *Bilophila*,*Campylobacter*, *Desulfovibrio*, *Helicobacter*, *Leminorella*, *Methylobacterium*,*Novosphingobium*, *Oceanospirillum*, *Oxalobacter*, *Sutterella*, *Xanthomonadaceae*
Clostridia (CLO)	*Anaerotuncus*, *Anaerostipes*, *Anaerovorax*, *Bulleidia*, *Clostridium*, *Catenibacterium*,*Coprobacillus*, *Coprococcus*, *Dialister*, *Dorea*, *Eubacterium*, *Faecalibacterium*, *Lachnobacillus*, *Lachnospira*, *Megamonas*, *Megasphaera*, *Mitsuokella*, *Papillibacter*, *Peptococcus*, *Peptostreptococcus*, *PhascolarctobacteriumRoseburia*, *Ruminococcus*, *Sporobacter*, *Subdoligranulum*

Chapter 8

Summary, conclusions, and general discussion

Summary of thesis

The link between prenatal maternal stress (PNS) and child outcomes has been studied extensively, and these studies showed that high levels of PNS are related to a broad range of child outcomes (e.g. health, behavior, and cognitive functioning). However, there is still much unknown about the persistence of these associations over time, and the underlying mechanisms. Previous studies on the links between prenatal physiological stress during pregnancy (i.e. maternal cortisol concentrations) and child outcomes are methodologically diverse, and their results are mixed. The **first aim of this thesis** was to review findings of studies on the links between prenatal maternal cortisol and child outcomes. Second, most studies on the links between PNS and infant health focused on birth outcomes and the first year of life. Research on long-term associations is scarce. Therefore, the **second aim of this thesis** was to address this gap by longitudinally examining the persistent and/or long-term associations of PNS with child outcomes. Finally, the **third research aim** was to gain more insight in a potential mechanism that may underlie the relation between PNS and child outcomes: the infant intestinal microbiota. The gut microbiota has recently been put forward as a mechanism potentially mediating the links between PNS and child outcomes, but has not yet been investigated in humans.

In the present chapter, the six studies included in the thesis (described in chapters 2 to 7) will be summarized, followed by the main conclusions of this thesis and a general discussion.

Chapter 2. Numerous studies have found links between mother-reported prenatal stress and anxiety and child outcomes. It is often proposed that heightened concentrations of maternal cortisol is the underlying mechanism in these links. If maternal cortisol concentrations are indeed a mediator, then there should be evidence from empirical studies that there are links between maternal prenatal cortisol concentrations and child outcomes. The designs of the studies conducted on these links are diverse, and the results are mixed. We conducted a systematic review following PRISMA guidelines on empirical studies of the association between maternal prenatal cortisol concentrations and child outcomes. Furthermore, an attempt was made to identify gestational periods in which the fetus is more susceptible to maternal cortisol.

The selection process of the empirical studies resulted in 28 papers, published between 2001 and 2012. The child outcomes were summarized in four main categories: physical/health, cognitive/motor, psychological/behavioral, and cortisol. The results of this review show that significant associations between maternal prenatal cortisol concentrations and child outcomes are not plentiful; 76% of all statistical analyses did not find a significant association. However, the majority of the studies that did find significant associations showed that higher levels of prenatal maternal cortisol concentrations are related to altered child outcomes in the expected direction: poorer physical/health outcomes, delayed cognitive/motor development, more

psychological/behavioral problems, and higher child cortisol concentrations. Furthermore, the results of the review show that fetal susceptibility to maternal cortisol probably varies over gestational periods for different child outcomes.

In conclusion, the fact that the statistical analyses yielded only few significant associations suggests that although maternal prenatal cortisol is related to (altered) child outcomes, cortisol may not be the sole or even main underlying mechanism in the relation between maternal prenatal stress/anxiety and child outcomes. Therefore, and also taking the limitations of the reviewed papers into account, we further conclude that the association between maternal prenatal cortisol and child outcomes merits attention in future studies. We highly recommend including other potential underlying mechanisms in these studies, as well as relevant confounding and moderating variables. These types of studies will help to further unravel the complex mechanisms between maternal prenatal stress/anxiety, maternal prenatal cortisol, and child outcomes.

Chapter 3. Previous studies have shown that PNS is positively related to child physical health problems in the first year of life. To examine if associations between PNS and child health outcomes are transient, persistent, or even progressive over time, it is important to examine these associations longitudinally. Therefore, in the second study of this thesis, the relation between PNS and health was examined longitudinally until middle childhood.

PNS was measured with maternal questionnaires and cortisol concentrations during late pregnancy. Children's illnesses and complaints were measured with the use of maternal reports at 30, 48, 60, and 72 months, covering the period from 18 months till age six. These illnesses and symptoms were divided into four main categories: respiratory, digestive, general, and skin. Furthermore, antibiotic use was obtained from medical records between one and six years of age. Multilevel models were used to examine the associations between PNS and child health (*N*=174).

The results showed a positive link between maternal reports of stress during pregnancy, in the form of prenatal general and pregnancy-specific anxiety, and offspring respiratory illnesses and symptoms. In addition, interaction effects with time showed that higher maternal general and pregnancy-specific anxiety during pregnancy was related to a higher number of illnesses until toddlerhood, but that these effects then tended to disappear. Additionally, a link was found between maternal prenatal cortisol concentrations and child health. A larger cortisol diurnal decline in late pregnancy was related to more child digestive illnesses until around the age of three. Also, higher levels of prenatal daily hassles were related to more prescribed antibiotic use in the period between one and six years of age. PNS was not related to general and skin illnesses between 18 months and six years of life.

The results of this study support the notion that the potential effects of PNS on child health persist after the first year of life, until toddlerhood, but disappear towards middle

childhood. Whether these or other associations between PNS and child health will reappear later in life remains to be determined in future prospective longitudinal studies.

Chapter 4. Animal studies in rat and primate models show that maternal PNS is related to their children's HPA axis reactivity. Only few studies examined this association in humans, and most focused on the first year of life. Also, to detect gradual changes of child HPA axis functioning over time, it is important to study HPA axis reactivity and the association with PNS at several time points during development. This type of research is hampered during early childhood due to the lack of an effective stress paradigm for this age group (toddlerhood till age seven). In order to examine the association between PNS and offspring HPA axis reactivity during early childhood, it was hence necessary to first develop a stress paradigm that elicited a significant cortisol response in 5- and 6-year-old children.

The Children's Reactions to Evaluation Stress Test (CREST) that we developed contains elements that have proven to be successful in increasing cortisol concentrations in older children and adults, namely social evaluative threat, unpredictability, and uncontrollability. The paradigm consists of three tasks, with a total duration of 20 minutes, in which the children (*N*=42 in this study) have to perform in front of a judge (social evaluative threat). Also, the tasks were rigged to provoke forced failure. Six saliva samples were taken to obtain measurements of baseline, reactivity, and recovery cortisol.

The results show that this paradigm is effective in increasing cortisol concentrations in 5- and 6-year-old children. Therefore, the CREST seems to be a suitable tool for future studies on stress reactivity in early childhood.

Chapter 5. Previous studies suggest that there may be developmental changes in stress reactivity during puberty and adolescence. However, existing laboratory stressors that were used in previous studies to examine stress reactivity at these ages are either not specifically aimed at adolescence, or their use requires a relatively high investment (i.e. several experimenters were needed to carry out the paradigm). Therefore, we aimed to develop a cost-effective stress paradigm that is specifically focused on puberty/adolescence, and to investigate cortisol responses and perceived stress in reaction to this test. Furthermore, sex and age differences in stress responses were examined.

Participants were 10- to-15-year old boys and girls (*N*=52; 23 boys; mean age=12.5 years). The new paradigm, the Social Evaluative Stress Test (SEST), is a computerized test that includes elements of social evaluation, unpredictability, and uncontrollability, which have been proven to be effective in eliciting a cortisol response in adolescents and adults. Furthermore, the paradigm focuses on social evaluation of personal characteristics instead of academic achievement, and includes comparisons/competition with peers. The test lasts 50 minutes. Seven saliva samples were taken to measure baseline, reactivity, and recovery cortisol. Additionally, perceived emotional stress was assessed with the use of questionnaires.

The results show that both boys and girls reported perceiving the paradigm as equally stressful, but only boys reacted with a significant cortisol increase (*M*=163%). There was no significant effect of age on cortisol responses. To our knowledge, this is the first computerized paradigm that elicits cortisol responses in 10- to 15-year old boys. In future studies it remains to be determined whether in girls other stress-related biological systems were activated as a consequence of the perceived stress.

Chapter 6. With the new effective stress paradigm described in chapter 4 we were able to examine the association between maternal PNS and cortisol and behavioral stress reactivity in 6-year-olds. As a behavioral measure we used gazing behavior towards the judge, who represents the social evaluative threat of the stress paradigm. Also, relations between cortisol reactivity and gazing behavior were examined to obtain more insight in the associations between physiological and behavioral stress responses.

PNS was measured with maternal questionnaires and cortisol concentrations during late pregnancy. Children (*N*=149; mean age=6.09 years) participated in a social evaluative stress paradigm: the Children's Reactions to Evaluation Stress Test (for stress protocol see Chapter 4). Six saliva samples were taken to obtain measures of cortisol reactivity and total cortisol secretion during the testing session. Also, gazing behavior towards the judge was observed.

The results showed that less maternal fear of giving birth, higher prenatal maternal cortisol concentrations, and more maternal postnatal anxiety were associated with higher child cortisol concentrations during the stress paradigm. Furthermore, there was a negative association between cortisol concentrations and gazing behavior towards the social evaluative threat (the judge) during the stress paradigm. These results point toward possible programming effects of PNS on child HPA axis functioning. Also, gazing behavior may serve as a coping strategy during a stressful situation for young children.

Chapter 7. Maternal PNS has often been associated with child outcomes. However, the mechanisms underlying this relation remain elusive. A previous study in rhesus monkeys showed a link between PNS and offspring intestinal microbiota and health. The goal of the present study was to investigate the development of the infant intestinal microbiota as a potential link between PNS and infant health in humans.

During late pregnancy, PNS was measured with the use of maternal questionnaires and cortisol samples in an ongoing longitudinal study in which 193 children are followed from pregnancy on. For the current study, a subset of participants was selected from this longitudinal sample. The selection was based on maternal reports on prenatal stress and anxiety. Two groups were created: the high prenatal stress group (*N*=28), and the low prenatal stress group (*N*=28). The development of the infant intestinal microbiota was followed over the first three months, and was determined in five fecal samples taken at 7, 14, 28, 80, and 115 days after

birth. Furthermore, mothers reported on their infants' gastrointestinal symptoms and allergic reactions on a monthly basis, by means of a semi-structured interview.

Results showed that both maternal reported stress and cortisol concentrations were related to dramatic shifts in the infant microbiota, persisting until 16 weeks of age. Secondary analyses showed that a combination of high reported stress and high cortisol concentrations was related to increased abundance of Proteobacteria, and decreased abundance of lactic acid bacteria and Actinobacteria. Also, this colonization pattern was associated with more maternal reports of gastrointestinal symptoms and allergic reactions in their infants.

In conclusion, associations were found between maternal prenatal stress and infant intestinal microbiota, as well as with infant gastrointestinal symptoms and allergic reactions. Although causality cannot be assumed, the results point towards a possible microbial mechanism by which PNS may influence child development.

Conclusions

1) Maternal prenatal cortisol is probably not the sole mediator of links between maternal stress and anxiety during pregnancy and child outcomes.
2) Maternal prenatal anxiety and cortisol are associated with children's respiratory and digestive illnesses until toddlerhood.
3) Pregnant mothers who reported more daily hassles have children who used more prescribed antibiotics between one and six years of age.
4) The Children's Reactions to Evaluation Stress Test (CREST) is a promising tool to be used in future studies on stress reactivity in 5- and 6-year-olds.
5) The computerized social evaluative stress test (SEST) is effective in increasing cortisol concentrations in 10- to 15-year-old boys.
6) Boys and girls perceive the SEST as equally stressful, but only boys react with significant cortisol increases.
7) Children of mothers who reported more prenatal and early postnatal anxiety show higher total cortisol secretions during the CREST, but their cortisol reactivity in reaction to the CREST is not higher.
8) Children who show higher cortisol reactivity during the CREST, gaze less towards the judge (social evaluative threat).
9) Infants of mothers who had more prenatal stress, have more potentially pathogenic bacteria and fewer beneficial bacteria in the first months of their life.

General Discussion

Studying prenatal stress and anxiety in the future: Towards an integrative model

In this thesis, maternal prenatal stress and anxiety (PNS) is examined as a main predictor of child outcomes. The concept of PNS is multidimensional, making it hard to obtain one measure of PNS that encompasses the broad range of feelings of stress and anxiety that can occur during pregnancy. For example, these feelings can be pregnancy-specific (e.g. fear of bearing a handicapped child) and/or of a more general nature (e.g. general feelings of anxiety). Also, what can be experienced by one person as stressful (e.g. bodily changes due to pregnancy), can be experienced as positive by others. These and other complexities described in the current thesis have led to an integrative model of the multidimensional mechanisms by which PNS may affect the developing fetus, and in turn the future of the child. Figure 1 presents this tentative model and in the next paragraphs the different associations presented in the model are explained.

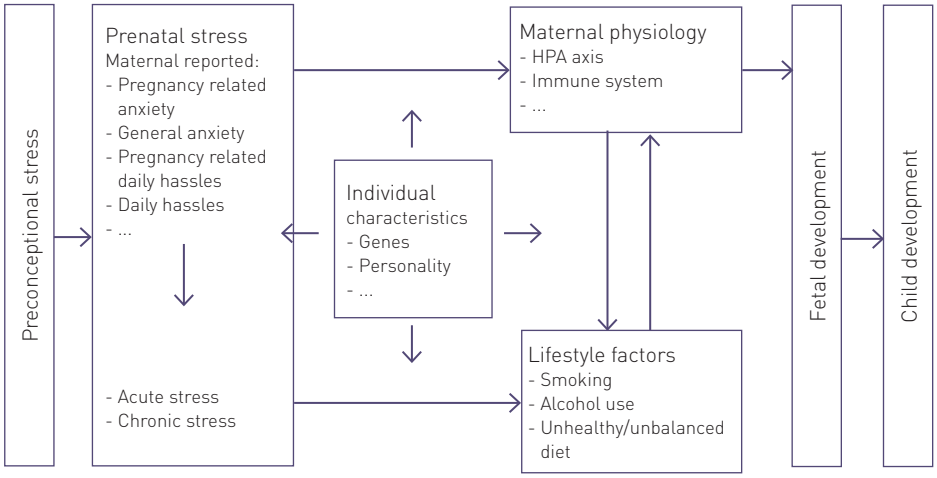

Figure 1. Integrative model of multidimensional mechanisms by which PNS may affect fetal and child development.

In the empirical studies that were included in this thesis, mother-reported PNS was assessed with the use of five stress variables: general anxiety, pregnancy-related anxiety (scales: fear of giving birth; fear of bearing a handicapped child), daily hassles, and pregnancy-related daily hassles, and physiological PNS was assessed with the use of salivary cortisol concentrations. The five mother-reported variables encompass a broad range of stress and anxiety and the chosen questionnaires meet good psychometric properties (Nast et al., 2013). Also, the correlations between the five variables are not high, indicating that they tap into

different forms of stress and anxiety. The results of the studies in this thesis showed that these different forms of PNS are related to different child outcomes. For example, prenatal general and pregnancy-specific anxiety (fear of handicapped child) were related to more child respiratory illnesses, prenatal daily hassles were related to more antibiotic use in children, and pregnancy-specific anxiety (fear of giving birth) was related to lower child cortisol concentrations during a social evaluative threat. These different associations could be due to different forms of PNS being related to child outcomes through different underlying mechanisms. For example, daily hassles (example items: 'you had a conflict with your partner', and 'family or friends were involved in a traffic jam') may result in acute stress and not necessarily in chronic stress. This may affect maternal physiology, and in turn fetal development, in different ways. Acute stress may lead to shorter periods of higher maternal cortisol secretion (Diego et al., 2009), whereas chronic stress has often been associated with a generally flatter diurnal cortisol curve (Cicchetti et al., 2010; Gunnar & Vazquez., 2001; Heim et al., 2008; Miller et al., 2007). These markers of cortisol physiological functioning may affect fetal development in different ways. On the one hand, glucocorticoids are essential for fetal organ development (Murphy et al., 2006; Mulder et al., 2002), but on the other hand prolonged heightened levels of cortisol could be detrimental for fetal development (Field and Diego, 2008; Weinstock, 2005). As mentioned above, in this thesis maternal cortisol concentrations are not studied as an underlying mechanism between reported PNS and child outcomes, but as a physiological stress marker in relation to child outcomes. The results of the studies on these links showed that maternal cortisol concentrations during late pregnancy were differently related to child outcomes. A larger cortisol decline during the day was related to more child digestive illnesses, higher maternal evening cortisol concentrations were related to higher child total stress cortisol concentrations, and higher maternal noon cortisol concentrations were related to more potentially pathogenic intestinal bacteria of infants. Hence, the dynamics of maternal stress physiology may affect fetal development differently depending on the exact aspect of maternal cortisol functioning that has changed.

There are studies showing that there is a low correlation between psychological stress and cortisol concentrations in pregnant women (Beijers et al., 2010; Bolten et al., 2011; D'Anna-Hernandez et al., 2012; Davis et al., 2007, 2011; Davis and Sandman, 2010, 2012; Goedhart et al., 2010; Gutteling et al., 2006, 2007; Ruiz et al., 2001; Tollenaar et al., 2011; Voegtline et al. 2013). This suggests that the HPA axis is most probably not the only physiological pathway through which reported stress and anxiety may affect fetal development (Beijers et al., 2014). Other physiological systems, such as the maternal immune system, might also be affected by PNS, and in turn affect fetal development. Again, these effects could be stressor-dependent. The experience of an acute, temporary stressor generally leads to enhanced immunity, whereas chronic stress may lead to immunosuppressive effects that can be severe enough to compromise defenses against infections and can in turn be pathogenic (Beijers et al., 2014; Sapolsky, 2005). Since intrauterine infections can be detrimental for fetal development and are found to be related to premature delivery (Goldenberg et al., 2008), chronic stress seems

especially harmful for later child development. Whether PNS will evolve into chronic stress or disappear rather quickly, will naturally depend on the nature and length of the PNS, but most probably also on the interplay between maternal individual characteristics such as genes, physiology, and personality. For example, in the development of post-traumatic stress disorders, introverted and less emotionally stable personality-types positively correlate with higher levels of PTSD and depression (Canetti et al., 2016).

Relations between different types of PNS, maternal physiology, and fetal/child development may additionally be mediated by maternal lifestyle factors. Studies showed that the exposure to chronic stressors (e.g. financial stress, parenting challenges, and living in disruptive home environments) is related to smoking during pregnancy (Yang et al., 2017). Also, symptoms of depression and/or anxiety during pregnancy are related to smoking, consumption of unhealthy and unbalanced diet, and excessive weight gain during pregnancy (see recent review by Ulrich and Petermann, 2016). These different types of lifestyle factors affected by PNS could potentially affect the fetus in different ways. For example, a study in humans showed that alcohol use in pregnancy is related to a decreased placental weight and placenta-to-birthweight ratio, whereas use of methamphetamine during pregnancy is associated with larger placental weight and placenta-to-birthweight ratio (Carter et al., 2016). Furthermore, excessive alcohol consumption during pregnancy is related to child mental retardation (Chomitz et al., 1995), while prenatal smoking retards fetal growth and increases the risk of preterm delivery (Yang et al., 2017).

As shown in the model in Figure 1, maternal individual characteristics potentially moderate the association between PNS and maternal lifestyle factors. For example, in contrast to maternal nicotine consumption, the consumption of low to moderate alcohol consumption during pregnancy was found to be more prevalent in women with a higher social-economic status and women who were older than 30 (Ulrich and Peternmann, 2016). Summarizing, PNS encompasses different types of stress and anxiety, which potentially activate different underlying physiological mechanisms and are related to different maternal lifestyle factors. Maternal individual characteristics would moderate these associations. Both changes in maternal physiology and lifestyle factors will affect fetal development.

Dynamics of change over pregnancy. To date most studies on PNS link discrete measures of PNS assessed during different periods of pregnancy to child outcomes. The dynamics of changes in PNS and corresponding physiological changes over pregnancy have received relatively little attention. Davis and Sandman (2010) showed that low concentrations of cortisol during early pregnancy in combination with high concentrations during late pregnancy were related to enhanced infant cognitive development. Also, they found that accelerated increases in cortisol during pregnancy were related to higher cognitive development (Davis and Sandman, 2010). Hence, the whole pregnancy trajectory of change in cortisol concentrations, instead of single measurements, may be most predictive of offspring development. Also, a study of

Sandman et al. (2012) showed that mothers who experienced congruent levels of depression before and after delivery, had infants with enhanced motor and mental development, even when depression was relatively severe. This indicates that the dynamics of psychological and physiological stress changes over pregnancy, as well as in the early postnatal period, are unexplored albeit promising areas for future research into fetal and child development.

Biological sampling of cortisol. Most studies including cortisol measurements in pregnant women use saliva, blood, or urine samples to determine cortisol concentrations. These measures reflect the cortisol concentrations of a limited time frame. To obtain measures of chronic stress, potentially indicating fetal cortisol exposure over longer periods of time, determination of cortisol in hair samples has emerged as a promising tool. Hair grows around 1 cm a month, and therefore the first centimeter of hair at the scalp represents the cortisol production during the past month, the second centimeter the month before that, and so on (Stalder and Kirschbaum, 2012). Hence, upon delivery, nine centimeters of maternal hair would indicate the cortisol production during pregnancy (e.g. Braig et al., 2016; Massey et al., 2016; Wikenius et al., 2016). This method may even make it possible to obtain measures of cortisol secretion *before* pregnancy. This period is often not included in prospective studies on PNS and child outcomes because women are mostly recruited when they visit the midwife or obstetrician for the first time. The periconceptional period is important in the development of gametes, embryonic organs and programming of the placenta. Poor nutrition and lifestyles during this period could affect pregnancy outcomes, and could have long-term health consequences through epigenetic programming (Steegers-Theunissen et al., 2013; Godfrey et al., 2010). Moreover, although periconceptional studies usually focus on nutrition and lifestyle factors, such as smoking and alcohol use, there are indications that stress during the preconception period may also affect fetal development. For example, a recent study by Strutz et al. (2014) showed links between preconception chronic stressors and lower birth weight, indicating that stress during this period may influence fetal development.

Interrelations between child health, HPA axis functioning, and intestinal microbiota

The findings of the three empirical studies using the same longitudinal research sample (BIBO study) showed that PNS is related to altered child outcomes. *First*, PNS was related to children's respiratory and digestive illnesses until around toddlerhood, and antibiotic use between age one and six (Chapter 3). *Second*, PNS was related to higher total cortisol secretion during a stressor in six- year-old children (Chapter 6). *Third*, PNS was related to the composition of the infant intestinal microbiota and gastrointestinal and allergic symptoms during the first 16 weeks of life (Chapter 7). Investigating links of PNS with separate outcome variables, is currently the most common method used in studies on PNS and child outcomes. However, the outcome variables just described (child health, HPA axis functioning, and intestinal microbiota) may be interrelated (see Figure 2). These potential associations, in addition to the relations

with PNS, were not studied in the current thesis due to insufficient statistical power, but are considered promising avenues for future research in large longitudinal population studies. Studying this will lead to a better understanding of possible multidimensional developmental pathways in offspring exposed to PNS.

First, the bidirectional communication between the gut microbiota and the brain (gut-brain axis) is mediated by neural, hormonal, and immunological routes, including the HPA axis. Previous studies showed that activity of the HPA axis, and the secretion of cortisol, could influence the gut permeability, barrier function, and composition of the intestinal microbiota (Cryan and Dinan, 2012). In rhesus monkeys, for example, exposure to an early life stressor, in the form of maternal separation between six and nine months of age, resulted in temporary alterations in the gut microbiota in the offspring (Bailey and Coe, 1999). In another study, in which rats were stressed by being daily separated from their mothers for 3 hours between 2 and 12 days after birth, showed long-term effects of stress in early life on the composition of the intestinal microbiota (O'Mahony et al., 2009). The communication between the gut and the HPA axis is bidirectional. Animal studies indicated that the intestinal microbiota influences HPA axis functioning. For example, mice that were raised in a germ-free environment, without gastrointestinal bacteria, showed an exaggerated HPA axis activity in reaction to stress (Sudo et al., 2004). After colonization with commensal bacteria, used from control mice, this reaction normalized. Timing during development seems to be important: the earlier the colonization, the greater the reversal of the effects (Cryan and Dinan, 2012).

Second, the HPA axis and the immune system are interrelated (Dumbell et al., 2016). The HPA axis represents an immunoregulatory system. Immune cells can activate the HPA axis via cytokines, and glucocorticoids can affect functioning of many immune cell types. The synthesis and release of cytokines is suppressed by glucocorticoids, and in turn protects the host from harmful consequences of long-term activation of the immune system (Dumbell et al., 2016). Especially in stressful situations the HPA axis plays an important role in balancing the immune response. It is suggested that appropriate HPA axis responsiveness is needed to control immunological processes, in turn preventing damaging immune responses and effects on health (Buske-Kirschbaum et al., 2003).

Third, the intestinal microbiota is related to health. One of the major functions of the intestinal microbiota is to protect humans from gut colonization by harmful species. This is also called the 'barrier-effect', and protects humans against invasion by pathogens (Vael & Deasager, 2009). Furthermore, the gut microbiota plays an important role in the maturation of a child's immunity. A late acquisition of gut bacteria or a reduced complexity of the microbiota may delay immune maturation (Adlerberth & Wold, 2008). In sum, the gut microbiome functions as an 'organ' that has an important influence on human health. This implies that distortions in any of the microbiota functions could potentially lead to a wide range of diseases, including diarrheal illness, food allergy, inflammatory diseases (atopic diseases and inflammatory bowel disease), irritable bowel syndrome, obesity, and diabetes (Sekirov et al., 2010). In line with this,

in this thesis links between the intestinal microbiota and infant health symptoms were found during the first sixteen weeks of life (chapter 7), indicating that there may be a link between intestinal microbiota and health already in early life.

From the above it becomes clear that the separate outcome variables (child health, HPA axis functioning, and intestinal microbiota) are most probably interrelated. The question remains how these outcomes are related, and if there are specific developmental pathways that can be defined for fetuses exposed to stress in the womb. For example, one could speculate that PNS affects fetal, and in turn infant, HPA axis functioning, which leads to changes in the composition of the intestinal microbiota, and in turn to adverse health outcomes in children. In humans, ethical constraints make it difficult to examine these causal developmental pathways between PNS, HPA axis functioning, microbiota, and health. However, studies with probiotic bacteria are promising for determining causal relations between these factors (e.g. Wilms et al., 2016; Kelly et al., 2016; Stojkoviç et al., 2016). For example, pooled data provides evidence that supplementation of infant formula with *B. lactis* is associated with a decreased risk of non-specific gastrointestinal infection in children (Bertelsen et al., 2016), suggesting a causal link between the intestinal microbiota and health of children. As described above, animal models have provided evidence for different parts of the model presented in Figure 2. To study these issues further in humans one may revert to the probiotic studies mentioned above, or, in the case of the HPA axis, to longitudinal research including frequent repeated measures of child intestinal microbiota and health. These types of studies are first steps to unraveling potential developmental pathways in which children might be programmed by PNS.

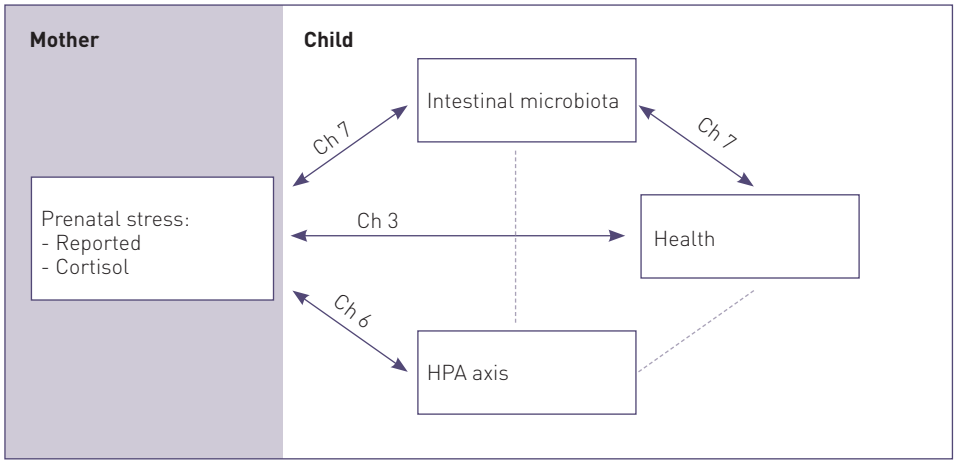

Figure 2. Overview of significant associations found in this thesis' empirical studies between maternal prenatal stress and child microbiota, HPA-axis functioning, and health. Note that the dotted lines represent proposed links that were not examined in this thesis.

Clinical implications

In this thesis, links were found between maternal PNS and child outcomes in a non-clinical sample. The participating women were not necessarily exposed to extreme forms of stress and anxiety, and the results hence indicate that more moderate levels of stress and anxiety during pregnancy are most probably already related to child outcomes. Maternal prenatal psychological distress is common. During pregnancy, mood symptoms increase, with more than 10% of pregnant women suffering from mood symptoms (Gentile, 2015). Also, pregnancy-specific fear, anxiety and depressive feelings are common in pregnant women, such as the fear of bearing a handicapped child or anxious feelings about the delivery (Martini et al., 2016). In order to protect children from the potentially negative effects of maternal distress, it is on the one hand imperative to find effective early intervention and prevention programs that can reduce the mother's psychological distress during pregnancy. Intervening when the child is still in the womb is an efficient way of giving the child a good start in life, and of positively and profoundly influencing the child's development (Conti and Heckman, 2013).

On the other hand, from the studies included in this thesis, we cannot conclude that 'effects' of PNS are long lasting. For example, the relation between PNS and child health persisted until toddlerhood, and disappeared in the ensuing study period from 4-6 years of age. The question that arises is if it is necessary to intervene when these potential 'effects' seem to disappear over time? The finding that the relations between PNS and child health persist only until toddlerhood, does not mean that children are better off for the rest of their lives. For example, studies on the effects of undernutrition during gestation on child outcomes, using the Dutch famine cohort, showed that malnutrition during gestation did not necessarily affect birth size of the baby, but did have adverse consequences for health in later life, such as coronary heart diseases. This implies that the fetus continued to grow, was born with a normal birth weight, but that the undernutrition nevertheless had adverse consequences for adult health (Roseboom et al., 2001). Moreover, maternal influences continue after birth. Studies have shown that mothers that are stressed and/or depressed during pregnancy are most likely to be also stressed and/or depressed after the child is born (Paulson et al. 2006). Growing up with a mother that is stressed and/or depressed can have severe detrimental effects on children's health and well-being (Sanger et al. 2015). Interventions that help reduce maternal distress during pregnancy may have important spillover effects to the postpartum period, reducing maternal distress in this period as well. In turn, this would contribute to improvements in health and well-being for the mother and to putting the child on a positive developmental pathway.

Besides prevention and intervention during pregnancy, it could be even more beneficial to prevent PNS *before* pregnancy. Preconceptional screening and care, in the form of risk assessment, health promotion, counseling, and interventions, is receiving increased attention in health care (Ramey et al., 2015), and might be a promising approach to improve both maternal and child health. Currently, effective interventions implemented in the

periconceptional period comprise maternal and paternal health behavior, such as weight loss, improving eating habits, using folic acid supplements, and decreasing tobacco use (van Dijk et al., 2016), but the inclusion of stress-reducing strategies has been largely neglected (Toivonen et al., 2016). Since studies show that PNS is also related to maternal and child health, also targeting PNS may be beneficial for pregnancy outcomes and should receive more attention in the current health care system.

Limitations and future directions

This thesis included several measures of maternal PNS: reported prenatal stress and anxiety, and cortisol concentrations as physiological stress markers. Furthermore, child outcomes were measured longitudinally in a relatively large sample and into middle childhood, and new effective stress paradigms were developed and used to examine stress reactivity and its relation with PNS in young children and adolescents. Besides these strengths, there are also some limitations. Mothers from the longitudinal sample were highly educated which could affect the generalizability of the studies. Note, however, that it is unrealistic to assume that highly educated pregnant women do not suffer from psychological stress and anxiety. Moreover, the fact that relations between PNS and child outcomes were found in a highly educated sample implies that in women suffering from more severe stress these associations may be even stronger. Another limitation is that only one endocrine parameter, child cortisol, was used as a marker of stress reactivity to the social evaluative paradigm. Endocrine factors, such as cortisol concentrations, do not function in isolation (Bos, 2016). Other hormones, such as oxytocin, are also part of the complex physiological stress system. Additionally, including measures of the sympathetic nervous system, such as cardiovascular measures, could render a more complete picture of the functioning of the physiological stress system of young children.

Summarizing the directions for future research discussed above, a first direction would be to obtain more insight into gestational periods in which the fetus is more sensitive to maternal cortisol concentrations and lower psychological wellbeing. Studying the impact of fetal exposure to maternal PNS during all trimesters of pregnancy is recommended, as well as studying the trajectory of PNS throughout pregnancy in relation to child outcomes. Second, future prospective research concentrating on the interrelations between the child outcomes included in this thesis should shed more light on potential underlying mechanisms in the relation to PNS. Third, more longitudinal studies with follow-ups at least into young adulthood are needed to uncover whether the associations found persist over time. To answer these future directions, intensive longitudinal studies that include comprehensive measures of PNS and child outcomes are needed. Sample sizes should be large enough to avoid power issues.

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NEDERLANDSE SAMENVATTING / SUMMARY IN DUTCH

SAMENVATTING

Eerdere studies naar maternale prenatale stress (PNS) hebben laten zien dat meer PNS is gerelateerd aan een breed scala van uitkomsten bij het kind, zoals meer gezondheids- en gedragsproblemen. Alhoewel er al veel onderzoek is gedaan naar de relatie tussen PNS en kinduitkomsten, ligt de focus van deze studies met name op het eerste levensjaar. Het is nog veelal onbekend of PNS ook is gerelateerd aan kinduitkomsten in de kindertijd. Ook is er nog steeds veel onbekend over de onderliggende mechanismen waardoor PNS is gerelateerd aan kinduitkomsten.

PNS bestaat uit zowel gerapporteerde stress als fysiologische stress. Studies naar de relatie tussen kinduitkomsten en fysiologische stress tijdens de zwangerschap, veelal gemeten door middel van cortisol concentraties bij de moeder, zijn methodologisch divers en laten gemengde resultaten zien. Het **eerste doel** van dit proefschrift was om een samenvatting te geven van alle eerdere studies naar de relatie tussen maternale prenatale cortisol en kinduitkomsten. Omdat onderzoek naar de lange termijn effecten van PNS op kinduitkomsten schaars is, was het **tweede doel** van dit proefschrift om dit gat te dichten door longitudinaal onderzoek te doen naar de lange termijn effecten van PNS. Tot slot was het **derde doel** van dit proefschrift om meer inzicht te krijgen in een potentieel onderliggend mechanisme in de relatie tussen PNS en kinduitkomsten: de darmmicrobiota. PNS zou mogelijk de ontwikkeling van de darmmicrobiota kunnen verstoren, waardoor onder andere gezondheidsproblemen bij het kind kunnen ontwikkelen. Een eerdere studie bij rhesus apen liet zien dat er een link is tussen PNS en de darmmicrobiota van hun nakomelingen, maar deze relatie is nog niet in mensen onderzocht.

In deze samenvatting worden de zes studies die onderdeel zijn van dit proefschrift (beschreven in hoofdstuk 2 t/m 7) samengevat, gevolgd door de conclusies die uit deze studies naar voren zijn gekomen.

Hoofdstuk 2. Eerdere studies hebben een relatie gevonden tussen stress en angst tijdens de zwangerschap (gerapporteerd door moeder) en kinduitkomsten. In deze studies wordt vaak gesuggereerd dat verhoogde cortisol concentraties van de moeder een onderliggend mechanisme is in deze relatie. Als maternale cortisol inderdaad een mediator is, dan zouden empirische studies naar de link tussen maternale cortisol tijdens de zwangerschap en kinduitkomsten dit moeten bevestigen. De studies die naar deze link hebben gekeken zijn qua design divers, en de resultaten zijn gemixt. Wij hebben een systematische review gedaan, met gebruik van PRISMA richtlijnen, naar de empirische gepubliceerde studies die de link tussen maternale prenatale cortisol en kinduitkomsten hebben onderzocht. Daarnaast hebben we onderzocht of er specifieke trimesters zijn waarin de foetus meer vatbaar is voor maternale cortisol concentraties.

Het selectieproces van empirische studies resulteerde in 28 artikelen, gepubliceerd tussen 2001 en 2012. De kinduitkomsten zijn samengevat in vier hoofdcategorieen: fysiek/gezondheid, cognitief/motorisch, psychologisch/gedrag en cortisol. De resultaten van deze review lieten zien dat de significante associaties tussen prenatale cortisol concentraties en kinduitkomsten niet erg groot zijn; 76% van alle statische analyses heeft geen significante associaties gevonden. De studies die wel een significante relatie vonden lieten zien dat hogere niveaus van maternale cortisol tijdens de zwangerschap gerelateerd zijn aan verslechterde kinduitkomsten: zwakkere fysieke/gezondheidsuitkomsten, vertraagde cognitieve/motorische ontwikkeling, meer psychologische/gedragsproblemen, en hogere cortisol concentraties van de kinderen. Daarnaast lieten de resultaten zien dat de foetale kwetsbaardheid voor maternale cortisol waarschijnlijk varieert over verschillende trimesters, afhankelijk van het type kinduitkomst.

Doordat uit deze systematische review blijkt dat er maar weinig significante relaties zijn gevonden tussen maternale prenatale cortisol en kinduitkomsten, concluderen we dat maternale cortisol waarschijnlijk niet het enige of zelfs hoofdmechanisme is in de relatie tussen gerapporteerde stress/angst van de moeder tijdens de zwangerschap en kinduitkomsten. Echter, gezien de limitaties van de geïncludeerde studies, concluderen we ook dat de relatie tussen maternale prenatale cortisol concentraties en kinduitkomsten verder onderzocht moet worden in toekomstige studies. Daarnaast adviseren we om ook andere potentiele mechanismen te onderzoeken in de relatie tussen PNS en kinduitkomsten. Deze type studies zullen helpen om de complexe mechanismen tussen maternale prenatale stress/angst, maternale prenatale cortisol en kinduitkomsten verder te ontrafelen.

Hoofdstuk 3. Eerdere studies hebben aangetoond dat hogere PNS gerelateerd is aan meer fysieke en gezondheidsproblemen in het eerste levensjaar van kinderen. Om te onderzoeken of deze relatie tussen PNS en gezondheidsuitkomsten van kinderen voorbijgaand, blijvend of zelfs progressief is, is het belangrijk om deze relatie longitudinaal te onderzoeken. Daarom werd in de tweede studie van dit proefschrift de relatie tussen PNS en de gezondheid van kinderen longitudinaal onderzocht tot de leeftijd van 6 jaar.

PNS werd gemeten aan het eind van de zwangerschap door het gebruik van vragenlijsten die ingevuld werden door de moeder, en de bepaling van cortisol concentraties in speeksel van de moeder in een longitudinaal onderzoek waarin 193 kinderen gevolgd worden vanaf de zwangerschap (BIBO onderzoek; Basale Invloeden in de Baby Ontwikkeling). Ziekten en gezondheidsklachten tijdens het afgelopen jaar werden gerapporteerd door de moeder toen de kinderen 30, 48, 60 en 72 maanden oud waren. De ziekten en symptomen werden onderverdeeld in vier hoofdcategorieën: ziekten van de luchtwegen, ziekten van de spijsvertering, algemene ziekten en ziekten van de huid. Daarnaast werd het antibiotica gebruik van de kinderen in de vroege kindertijd (1-6 jaar) verkregen via medisch dossiers van de huisarts. Multilevel modellen en regressieanalyses werden gebruikt om de associatie tussen PNS en de gezondheid van kinderen te onderzoeken (N=174).

Deze studie liet een positieve relatie tussen gerapporteerde stress tijdens de zwangerschap, in de vorm van prenatale algemene en zwangerschaps-specifieke angst, en ziekten van de luchtwegen van kinderen zien. Daarnaast lieten interactie effecten met tijd zien dat deze relatie bleef bestaan tot de kleutertijd, maar daarna verdwijnt. Ook laat deze studie zien dat maternale prenatale cortisol concentraties gerelateerd zijn aan ziekten van de kinderen. Een grotere afname van cortisol gedurende de dag aan het eind van de zwangerschap was gerelateerd aan meer ziekten van de spijsvertering. Ook deze relatie hield stand tot de leeftijd van ongeveer drie jaar. Verder waren meer dagelijkse problemen gerelateerd aan meer voorgeschreven antibiotica kuren. PNS was niet gerelateerd aan algemene ziekten en ziekten van de huid in de vroege kindertijd.

De resultaten van deze studie bevestigen deels dat potentiele effecten van PNS op de gezondheid en antibiotica gebruik van kinderen blijven bestaan na het eerste levensjaar, tot de kleutertijd, maar verdwijnt tijdens de kindertijd. Of deze of andere associaties tussen PNS en de gezondheid van kinderen weer zullen verschijnen op een later moment in het leven zal in toekomstige longitudinale studies onderzocht moeten worden.

Hoofdstuk 4. Om te kunnen onderzoeken of de relatie tussen PNS en cortisol reactiviteit van baby's en kinderen voorbijgaand, blijvend of zelfs progressief is, is het belangrijk om de ontwikkeling van cortisol reactiviteit over tijd te bestuderen. Onderzoek naar stress reactiviteit is afwezig tijdens de vroege kindertijd doordat er geen effectieve stress taak is voor deze leeftijdsgroep (peutertijd tot 5 jaar). Om de relatie tussen PNS en stress reactiviteit tijdens de kleutertijd te kunnen onderzoeken, was het allereerst nodig om een stress taak te ontwikkelen die effectief is in het uitlokken van een cortisol reactie bij 5- en 6-jarigen.

In deze studie hebben we een nieuwe stress test, genaamd De 'Children's Reactions to Evaluation Stress Test (CREST)' ontwikkeld voor 5- en 6-jarigen. Deze test bevat elementen waarvan we uit eerder onderzoek weten dat ze succesvol zijn in het verhogen van cortisol concentraties in oudere kinderen en volwassenen, namelijk: sociale evaluatie, onvoorspelbaarheid en oncontroleerbaarheid. De CREST bestaat uit drie taken, met een totale duur van 20 minuten, waarin de kinderen taken moeten uitvoeren die beoordeeld worden door een jury. Deze taken waren zo opgezet dat de kinderen sowieso zouden falen. Zes speekselsamples werden afgenomen om cortisol te kunnen bepalen tijdens de hele CREST, zowel de stress reactie als ook het herstel. De test is goedgekeurd door de ethische commissie van de Radboud Universiteit, die de Helsinki richtlijnen heeft gevolgd.

De resultaten lieten zien dat de CREST effectief is in het verhogen van cortisol concentraties van 5- en 6-jarigen (N=42) Daarom kan de CREST gezien worden als een bruikbare stress taak voor toekomstig onderzoek naar stress reactiviteit in de kleutertijd.

Hoofdstuk 5. Eerdere studies suggereren dat er ontwikkeling is in stress reactiviteit tijdens de puberteit en adolescentie. De bestaande stress taken die gebruikt worden in deze leeftijdsgroep

hebben verschillende nadelen: ze zijn òf niet specifiek gericht op de adolescentie, òf ze vragen een relatief hoge investering (meerdere testleiders nodig om de stress taak uit te kunnen voeren). Het doel van deze studie was om een kosten efficiënte stress taak te ontwikkelen die specifiek focust op de adolescentie, en die mogelijk ook in MRI studies gebruikt kan worden. De cortisol reacties, gerapporteerde stress, en de sexe- en leeftijdsverschillen in stress reacties op de nieuw ontwikkelde stress taak werden onderzocht.

De nieuwe stress taak, de 'Social Evaluative Stress Test (SEST)', is een computertaak bestaande uit elementen waarvan we uit eerder onderzoek weten dat ze voor stress verhoging zorgen in adolescenten en volwassenen, namelijk: sociale evaluatie, onvoorspelbaarheid en oncontroleerbaarheid. Daarnaast focust de stress taak op sociale evaluatie van persoonlijke aard in plaats van academische prestaties, en bevat het vergelijking en competitie met leeftijdgenoten. De taak duurt 50 minuten. Zeven speekselsamples werden afgenomen om cortisolconcentraties te kunnen bepalen, zowel de stressreactie als ook het herstel. Ervaren emotionele stress werd gemeten door middel van vragenlijsten die ingevuld werden na het uitvoeren van de SEST. De test is goedgekeurd door de ethische commissie van de Radboud Universiteit, die de Helsinki richtlijnen heeft gevolgd.

De SEST werd afgenomen bij 52 10- tot 15-jarige jongens en meisjes (gemiddelde leeftijd=12.5 jaar). De resultaten laten zien dat zowel jongens als meisjes de taak stressvol vonden, maar dat alleen jongens met een cortisol verhoging reageerden (gemiddelde verhoging van 163%). Er was geen significant effect van leeftijd op de cortisol reactie. Voor zover we weten is dit de eerste computergestuurde stress taak die resulteert in cortisol verhoging in 10- tot 15-jarigen jongens. Aangezien meisjes wel stress ervaren in reactie op de SEST, zal in toekomstig onderzoek verder onderzocht moeten worden welke andere stress-gerelateerde biologische systemen geactiveerd worden bij meisjes in reactie op de stress taak.

Hoofdstuk 6. Eerdere studies hebben aangetoond dat PNS is gerelateerd aan cortisol reactiviteit van nakomelingen van ratten en primaten. Er zijn maar weinig studies die naar deze relatie hebben gekeken in mensen, en de studies die deze relatie hebben onderzocht hebben zich vooral gefocust op cortisol reactiviteit in het eerste levensjaar. Door de nieuw ontwikkelde effectieve stress taak, zoals beschreven in hoofdstuk 4, werd het mogelijk om de relatie tussen PNS en de stress reactie van 6-jarigen te onderzoeken. Als fysiologische stress maat hebben we de cortisol reactie gebruikt. Als gedragsmaat hebben we 'kijkgedrag' ten opzichte van de stressor gebruikt. Hierbij verwachtten we dat het niet aankijken van de jury mogelijk meer gerapporteerde stress weergeeft. Ook de relatie tussen kijkgedrag en cortisol reactie tijdens de stress taak werd onderzocht om op deze manier meer inzicht te krijgen in mogelijke associaties tussen fysiologische en gedragsmatige stress reacties.

PNS werd aan het eind van de zwangerschap gemeten door middel van vragenlijsten en cortisol concentraties (BIBO-studie). De Children's Reactions to Evaluation Stress Test (CREST) werd afgenomen bij de kinderen (N=149; gemiddelde leeftijd=6.1 jaar). Zes

speekselsamples werden afgenomen om cortisol reactiviteit en totale cortisol afgifte tijdens de CREST te bepalen. Ook werd het kijkgedrag van de kinderen ten opzichte van de jury geobserveerd.

Uit de resultaten blijkt dat de kinderen van moeders die tijdens de zwangerschap minder angst voor de bevalling hadden, hogere cortisol concentraties tijdens de gehele stress taak hadden, maar geen hogere cortisol reactie. Ook hadden de kinderen van moeders die hogere cortisol concentraties tijdens de zwangerschap hadden, hogere cortisol concentraties tijdens de stress taak, maar geen hogere cortisol reactie. Er was geen relatie tussen PNS en kijkgedrag van het kind tijdens de stress taak. Deze resultaten wijzen op mogelijke programmerende effecten van maternale prenatale stress en HPA-as functioneren van kinderen.

Daarnaast was er een negatieve associatie tussen de cortisol concentraties in reactie op de stress test en kijkgedrag naar de jury tijdens de stress test: kinderen met hogere cortisol concentraties tijdens de taak keken minder vaak naar de jury. Kijkgedrag zou mogelijk als coping strategie kunnen dienen tijdens een stressvolle situatie voor jonge kinderen.

Hoofdstuk 7. Veel studies hebben laten zien dat PNS is gerelateerd aan kinduitkomsten, maar de onderliggende mechanismen in deze relatie zijn nog vrijwel onbekend. Een eerdere studie bij rhesus apen liet zien dat er een relatie is tussen PNS en de darmmicrobiota van hun nakomelingen. De darmmicrobiota is een complex ecosysteem in de darmen, en vervult vele fysiologische functies, de ontwikkeling van het immuunsysteem en de afweer tegen pathogenen. De ontwikkeling van de darmmicrobiota is dus belangrijk voor de gezondheid van het kind. Het doel van deze studie was om in mensen te onderzoeken of de ontwikkeling van de darmmicrobiota van baby's een mogelijk onderliggend mechanisme zou kunnen zijn in de relatie tussen PNS en de gezondheid van baby's.

PNS was aan het eind van de zwangerschap gemeten door middel van vragenlijsten en cortisol metingen. Voor de huidige studie is een deel van de participanten geselecteerd van de totale BIBO sample. Op basis van de gerapporteerde stress en angst tijdens de zwangerschap werden twee groepen gevormd: de hoge prenatale stress groep (N=28) en de lage prenatale stress groep (N=28). De ontwikkeling van de darmmicrobiota van baby's werd gevolgd tijdens de eerste vier maanden en werd bepaald in vijf ontlasting samples die verzameld werden op de leeftijd van 7, 14, 28, 80 en 115 dagen. Daarnaast werden moeders maandelijks geïnterviewd over de spijsverteringsziekten en symptomen en allergische reacties van hun baby.

De resultaten lieten zien dat zowel maternale gerapporteerde stress en cortisol concentraties tijdens de zwangerschap gerelateerd zijn aan de darmmicrobiota van de baby's. Secundaire analyses lieten zien dat baby's van moeders met zowel hoge gerapporteerde stress als hoge cortisol concentraties tijdens de zwangerschap, meer Proteobacteriën en minder melkzuurbacteriën en actinobacteriën hebben. Ook waren deze bacteriën geassocieerd met meer spijsverteringsziekten/symptomen en allergische reacties van de baby's.

Samenvattend, er zijn associaties gevonden tussen PNS en baby's darmmicrobiota, spijsverteringsziekten/symptomen en allergische reacties. Baby's van moeders die meer PNS hadden, hebben meer mogelijk pathogene darmbacteriën en minder gunstige darmbacteriën in de eerste maanden van hun leven. Alhoewel met dit type onderzoek weinig gezegd kan worden over causaliteit, wijzen de resultaten op een mogelijk microbieel onderliggend mechanisme in de relatie tussen PNS en de gezondheid van kinderen.

CONCLUSIES

- Maternale prenatale cortisol is mogelijk niet de enige mediator in de link tussen maternale stress en angst tijdens de zwangerschap en kinduitkomsten.
- Maternale prenatale angst en cortisol zijn geassocieerd met ziekten aan de luchtwegen en spijsverteringsziekten van kinderen, tot aan de peutertijd.
- Zwangere vrouwen die meer dagelijkse problemen rapporteren tijdens de zwangerschap hebben kinderen die meer antibiotica voorgeschreven krijgen in de vroege kindertijd.
- De 'Children's Reactions to Evaluation Stress Test (CREST)' is een veelbelovende test voor toekomstig onderzoek naar stress reactiviteit van 5- en 6-jarigen.
- De computer-gestuurde 'Social Evaluative Stress Test (SEST)' is effectief in het verhogen van cortisol concentraties van 10- tot 15-jarige jongens.
- Jongens en meisjes ervaren beide de SEST als even stressvol, maar alleen jongens reageren met een verhoging in hun cortisol concentraties.
- Kinderen van moeders die meer prenatale en vroege postnatale angst ervaren hebben hogere cortisol concentraties tijdens de gehele CREST, maar hun cortisol reactiviteit in reactie op de CREST is niet hoger.
- Kinderen die een hogere cortisol reactiviteit hebben tijdens de CREST, kijken minder vaak naar de jury (de stressor).
- Baby's van moeders die meer prenatale stress hadden, hebben meer mogelijk pathogene darmbacteriën en minder gunstige darmbacteriën in de eerste maanden van hun leven.

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CURRICULUM VITAE AND PUBLICATIONS

CURRICULUM VITAE

Maartje Zijlmans was born on April 22th, 1985, in Gilze-Rijen, the Netherlands. She studied Social Work at Avans Hogeschool, where she obtained her B.Sc. in 2007. Subsequently, she studied Child and Family studies at Radboud University, and obtained her B.Sc. in 2009. In 2011, she finished the Research Master in Behavioural Science at the Radboud University (Cum Laude).

In September 2011, Maartje started her PhD project at the department of Developmental Psychology at the Behavioural Science Insitute (Radboud University), under supervision of professor Carolina de Weerth and professor Marianne Riksen-Walraven. She studied the relation between prenatal stress and child outcomes, with a focus on physical outcomes and stress reactivity.

Currently, she is working as a postdoctoral researcher on a project about how early life experiences impact childhood development (department of developmental psychology, Radboud University), supervised by Dr. Roseriet Beijers. Besides her scientific work, she is the co-owner of Baby Proof, and prepares expectant parents towards parenthood.

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