A multi-systems approach to understanding the effects of antenatal distress: biological underpinnings of perinatal depressive symptoms and biobehavioural outcomes in the child

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Personal Statement

I, Sarah Nazzari, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Evidence linking maternal antenatal depression with an increased risk of altered biological and behavioral outcomes in offspring is substantial. However, knowledge concerning the underlying mechanisms is strikingly less advanced. The current dissertation addresses this gap by adopting a multi-systems approach and prospectively investigating three main biological pathways, involving stress and inflammation, possibly underlying the effects of maternal antenatal depression on infants' early development, taking into account the potential buffering role of postnatal maternal care.

One-hundred-ten healthy pregnant women, together with their offspring, were studied from late pregnancy to three months after delivery as part of the Effects of Depression on Infants (EDI) Study. In Chapter 3, cross-sectional associations between maternal depressive symptoms and stress-related biology in late pregnancy and soon after delivery were examined. Chapters 4 and 5 investigated the prospective associations between antenatal variations in maternal depressive symptoms, stress and inflammation and infant behavioral and biological outcomes at birth (Chapter 4) and 3 months after delivery (Chapter 5). Lastly, Chapter 6 explored the moderating role of maternal caregiving in the association between prenatal maternal influences and 3-month-olds' bio-behavioral outcomes.

Current findings indicate that antenatal depressive symptoms are associated with an altered diurnal cortisol pattern and heightened inflammation in late pregnancy and independently predict 3-month-olds' negative affectivity. Additionally, this thesis provides evidence that variations in maternal stress-related biology during pregnancy are associated with offspring physiological and behavioural outcomes and that the impact of antenatal maternal cortisol on infant cortisol stress reactivity may be moderated by maternal sensitive caregiving. Despite the fact that replication of these

findings in larger and different samples is needed, our results are in keeping with the hypothesis that maternal antenatal stress exerts a programming effect on offspring bio-behavioral development and that the impact partially depends on the quality of the early rearing environment.

Impact statement

Caring for our children is caring for the future of our society. It is estimated that 250 million children younger than five years old are at risk of not reaching their full developmental potential and there is increasing realisation that the roots of abnormal bio-behavioral functioning can often be traced early in life. It is therefore essential for the future well-being of our society that we thoroughly understand the factors that can affect child development from conception to toddlerhood, as this represents a unique window of opportunity for a “good start in life”.

In the last decades, a tremendous amount has been learnt about the negative long-term influences of maternal depression during pregnancy on child development. However, research in humans has not yet successfully demonstrated how maternal antenatal depression is “communicated” to the fetus and might affect its development.

The “Effects of Depression on Infants” (EDI) Study was conceived to address this gap by prospectively investigating three stress-related biological pathways, possibly underlying the association between maternal antenatal depression and infant outcomes, in a sample of 110 women and infants.

Findings indicated that maternal antenatal depressive symptoms and variations in stress-biology are associated with offspring’s bio-behavioral outcomes and that a postnatal sensitive rearing environment can reverse some of these effects. These are promising and largely unexplored areas for future scientific endeavours. Findings will be disseminated to the scientific community through scientific publications. Furthermore, they have important clinical implications. Indeed, although prevalence estimates suggest that up to 30% of pregnant women experience depressive symptoms, they are mostly undetected by health practitioners with possible negative consequences for both women and their children. The EDI Study was an occasion to open dialogue between researchers and both health professionals working with

pregnant women and the public, on the importance of maternal perinatal psychological health, thus fostering closer connections between research and practice. Although there is still a long way to go before maternal psychological health during pregnancy can be addressed as fully as any other medical aspects of antenatal care, the EDI study promotes awareness, at least at local level, about the importance of maternal emotional state during pregnancy. Furthermore, our findings underline the role of a sensitive caregiver in buffering some effects of prenatal risk exposure, thus extending the window of opportunities for preventive interventions beyond the first nine months of life and suggesting that the quality of maternal caregiving should be a major target for intervention. If the current findings are replicated, they suggest that researchers and clinicians should join their efforts to find the best way to effectively identify women experiencing antenatal depressive symptoms and provide them with accessible and effective antenatal and postnatal treatment in order to help all mothers and babies have the best start in life.

Lastly, the EDI study constituted a unique opportunity to critically extend an existing collaboration between UCL and the Scientific Institute Medea in Italy, which, besides promoting scientific exchange, created the basis for a long-lasting network to significantly move forward research in psychobiological development.

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Abbreviations

11β-HSD2: 11b-hydroxysteroid-dehydrogenase type 2

ACTH: Adrenocorticotrophic Hormone

ADHD: Attention Deficit and Hyperactivity Disorder

ANOVA: analysis of variance

ANS: Autonomous Nervous System

AUCg: Area Under the Curve with respect to the ground

AVP: Arginine-Vasopressin

Bayley III: Bayley Scales of Infant and Toddler Development – Third Edition

BMI: Body Mass Index

CAR: Cortisol Awakening Response

CRH: Corticotrophin-Releasing Hormone

CRP: C-Reactive Protein

C-Section: Caesarean Section

CV: Coefficient of Variation

DOHaD hypothesis: Developmental Origins of Health and Disease hypothesis

E: Epinephrine

EA: Emotional Availability

EDI Study: Effects of Depression on Infants Study

EEG: Electroencephalography

EPDS: Edinburgh Postnatal Depression Scale

F: F statistic in analysis of variance (ANOVA)

GR: Glucocorticoid Receptor

hCRH: hypothalamic CRH

HLM: Hierarchical Linear Models:

HPA axis: Hypothalamic-Pituitary-Adrenal axis

HRV: Heart Rate Variability

IBQ: Infant Behavior Questionnaire

IFNγ: interferon gamma

Ig: Immunoglobulin

IL-1: Interleukine-1

IL-6: Interleukine-6

IQ: Intelligence Quotient

IRS: Inflammatory Response System

LN: natural logarithmic

MDD: Major Depressive Disorder

µg/dl: microgram per deciliter

µL: microliter

MR: Mineralocorticoid Receptor

NA: Negative Affectivity

NBAS: Neonatal Behavioural Assessment Scale

NE: Norepinephrine

ng/ml: nanograms per millilitre

p: probability statistic (significance level)

PAR: Predictive Adaptive Response

pCRH: placental CRH

pg/mL: picograms per millilitre

PNS: Parasympathetic Nervous System

PTSD: Posttraumatic Stress Disorder

PVN: Paraventricular Nucleus

r: pearson product moment correlation coefficient

RCTs: Randomized Controlled Trials

RSA: Respiratory Sinus Arrhythmia

RSPM: Raven Standard Progressive Matrices

sAA: salivary Alpha Amylase

SAM system: Sympathetic AdrenoMedullary system

SD: Standard Deviation

SE: Standard Error

SES: Socioeconomic Status

SNS: Sympathethic Nervous System

SRS: Stress Response System

STAI: State-Trait Anxiety Inventory

Th1: T-helper 1 cells

Th2: T-helper 2 cells.

TNF-α: Tumor Necrosis Factor alpha

U/mL: units per millilitre

χ^2: Chi-Square Independence Test

Chapter 1: Introduction

Early problems in behavioural, cognitive and physiological regulation in infancy may represent vulnerability factors for the development of later psychopathology (Schlotz & Phillips, 2009). An important step toward the early identification and treatment of children at risk is the understanding of potential pathways that may lead to early deficits in regulatory capacities. The last decades have seen a growing appreciation for the roles of environmental exposures in shaping brain development and influencing later risk for psychopathology, with increasing attention directed toward prenatal exposures (Grossman et al., 2003). Indeed, the rapid fetal growth and brain development occurring across gestation make the fetus particularly vulnerable to any insults and changes in the intrauterine environment, with possibly long-term consequences (Gluckman, 2008). This has led to the hypothesis that stress or psychological distress experienced by the mother during pregnancy, including major life events or psychiatric disturbances such as depression, can induce alterations in the intrauterine environment that in turn can affect fetal development and later risk for lifetime physical and mental health problems (Maccari et al., 2017, Weinstock, 2005). Since this hypothesis has been proposed, many prospective studies have provided evidence of an association between maternal stress or distress during pregnancy and long-term altered behavioral and physiological outcomes in the offspring (reviewed in Van den Bergh et al., 2017b). However, research in humans has not yet successfully demonstrated how maternal antenatal stress is "communicated" to the fetus and might affect its development. The current dissertation focuses on symptoms of psychological distress, particularly depression, during pregnancy and investigates three main biological pathways, involving the two main stress response systems and the inflammatory response system, possibly underlying the effects of maternal antenatal

depressive symptoms on infants' early development, taking into account the role of postnatal maternal care.

This chapter begins by delineating the theoretical foundation of the Developmental Origins of Health and Disease (DOHaD) hypothesis and summarizing illustrative evidence. Then the focus of interest is restricted on the effects of maternal depression on child development and empirical evidence for it as well as critical conceptual and methodological issues is reviewed. In the second half of this chapter, possible biological mechanisms underlying the effects of antenatal depression on fetal development involving the Hypothalamic-Pituitary-Adrenal (HPA) axis, the Sympathetic Nervous System (SNS) and the inflammatory response system (IRS) are examined. For each of these, a brief overview of system functioning and of the main pregnancy-related changes these systems undergo is provided, jointly with a description of what is known about associations with perinatal depressive symptomatology. Finally, possible mechanisms of fetal programming by stress and inflammatory response systems are proposed and available evidence for these are reviewed.

1.1 The Developmental Origins of Health and Disease (DOHaD) hypothesis

The notion that maternal health during pregnancy may affect the developing child has a long lineage across cultures and is strongly embedded in folk psychology. Over the last decades, this belief has attracted considerable research interest and several models have been proposed such as, the 'Fetal programming hypothesis' (Seckl & Holmes, 2007), the 'Developmental programming hypothesis' (Barker, 2004, Langley-Evans, 2006), and the 'Developmental Origins of Health and Disease (DOHaD) hypothesis' (Wahdwa et al., 2009; Hanson & Gluckman, 2008). Despite some differences, all these models emphasize fetal plasticity and share the basic idea that intrauterine exposure to an altered environment, characterized for example by high levels of glucocorticoids, either synthetic or endogenous, or by nutrient

restrictions, during sensitive windows of development might alter the structure and/or function of fetal biological systems, influencing later health and susceptibility to disease (Gluckman, 2008; Hanson & Gluckman, 2014; Seckl & Holmes, 2007; Van den Bergh, 2011). More specifically, antenatal exposure to environmental challenges has been hypothesized to affect the fetal set-point of the stress response systems as well as induce changes in proteins and neurotransmitters involved in fetal brain development and function (McGowan & Matthews, 2018; Wyrwoll & Holmes, 2012). These prenatally-induced changes are thought to heighten the offspring's susceptibility to later physical and mental health problems which, in interaction with genetic and postnatal environmental factors, will determine ultimate individual outcomes (Schlotz and Phillips, 2009).

1.1.1 Epidemiological studies

Although the notion that early-life exposures can significantly impact subsequent development has a long-standing tradition in psychology (e.g. Lorenz, 1935; Watson & Rayner, 1920), the greatest impetus to the emergence of the fetal programming hypothesis came from the original work of David Barker and colleagues. Barker and collaborators, from the late 1980s onward, conducted a series of epidemiological studies demonstrating an association between nutrition early in life and health problems in adulthood (Barker, 1995; Barker & Osmond, 1986). In particular, they showed that low birthweight was associated with a higher prevalence of a number of adult diseases such as type 2 diabetes (Hales & Barker, 1992), heart disease (Barker, 1997), hypertension (Barker & Osmond, 1988) and metabolic syndrome (Barker, 1995). Based on this evidence, Barker suggested that prenatal exposure to malnutrition, substance use or poverty, could affect fetal development and these alterations, in turn, could confer a higher risk for certain diseases in later life in a process that he described as "fetal programming". Despite the fact that birth weight was only a proxy of prenatal development and that later meta-analyses challenged

some of his results (e.g. Huxley, Neil, & Collins, 2002), Barker's original work inaugurated a wave of research on the effects of prenatal experiences on health and risk for disease across the lifespan. In addition, while the initial focus was exclusively on the role of the intra-uterine environment for offspring's later outcomes, life experiences occurring during periods of high developmental plasticity, such as the early postnatal period, as well as possible pre-conception and intergenerational influences, were later included in the DOHaD hypothesis.

Independently of the work on fetal malnutrition, accumulating evidence in support for the fetal programming hypothesis came from studies highlighting a link between prenatal exposure to toxic agents, such as smoking, alcohol, radiation, lead and methyl mercury, and later adverse outcomes in the offspring (e.g. Thayer et al., 2012; Grandjean & Landrigan, 2014). Additionally, these studies showed that timing and severity of the exposure during gestation influence the nature and degree of these effects (e.g. Eberhart-Phillips, Frederick, Baron, & Mascola, 1993), providing further support for the notion that sensitive periods exist during fetal development, when specific biological structures and systems, usually undergoing rapid developmental change at that given point, are particularly vulnerable to environmental influences.

1.1.2 The effects of antenatal maternal stress: observational studies

In the last decades, mounting evidence has indicated that stress experienced by the mother during pregnancy is another powerful environmental factor that could be involved in fetal programming (reviewed in Talge, Neal, & Glover, 2007; Glover, 2011; Van den Bergh et al., 2017). This includes maternal exposure to negative life events or bereavement, self-reports of daily hassles, conflictual relationship with the partner, anxiety and depressive symptoms. In particular, several independent prospective studies in humans showed an association between maternal antenatal distress and altered child outcomes in several domains such as: 1) poor birth outcomes, including an higher risk of preterm delivery and smaller size at birth (e.g. Copper et al., 1996;

Dole et al., 2003); 2) regulation and sleep problems (e.g. Gerardin et al., 2011; Pacheco & Figueiredo, 2012; Räikkönen et al., 2015); 3) poor cognitive outcomes (Tarabulsy et al., 2014 meta-analytical findings) and, to a lesser extent, motor outcomes (Lin et al., 2017; Gerardin et al., 2011); 4) mixed-handedness during childhood (Glover et al., 2004); 5) altered behavioral stress reactivity (Davis et al., 2011) and more "difficult" and fearful temperament (e.g. Madigan et al., 2018; Braithwaite et al., 2017; Bergman et al., 2007); 6) behavioral problems in childhood and adolescence (Madigan et al., 2018 meta-analytical findings), including externalizing (e.g. O'Donnell et al., 2014; Leis et al., 2014; Loomans et al., 2011), internalizing (e.g. Capron et al., 2015, Gerardin et al., 2011), social behavioral problems (Loomans et al., 2011) and autistic traits (Rijlaarsdam et al., 2017); 7) neuroendocrine alterations, including an altered pattern of cortisol stress reactivity (K. A. Grant et al., 2009), an altered diurnal cortisol rhythm (e.g. O'Connor et al., 2005; O'Donnell et al., 2013; Van Den Bergh, et al., 2008) and altered autonomic stress reactivity (Nancy Aaron Jones et al., 1998); 8) brain structural and functional alterations such as cortical thinning (Sandman et al., 2015; Lebel et al., 2016), altered patterns of connectivity of the amygdala (Qiu et al., 2015; Favaro et al., 2015), microstructural brain changes from the neonatal period to age 9 years (reviewed in Franke et al., in press).

The magnitude of these effects varies widely across studies and appears to depend on infant's age and gender, as well as on the type of maternal stress measured and gestational time window. Additionally, studies vary considerably for sample size and methods of assessment and analyses. Nonetheless, despite all methodological variations, findings collectively support the notion that maternal stress during pregnancy is a non-specific risk factor for negative outcomes during childhood. However, the observational design of these studies does not enable to rule out the contribution of factors, such as genetic, postnatal environmental factors or potential third variables (e.g. maternal smoking), that might confound the observed

associations, thus limiting the possibility to draw causal inferences and really establish whether fetal programming is occurring.

1.1.3 The effects of antenatal maternal stress: animal studies

Animal research has allowed for further investigation of the direct effects of prenatal experiences on offspring development in experimentally controlled settings. Animal experiments offer several advantages, including shorter life spans and breeding cycles as well as the possibility to control the intensity, duration and timing of the stressor (Maccari et al., 2003; Weinstock, 2008). Additionally, as stress experiences during pregnancy might induce changes in early maternal care in the postnatal period, which might in turn also influence offspring development, cross-fostering procedures can be adopted in animal models that allow researchers to disentangle the contribution of prenatal factors versus postnatal rearing effects on offspring development.

The majority of studies on the effects of prenatal stress on brain development has been conducted in rat strains providing, to date, the most comprehensive information on the behavioural, morphological and histological effects of prenatal stress (Boersma & Tamashiro, 2015; Weinstock, 2017). Several protocols have been employed to induce stress in pregnant dams including drugs administration, infections, hormonal manipulations, undernutrition, noise and sleep deprivation, and physical or social stressors that induce strong psychoneuroendocrine responses in the mothers (e.g. Mastorci et al., 2009; Bock et al., 2011; Muhammad & Kolb, 2011; Mychasiuk, Gibb, & Kolb, 2011). Taken together, this body of research shows that exposure to prenatal stress has a long-lasting impact on the physiological and behavioral development of the resulting offspring (Frasch et al., 2018) and provides a possible biological basis for prenatal stress-induced behavioral and physiological alterations in offspring. In particular, exposure to different stressors in late pregnancy has been found to alter offspring neurogenesis and brain morphology and this, in turn, appears

to affect offspring behavior (Charil et al., 2010; Mastorci et al., 2009; Weinstock, 2001). In addition, offspring of prenatally stressed dams are characterized by lower birth weight, impaired stress reactivity, learning and memory, motor delay, alterations in neurotransmitter pathways including dopaminergic, gabaergic and glutamate systems (reviewed in Frasch et al., 2018). In adulthood, prenatal stress exposed offspring show diminished propensity for social interactions, increased anxiety and depressive-like behaviors, altered sexual behaviors and gonadal dysfunction (Darnaudery & Maccari, 2008; Weinstock, 2008; Yaka et al., 2007; Yang et al., 2006).

Because animal models allow the duration and timing of prenatal stress to be controlled, they allow us to differentiate the effect of prenatal stress at different stages of development. Evidence to date suggests that the characteristics of the behavioral alterations associated with antenatal stress depends on the gestational timing of maternal stress (Frasch et al., 2018). In particular, it has been shown that fetal brain development is characterized by a sequence of vulnerability time windows during which specific organ systems are developing and that specific cellular events and neuronal systems in fetal brains can be affected by environmental stress depending on the time of exposure (Weinstock, 2007).

Lastly, animal models have provided evidence that prenatal stress-related vulnerability can be transmitted across generations, so that the effects of prenatal stress exposure on the phenotype are apparent also in subsequent generations of offspring that were not exposed to environmental stressors at any time (Aiken & Ozanne, 2014; Heard & Martienssen, 2014). Despite the mechanisms underlying transgenerational transmission being far from elucidated, some have argued that they may involve transmission of epigenetic information across generation (Skinner, 2014).

1.1.4 The effects of antenatal maternal stress: natural disasters studies

While in human studies rigorous experimental control is difficult to achieve, as pregnant women cannot be randomized to different stress conditions, research on the

consequences of major stressful events, such as wars or natural disasters, in humans are natural experiments that allow for an approximation of the randomization adopted in animal studies. This body of research gives additional support to the notion that stressful events occurring during prenatal life are associated with altered outcomes in childhood and beyond.

Early research into the consequences of prenatal exposure to the Dutch Hunger Winter of 1944–1945 revealed that offspring of women who were pregnant during the famine showed lower birth weight and impaired glucose tolerance in adulthood, as compared with the unexposed group of individuals born in the year before or conceived in the year after the famine (Ravelli et al., 1998). Additionally, associations between conception during the famine and both a higher rate of neural tube defects such as spina bifida and anencephaly (Brown & Susser, 1997) and a higher risk of schizophrenia in adulthood (Susser, Hoek, & Brown, 1998), as compared to the unexposed cohorts, were reported. Similar associations were found in studies on the consequences of a famine in China, suggesting an impact of severe caloric restriction during pregnancy on offspring's later risk for schizophrenia (Song, Wang, & Hu, 2009). Kinney and colleagues (2008) conducted historical analyses exploring the impact of prenatal exposure to several severe storms in Louisiana on autism risk by examining changes in prevalence rates before and after the exposure. The authors reported a significant increase in autism rates after prenatal exposure to the storms (from a prevalence of 5 per 10,000 births to 13 per 10,000 births) and highlights a role of storm severity and timing of exposure in predicting the risk for psychopathology.

The first prospective study on natural disaster was Project Ice Storm in Canada (King et al., 2012). This study found that prenatal exposure to the worst natural disaster in Canadian history significantly affected outcomes in the resulting offspring in nearly every developmental domain examined. In particular, researchers were able to differentiate between the objective severity of stress exposure (for example greater loss or changes) and woman's perceived stress (King et al., 2015) and showed that

greater objective stress exposure was associated with poor birth outcomes (Dancause et al., 2011), increased obesity risk in preschool (Dancause et al., 2012) and school age (King et al., 2012), more difficult temperament (Laplante, Brunet, & King, 2016), poorer cognitive and language development (Laplante et al., 2004) and poorer bilateral coordination and visual motor integration (Cao et al., 2014), with effects depending on timing of exposure and baby's gender (King et al., 2012; Dancause et al., 2011). Additionally, maternal perceived stress (and to a lesser extent, objective exposure) was found to influence fingerprint asymmetry (King et al., 2009), autism traits (Walder et al., 2014) and childhood asthma in girls (Turcotte-Tremblay et al., 2014). Interestingly, the severity of objective exposure and subjective distress in response to the storm were found to interact to predict child outcomes so that the poorer outcomes were found in children of women who showed a mismatch between objective and subjective exposure (i.e. high objective stress versus low subjective distress or low objective stress versus high subjective distress; King et al., 2012).

Similarly, results from the QF2011 Queensland Flood Study in Australia showed an association between higher levels of objective flood exposure and both problem-solving skills at 6 months of age (Simcock et al., 2018) and lower cognitive and motor scores at 16 months of age, especially when the flood occurred in late gestation (Moss et al., 2017). Additionally, infant's socio-emotional development was lower the later in gestation they were exposed to the flood and the greater maternal objective exposure (Simcock et al., 2017).

The effects of acute disasters have also been studied. For example, it was found that exposure to the Chernobyl disaster in the second or third trimester of pregnancy was associated with an increased prevalence of depressive and attention deficit and hyperactivity disorder (ADHD) symptoms in adolescence in the offspring (Huizink et al., 2007). Work on infants prenatally exposed to the World Trade Center Attacks (Yehuda et al., 2005) showed decreased salivary cortisol levels in infants of mothers who developed posttraumatic stress disorder (PTSD) in response to the

attacks, as compared to infants of exposed mothers who did not develop PTSD. Additionally, effects were most apparent when the exposure occurred in the third trimester of pregnancy.

Collectively these studies represent a unique opportunity to quasi-experimentally investigate the impact of stress exposure upon offspring outcomes. However, results are still likely to be confounded by several factors. For example, the association between calorific restriction or malnutrition during pregnancy in wartime and later offspring outcomes is confounded as the population was clearly exposed to considerable stress and significant life changes in addition to malnutrition. Additionally, depending on the characteristics of the disaster under examination, the proportion of population affected by it might vary, thus leading to considerable variability in sample sizes, as well as in the severity of stress exposures (Harville, Xiong, & Buekens, 2010). Similarly, the measurement of such exposure varies widely across studies, ranging from exposure defined simply by being resident in an area affected by the disaster (e.g. Xiong et al., 2008) to more formal assessment of maternal perception of stress or mental health (e.g. Engel et al., 2005). Furthermore, duration and timing of the effects are confounded, as women who experience the event at an earlier stage of pregnancy are affected by it and its consequences for a greater proportion of the gestation than those later exposed (O' Connor et al., 2014). Lastly, direct replication of the results is difficult and findings are not likely to generalize to different forms of stress experienced by pregnant women such as more normative chronic stress conditions.

1.1.5 Evolutionary significance

It is important to note that a strong evolutionary connotation is inherent in the fetal programming or DOHaD hypotheses. In particular, early alterations in fetal biological systems are thought to represent fetal adaptations to the intra-uterine environment that are triggered by maternal signals, such as nutrient restriction or

stress hormones, and are thought to serve the function of preparing the developing fetus for optimal functioning in the postnatal environment (e.g. Bateson et al., 2004; Ellis & Del Giudice, 2018; Gluckman, 2008; Glover, 2011). Whether such adjustments, based on prenatal maternal hormonal cues, will be dysfunctional rather than adaptive in later life is thought to depend on how they suit actual characteristics of the postnatal environment (Monaghan & Haussmann, 2015).

Hales and Barker (1992) first proposed the "Thrifty phenotype" hypothesis, according to which cardiovascular and metabolic alterations associated with prenatal exposure to adversity (e.g. stress, undernutrition, higher levels of glucocorticoids) constitute an organism's anticipatory preparations to conditions of reduced nutrient supply and/or the increased metabolic demands of postnatal life. Later, this was further extended within the predictive-adaptive-response (PAR) model (Gluckman, Hanson, & Spencer, 2005), also known as the weather-forecasting model (Bateson et al., 2004) or developmental match/mismatch hypothesis (Gluckman, Hanson, & Beedle, 2007), which questions the initial disease/dysfunction emphasis of fetal programming models and proposes that under certain conditions, prenatally stressed individuals may have an adaptive advantage if they have to cope with stress later in development, while they might have an increased risk for disease in a non-stressful postnatal environment (Gluckman et al., 2005). Thus, it is the mismatch between prenatal and postnatal environments that render offspring stressed in utero more susceptible to the development of physical and mental problems later in life. In this vein, the effects of maternal antenatal stress on fetal stress reactivity might be adaptive in that they help the offspring succeeding in an environment characterized by high levels of stress. However, the fetal stress-induced adjustments might prove to be dysfunctional in a non-stressful postnatal environment.

The majority of studies investigating the consequences of discordance between prenatal and postnatal environments have been limited to prenatal nutritional adversity and provide support for the PAR model. For instance, offspring of dams

exposed to nutrient restriction during pregnancy showed an excessive weight increase and atypical percentage of body fat, if they received ad libitum food postnatally (Desai et al., 2005). Similarly, increasing the discrepancy between prenatal and postnatal nutrition enhanced the risk for altered cardiovascular function in sheep (Cleal et al., 2007), while children provided with adequate nutrition after being exposed to nutritional restriction in utero have an increased risk of developing metabolic diseases in later life (Metcalfe & Monaghan, 2001).

To our knowledge, only two studies have tested the predictions of the PAR model on neurodevelopmental outcomes in humans and provided mixed results. Specifically, Sandman and colleagues (2012) reported improved motor and mental development across the first year of life among infants whose mothers experienced high depressive symptoms both prenatally and postnatally, while infants prenatally exposed to maternal depression did not benefit from maternal postpartum euthymia, in line with the PAR model predictions. However, Grant and colleagues (2009) found that children exposed to synthetic glucocorticoids during pregnancy showed impaired memory performance if they were reared in a postnatal environment at high psychosocial risk, in line with a vulnerability-stress model. More empirical work is clearly needed, however these findings highlights the fact that, although the notion of fetal programming is often related to adverse developmental outcomes, pathology is not inherent to this concept (Schwartz & Morrison, 2005).

1.1.6 Defining the risk phenotype

During pregnancy women can be exposed to several endogenous and exogenous challenges that may represent a threat to their health and wellbeing and be subjectively perceived as unpleasant or distressing (Glover, 2014). Prenatal stress is an all-encompassing term for a wide range of exposures, varying from very severe to mild, that has been investigated and was found to be associated with later child developmental outcomes (reviewed in Van den Bergh et al., 2017). These include

exposure to major life events, such as natural disasters or wars, bereavement, marital conflict, symptoms of depression and anxiety as well as clinically diagnosed mood or anxiety disorders, pregnancy-specific anxiety and daily hassles. Taken together, available findings suggest that the "prenatal risk" phenotype is fairly broad and that the programming effects of antenatal stress on fetal development are not limited to extreme levels of stress or severe maternal disturbance and are not unique to clinical samples. Rather, the effects of antenatal maternal stress appear to occur across the whole spectrum of stress exposures and symptom severity that women all around the world might experience at some time during their pregnancies (reviewed in Talge et al., 2007; Van Den Bergh et al., 2017). A fairly linear or dose-response relationship across the range between prenatal stress and child outcomes has often been reported (e.g. MacKinnon et al., 2018; Kingston et al., 2018; O'Connor et al., 2002), suggesting that prenatal maternal distress might have a detectable impact even at subclinical levels and that maternal mental health should be considered a population health issue (O'Donnell & Meaney, 2017). Nevertheless, it has been hypothesized that some outcomes, such as a higher risk of schizophrenia, might be observed only in offspring prenatally exposed to severe stress experiences, such as the death of a close relative (Khashan et al., 2008). Additionally, it has also been shown that mild levels of antenatal maternal stress might actually promote, rather than adversely affect, offspring's development, as highlighted in a study showing a positive association between prenatal maternal distress and infants' mental and motor development (DiPietro et al., 2006). Some researchers proposed that the association between maternal prenatal stress and children's outcomes might be U-shaped, with mild to moderate levels of maternal distress during pregnancy actually promoting fetal development in healthy samples and being associated with more optimal outcomes, as compared to too little or too much prenatal maternal stress (Fernandes et al., 2014; DiPietro et al., 2006; Davis et al., 2017).

While a wide range of prenatal stress measures has been found to be associated with child outcomes, it has been shown that the significance and magnitude of the observed associations varies according to the prenatal maternal measure investigated. For example pregnancy-specific stress, such as fear of bearing a handicapped child, rather than daily hassles or anxiety symptoms, has been found to be significantly associated with infants' cortisol reactivity in some studies (Tollenaar et al., 2011; Gutteling, De Weerth, & Buitelaar, 2005).

While methodological heterogeneity among studies might account for different findings, discrepancies still reflect the poor understanding of the mechanisms underlying the effects of prenatal maternal distress on child development that might also explain why a significant association with child outcomes is sometimes detected for one stress measure but not another. For example, one potential source of confusion is the assumption that different forms of maternal antenatal stress share the same distress-linked biological alterations, such as increased glucocorticoid levels, which might not be always the case. The field of prenatal stress in humans suffers from a lack of specificity and imprecision in the definition and operationalization of prenatal maternal stress and, in turn, of the underlying biological mechanisms that might link maternal distress during pregnancy to altered child outcomes (O'Donnell & Meaney, 2017; O'Connor, Monk, & Fitelson, 2014). Recent reviews have called for more research into the effects of specific maternal conditions on specific neurodevelopmental outcomes and for more studies integrating subjective and objective measures of maternal stress and investigating their effects on child development (Graignic-Philippe et al., 2014; O'Donnell & Meaney, 2017). For the purposes of the current thesis, the prenatal risk phenotype is narrowed and both psychological (i.e. depressive symptoms) and neuroendocrine (i.e. stress and inflammatory markers) measures of maternal antenatal stress experience are included. Furthermore, as anxiety symptoms are often found in comorbidity with depression (Dindo et al., 2017; Falah-Hassani, Shiri, & Dennis, 2017), in order to

evaluate the specificity of the associations investigated, measures of maternal anxiety will be included. In addition, studying a low risk community sample of pregnant women allows to limit confounding influences of additional risk factors associated with psychosocial adversity (e.g. teenage motherhood, unemployment, financial problems etc.). We believe that a clear and unambiguous definition of the type of stress measured both in terms of psychological construct, magnitude of exposure (i.e. how much distress can significantly affect offspring development) and biological underpinnings is pivotal in order to bring the field of fetal programming a step forward.

The rationale for focusing on depressive symptomatology experience during pregnancy include: 1) the significant prevalence of such symptoms during pregnancy and after delivery (e.g. Woody et al., 2017); 2) a considerable body of findings suggesting an association between maternal antenatal depressive symptoms and an increased risk for altered outcomes in the offspring (e.g. Gentile et al., 2017); 3) the great wealth of evidence suggesting specific associations between depressive symptomatology and altered functioning of biological systems that might be involved in fetal programming (e.g. Knorr et al., 2010; Miller & Raison, 2016; Vreeburg, Sophie; Hoogendijk, 2009). Points 1) and 2) will be further discussed in what follows, while point 3) will be extensively examined in section 1.4.

1.2 The effects of antenatal maternal depression on child development

1.2.1 Maternal depression during the perinatal period

The perinatal period, encompassing both gestation and childbirth, is inevitably accompanied by physiological, psychological and social challenges and it is a time of high risk for new onset or re-lapse of mental disorders (Johnson et al., 2012). In particular, depression, a leading cause of the disease burden for women of childbearing age, is one of the most common complications during the perinatal period in developed countries (Woody et al., 2017; Dimidjian & Goodman, 2009).

Historically, maternal mental health in the postpartum period has attracted greater attention both from researchers and clinicians as compared to the antenatal period; however, prevalence estimates do not justify this bias. Between 7 and 12.7% of women in high-income countries experience a major depressive disorder during the gestational period (Woody et al., 2017; Melville et al., 2010; Gavin et al., 2005; Banti et al., 2011) and between 7 and 13.6% in the first year post-partum (Banti et al., 2011; Gavin et al., 2005, Woody et al., 2017). In addition, depressive symptoms affect up to 30% of women during the perinatal period, with significant variation in prevalence rates depending on time and method of assessment (Banti et al. 2011; Le Strat et al., 2011; Verreault et al., 2014; Waldie et al., 2015). Antenatal depression has been shown to represent one of the strongest risk factors for postnatal depression (Underwood et al., 2016; Robertson et al., 2004; Norhayati et al., 2015), with only a minority of women experiencing depression for the first time in the postpartum period (e.g. Verrault et al., 2014).

The clinical features of depression during pregnancy are essentially identical to those appearing in other life periods and include persistent feelings of sadness and/or loss of interest in things once pleasurable, as well as emotional problems, such as feeling worthless or guilty, difficulty concentrating or decision-making, suicidal thoughts, and somatic complaints, such as change in appetite, sleep and physical activity. However, some of these symptoms overlap with the changes that accompany pregnancy and are often misattributed to gestation, both by women and clinicians, and therefore often go unrecognized (Marcus & Heringhausen, 2009). In addition, a widespread stigma associated with mental illness, particularly occurring during a time that is expected to be one of the most joyful in a woman's life, make it difficult for women to report. Thus, although a considerable proportion of women experience mental health problems across gestation, only a small portion is detected and receive mental health treatment (Andersson et al., 2006; Goodman & Tyer-Viola, 2010),

indicating that adequate screening instruments and assessment procedures are still lacking or poorly implemented.

Anxiety is often found in comorbidity with depression during the perinatal period (e.g. Dindo et al., 2017; Lancaster et al., 2010; Biaggi et al., 2016), with a prevalence rate of around 10% antenatally and 8% postnatally for anxiety and depressive symptoms, while 4.1% during pregnancy and 6.6% postnatally for comorbid anxiety and mood disorder, according to recent meta-analytical estimates (Falah-Hassani et al., 2017), potentially exacerbating negative outcomes both in women (Fichter et al., 2010) and their children (Field et al., 2011; although see Leis et al., 2014 for contradictory results).

As will be further discussed in section 1.4, the background of neuroendocrine changes occurring during pregnancy and after delivery has been held responsible for increasing women's vulnerability to developing depression during the peripartum period (e.g. Corwin & Pajer, 2008). However, perinatal depression has a multi-factorial aetiology and, although hormonal and immune alterations are thought to play a role, genetic vulnerability, life experiences, stress and psychosocial factors all contribute to determine individual risk (Leigh & Milgrom, 2008; Lancaster et al., 2010). Concerning the latter ones, the factors most consistently related to maternal depression both during pregnancy and after delivery are a personal and family history of psychiatric illness, lack of social or partner support, adverse life events, negative attitude towards pregnancy or unplanned/unwanted pregnancy and pregnancy complications (reviewed in Norhayati et al., 2015 and Biaggi et al., 2016).

1.2.2 Antenatal maternal depression and offspring outcomes

Several prospective studies have now demonstrated that, if left untreated, maternal depression during pregnancy is associated with an increased risk for several adverse pregnancy-related outcomes and behavioural, cognitive, emotional and neurophysiological outcomes in the offspring (reviewed in Gentile et al., 2015; Glover

et al., 2014). Despite great variations among studies in terms of gestational timing, assessment of maternal depression and which outcome is evaluated, the vast majority of findings converge to suggest that maternal antenatal depression influences, or at least is associated with, fetal development, resulting in altered offspring outcomes that are summarized in what follows.

1.2.2.1 Pregnancy and fetal outcomes

There is considerable evidence that maternal depression during gestation is associated with poorer maternal health and pregnancy outcomes. In particular, maternal depression have been associated with unhealthy life style during pregnancy, including increased alcohol or substance use, smoking, inadequate diet and weight gain (Marcus, 2009). Additionally, antenatally depressed women are more likely to access antenatal care services late and less frequently, to miss regular scans (Kim et al., 2006; Redshaw & Henderson, 2013), to experience greater fear of childbirth and to show a preference for elective caesarean section (Andersson et al., 2004). Maternal depression has also been shown to relate to a heightened risk for medical complications during pregnancy, such as hypertension, preeclampsia, gestational diabetes and severe forms of hyperemesis gravidarum (e.g. Qiu et al., 2009; Alder et al., 2007; Kozhimannil, Pereira, & Harlow, 2009; Kurki et al., 2000), and is associated with prolonged sick leave (Gentile et al., 2017).

Recent systematic reviews and meta-analyses indicate that maternal depression during pregnancy is related to a modest but significantly greater risk of preterm delivery (Staneva et al., 2015; Alder et al., 2007; Grote et al., 2010; Szegda et al., 2014) and low-birth weight (Alder et al., 2007; Grote et al., 2010; Jarde et al., 2016). Nonetheless, findings vary widely depending on timing of assessment, with greater effects observed in mid-late pregnancy as compared to earlier exposure, and operationalisation of maternal depression, with stronger effects for categorical clinical diagnosis of depression, rather than continuous measure of depressive symptoms.

Few studies have directly examined the impact of antenatal depressive symptomatology on fetal behaviour and physiology. Generally, a greater amount of fetal activity in foetuses of depressed pregnant women as compared to a control group of non-depressed women has been reported (Emory & Dieter, 2006; Dieter et al., 2001; Field, Diego, & Hernandez-Reif, 2004). In contrast, the association between maternal antenatal depression and fetal heart rate baseline and reactivity is less consistent. Allister and colleagues (2001) found a significant association between maternal depression and both fetal heart rate and reactivity to external stimuli, with foetuses antenatally exposed to maternal depression showing a higher baseline heart rate, and a slower and reduced responsivity to an external vibroacustic stimulus. Similarly, lower fetal heart rate during stimulation (Emory and Dieter, 2006) and lower heart rate variability in response to a familiar speech stimulus (Figueiredo et al., 2017) have been reported in fetuses of prenatally depressed women. In contrast, Monk and colleagues (2004) reported no differences in baseline fetal heart rate according to women's psychiatric condition, however foetuses of depressed women showed a larger increase in fetal rate in response to a laboratory challenge (i.e. mothers completing a Stroop Task), independent of women's cardiorespiratory activity. Taken together, findings seem suggestive of an association between maternal symptomatology and offspring's behaviour and physiological reactivity even before birth, although the limited number of available studies and considerable differences in assessment procedures and timing of assessment, do not allow to draw firm conclusions.

1.2.2.2 Neurodevelopment and psychomotor development

Infants of prenatally depressed mother have been observed to obtain lower scores on the Brazelton Neonatal Behavioural Assessment Scale (NBAS; Brazelton & Nugent, 1995), indicating less neonatal neurobehavioral maturity (Osborne et al., 2018; Field et al., 2004; Lundy et al., 1999; Pacheco & Figueiredo, 2012; Figuereido et

al., 2017). Negative effects of maternal depression during pregnancy were also seen on infants' attentiveness and responsiveness to faces and voices, as early as the neonatal period (reviewed in Field, Diego, & Hernandez-Reif, 2009), and on infants early regulatory behaviors and sleep problems (Diego et al., 2004; Gerardin et al., 2011; Pacheco and Figuereido, 2012; Räikkönen et al., 2015).

Findings of an association between maternal depression during pregnancy and infants' psychomotor development are controversial. In particular, a significant association between maternal antenatal depression and lower cognitive development independent of postnatal depression has been reported at 18 months of age (Koutra et al., 2013), 24-30 months of age (Lin et al., 2017) and in a large sample of 8 years-old children of the Avon Longitudinal Study of Parents and Children (ALSPAC) in the UK (Evans et al., 2012). However, a subsequent analyses of data from the ALSPAC sample showed no direct effect of maternal antenatal depression on children cognitive functioning at 8 years of age, while the effects of maternal depressive symptoms during pregnancy were mediated by a poor maternal prenatal nutrition, which in turn predicted reduced cognitive function (Barker et al., 2013). In addition, a positive association between maternal depression assessed in mid-pregnancy and children motor and mental development at 2 years of age, after controlling for several covariates including postnatal depression has also been reported (DiPietro et al., 2006). Furthermore, Sandman and colleagues (2012) reported greater cognitive and motor development scores in 3-6 month old infants of prenatally and postnatally depressed mothers or in infants of never-depressed mother, as compared to infants where prenatal and postnatal symptomatology were discordant (i.e. high prenatal depression versus low postnatal depression or low prenatal depression versus high postnatal depression), suggesting that it is the continuity in prenatal and postnatal environment that might confer an advantage for infants' development, consistent with the PAR model. Lastly, a number of studies failed to detect any significant association

between maternal antenatal depression and child psychomotor development (Osborne et al., 2018; Santucci et al., 2014; Tse et al., 2010).

1.2.2.3 Temperament and behavioural reactivity

Most evidence points to an association between maternal antenatal depression and caregiver-reports measures of infants' early temperamental traits, and in particular difficult temperament and negative affectivity (e.g. Rode & Kiel, 2016; Nomura et al., 2014; Davis et al., 2007; Rouse & Goodman, 2014) between 2 and 6 months of age, after accounting for postnatal depression. However, null associations have also been reported (Rothenberger et al., 2011; Braithwaite et al., 2017; Werner et al., 2013; Kantonen et al., 2015). Furthermore, the majority of studies conducted on children aged 24-36 months failed to detect any significant effect of prenatal symptomatology (Lin et al., 2017; Bekkhus et al., 2011; Blair et al., 2011; though not all, Stroustrup et al., 2016).

Very few studies examined the association between maternal depressive symptoms during pregnancy and observational measures of infants' temperament and behavioral reactivity and these yielded mixed findings. Maternal depressive symptoms during pregnancy, but not postnatally, have been found to predict either greater (Davis & Schetter, 2004; Werner et al., 2007) or lower (Rothenberg et al., 2011) infant behavioral reactivity to novelty at 4-5 months of age, although non-significant associations have also been reported (Werner et al., 2013a). Moreover, Davis and colleagues (2011) found maternal depression in mid-late pregnancy to predict a slower behavioural recovery after the heel-stick soon after birth, while Swales and colleagues (2018) reported no effects of antenatal depression on pre-schoolers behavioural response to a stressful laboratory procedure.

1.2.2.4 Emotional and behavioural problems

Maternal antenatal depression is considered a risk factor for children's socio-emotional development and mental health. Several observational studies have now provided evidence for a significant association between prenatal depressive symptoms and children's increased risk for attention and behavioural problems, internalizing and externalizing disorders and psychopathology from 1 to 10 years of age (Barker et al., 2011; Luoma et al., 2001; Leis et al., 2014; Nulman et al., 2012; Gerardin et al., 2011; Lahti et al., 2017), after controlling for postnatal symptomatology. However, in a study by Leech and colleagues (2006) the association between prenatal depression and children's depression and anxiety symptoms was no longer significant after accounting for postnatal predictors of children's mental health. Similarly, in a large combined dataset from the Generation R and ALSPAC studies, the association between antenatal maternal depression and child inattention problems at 3-4 years of age was strongly attenuated (and in Generation R sample became non-significant) when postnatal depressive symptoms were taken into account (Van Batenburg-Eddes et al., 2013). In addition, gender differences and timing effects in the association between prenatal maternal depression and anxiety and child behavioural problems have been reported (de Bruijn, van Bakel, & van Baar, 2009), with significant association early in pregnancy for boys, while later in pregnancy for girls. A recent meta-analyses by Madigan and colleagues (2018) including 73 studies showed that maternal depression during pregnancy is associated with children socio-emotional problems (i.e. difficult temperament, behavioral problems, behavioral dysregulation). In particular, children prenatally exposed to maternal depression were 1.5-2 times at greater risk of experiencing behavioral problems as compared to unexposed children. The effects were stronger for clinical depression as compared to self-reported symptoms and for children from socio-economic disadvantaged families. However, it is important to note that meta-analytic work only estimates the overall unadjusted effect size, and was not able to control for relevant covariates.

Available evidence suggests that the relationship between maternal antenatal depression and offspring psychopathology might persist into adolescence and adulthood. In a study based on the large dataset from the ALSPAC study maternal prenatal depression or anxiety was found to predict persistently higher behavioral and emotional problems from 4 to 13 years of age, independent of postnatal mood, with no attenuation of the effects into adolescence (O'Donnell et al., 2014). In addition, a higher risk for depression (Pawlby et al., 2009; Pearson et al., 2013), anxiety and co-morbid anxiety and depression (Capron et al., 2015) in young adults aged 16-25 prenatally exposed to maternal depression, independently of postnatal depression, has been reported. Similarly, findings from a large cohort of 3,099 mother–offspring pairs from the Mater University Study of Pregnancy in Australia showed that high levels of depressive symptoms in pregnancy predicted higher levels of internalizing problems at 14 years (Betts et al., 2014), internalizing and externalizing behavioral problems and greater self-reported symptoms at 21 years of age (Betts et al., 2015). In addition, exposure to maternal depression during pregnancy has been associated with a greater risk for conduct disorders and violent acts at 16 years of age (Hay et al., 2010), after controlling for postnatal symptoms and parents' antisocial behaviors, and with greater offspring externalizing, but not internalizing, problems at age 16 (Korhonen et al., 2014).

Gender effects in the association between antenatal depression and emotional problems have been reported also in adolescence, with Hay and colleagues (2008) and Quarini and colleagues (2016) showing significant and stronger associations in girls, as compared to boys. Furthermore, some studies showed that antenatal depression alone did not increase the risk of developing psychopathology in adolescence, while the exposure to both antenatal depression and childhood maltreatment (but not maltreatment alone) increased around 12 times the risk for a depressive or conduct disorder (Pawlby et al., 2011; Plant et al., 2013).

To sum up, mounting evidence points to a significant association between maternal antenatal depression and attention, emotional and behavioral problems in the offspring from early childhood to adulthood, although postnatal factors, including postnatal maternal depression, appear to account for some of the effects in a number of reports (e.g. Hay et al., 2010, Leech et al., 2006; Van Batenburg-Eddes et al., 2013).

1.2.2.5 Stress-related physiology and inflammation

The fetal programming hypothesis proposes that maternal prenatal stress might alter the development of fetal biological systems and this in turn might be associated with altered behavioural and physiological outcomes later in life (Talge et al., 2007). In particular, it has been hypothesized that maternal antenatal stress might influence child development and increased later vulnerability for physical and mental health problems by altering the developing fetal stress response systems and immune system (Seckl & Holmes, 2007; Van den Bergh et al., 2017). A number of studies have assessed the effects of maternal antenatal depression on offspring stress-related physiology and, more specifically, on the functioning of the two main components of the stress response system, namely the Hypothalamic-Pituitary-Adrenal (HPA) axis and the Autonomic Nervous System (ANS).

Several studies reported higher baseline cortisol levels in infants of prenatally depressed women (e.g. Brennan et al., 2008; Diego et al., 2004; Lundy et al., 1999; Osborne et al., 2018). Few prospective studies have evaluated samples of older children and these have found lower basal cortisol levels in children prenatally exposed to maternal depression (Laurent et al., 2013; Stonawsky et al., 2019), thus possibly suggesting that age-dependent effects might exist (Essex et al., 2011). This is also in line with the notion that, as part of an allostatic process, the “costs” associated with chronic HPA-axis activation may lead over time to an exhausted and down-regulated HPA-axis, with a reduced cortisol output (Miller et al., 2007). While this has

been well-demonstrated in children and adults with a history of maltreatment (Fries et al., 2008; Miller et al., 2007), it still need to be empirically investigated in children exposed to maternal depression early in life.

Evidence concerning an association between in utero exposure to maternal depression and children's cortisol reactivity to stressors is inconsistent. Either positive (i.e. greater reactivity, e.g. Brennan et al., 2008; Swales et al., 2018) or negative (i.e. blunted reactivity, e.g. Vedhara et al., 2012; Waters et al., 2013) or even U-Shaped (i.e. greater reactivity in infants exposed to the highest and lowest levels of maternal depression, Fernandes et al., 2014) associations have been reported, while a number of studies failed to detect any significant associations (Azar et al., 2007; Davis et al., 2011; Werner et al., 2013). In addition, two studies reported that maternal antenatal depression predicted heightened cortisol levels in girls but not in boys (De Brujin et al., 2009; Giesbrecht et al., 2017), while recent findings by Osborne and colleagues (2018) showed greater cortisol response to inoculation in infants prenatally exposed to maternal depression at 12 months, but not at 2 months, suggesting that both infants' gender and age might play a role in the association. Two recent investigations reported an association between maternal depression during pregnancy and greater infant cortisol reactivity, possibly mediated by epigenetic mechanisms involving, respectively, methylation of NR3C1 gene (Oberlander et al., 2008) and methylation of placental 11β-HSD2 (Stroud et al., 2016).

To date, only two prospective studies have evaluated the association between maternal prenatal depression and offspring's cortisol physiology at older ages. Data on a large sample from the ALSPAC study showed that maternal depression in late pregnancy was significantly associated with an alteration of cortisol diurnal patterning, as indexed by a reduced cortisol awakening response (CAR, O'Donnell et al., 2013). In contrast, a recent study, based on a smaller sample, provided no evidence for an association between maternal prenatal depression and CAR in 25 year olds adults (Plant et al., 2016).

Evidence concerning the functioning of the ANS in children prenatally exposed to maternal depression are scarce. Increased norepinephrine (noradrenaline) and decreased dopamine levels soon after birth have been found in infants of prenatally depressed women (Lundy et al., 1999, Field et al., 2004) and in newborns of women depressed both prenatally and postnatally (Diego et al., 2004). Cardiac measures of ANS functioning have been evaluated in a handful of studies. Lower Respiratory Sinus Arrhythmia (RSA), an index of Parasympathetic Nervous System (PNS) activity, in newborns of mothers with depressive symptoms in late gestation have been reported in an early study (Jones et al., 1997). However, no associations between maternal negative emotionality (measured by combining anxiety- and depression-related variables) both in early and late pregnancy and infant RSA were reported in a different study (Ponirakis, Susman, & Stifter, 1998). In addition, no associations between maternal antenatal depression and infants' heart rate variability have been reported at 4 months (Kaplan, Evans, & Monk, 2009) and 14 months of age (Dieter et al., 2008). Similarly, in a large sample of mother-child dyads from the Generation-R study, no significant associations were found between maternal depressive symptoms at 16 gestational weeks and preschoolers' baseline heart rate and RSA (Van Dijk et al., 2012). Thus, although the evidence is only preliminary, they do not seem suggestive of an altered functioning of the ANS in children of prenatally depressed women.

Only a small number of studies to date has investigated the impact of maternal depression during pregnancy on the immune system functioning in offspring. Two studies reported an association between antenatal depression and altered immune parameters in newborns soon after birth (Kianbakht et al., 2013; Mattes et al., 2009). In particular, Kianbakht and colleagues (2013) reported an increased lymphocyte count and a decreased ratio of cord blood levels of immunoglobulins to maternal blood levels of immunoglobulins in newborns of prenatally depressed as compared to controls. Mattes and colleagues (2009) showed an association between maternal depressive symptoms at 20 gestational weeks and heightened immune

responsiveness in neonates, as indicated by higher cytokine production both spontaneously and stimulated by bacterial antigens and allergens. Furthermore, Plant and colleagues (Plant et al., 2016) found that antenatal exposure to maternal depression predicted increased offspring inflammation, as indexed by higher levels of C-Reactive Protein (CRP), at 25 years and that the effect was independent of later adverse experiences such as child maltreatment and adult diagnosis of mood disorder.

In summary, while a dysregulation of the stress and inflammatory response systems in the offspring might be a mechanism underlying the impact of antenatal maternal stress on later mental and health outcomes, evidence for an association between prenatal maternal depression and neuroendocrine alterations in the offspring is complex and inconsistent. Although there is little research on immune functioning in offspring prenatally exposed to maternal depression, preliminary evidence suggest that this might represent a promising route to be explored.

1.2.2.6 Functional and structural brain alterations

A number of studies have evaluated whether antenatal exposure to maternal depression is associated with functional and structural alterations of the brain in offspring that, in turn, might explain the greater risk for behavioral and mental health problems.

Early studies showed an association between antenatal maternal depression and an altered pattern of resting brain electrical activity as measured through electroencephalography (EEG) in infants soon after birth until 6 months of age (Field et al. 1995; Jones et al. 1997; Diego et al., 2004). In particular, greater activation in right-frontal cortex relative to the left, which is considered an early marker of behavioural inhibition and negative affectivity (Fox & Davidson, 1986), was observed in infants of prenatally depressed mother as compared to infants of healthy women. In addition, greater right frontal EEG asymmetry was particularly evident in newborns of depressed mothers showing concomitant severe anxious symptoms (Field et al.,

2003). A recent study showed that not only a clinical diagnosed depressive disorder but also depressive symptoms in late pregnancy are associated with greater relative right frontal asymmetry in 2-day-old infants (Gustafsson et al., 2018). In contrast, prenatal depressive symptoms were not found to predict frontal EEG activity and functional connectivity in 6 and 18-month-olds, although an increase in maternal symptoms from pregnancy to 3 months after delivery was related to greater right frontal activity and relative right frontal asymmetry amongst 6-month-old infants (Soe et al., 2016).

More recently, magnetic resonance imaging studies have begun to shed light on the neurobiological correlates of prenatal exposure to maternal depression. In particular, after controlling for postnatal maternal mood, women's self-reported depressive symptoms during pregnancy have been associated with cortical thinning, particularly over the frontal lobe, in 2-5 year olds (Lebel et al., 2016) and in 6-9 year olds children (Sandman et al., 2015). Furthermore, maternal depressive symptoms during pregnancy were related to alterations of the microstructure of the amygdala (Rifkin-Graboi et al., 2013) and of amygdala functional connectivity with dorsal prefrontal cortex, bilaterally, in neonates at birth (Posner et al., 2016) and with cortico-limbic circuits in 6-month-olds (Qiu et al., 2015) and 4.5 year-old girls (Soe et al., 2018), as well as with greater right amygdala volume in preschool girls but not in boys (Wen et al., 2017). In contrast, a recent study did not show any gender differences in the association between prenatal depression exposure and larger right amygdala volume, but found that infant genotype moderated the association (Qiu et al., 2017).

In summary, converging evidence demonstrates that in utero exposure to maternal depression (both alone and in interaction with infant gender and genes) is associated with different aspects of offspring brain development. In particular, antenatal maternal depression seems to be associated with differences in the development of frontal and limbic circuits, with possible implications for later emotion regulation and the development of psychopathology. In addition, initial data suggest

that gender and timing effects deserve further exploration, with preliminary evidence of stronger associations in girls (Soe et al., 2018, Wen et al., 2017), and in mid pregnancy (i.e. in gestational week 17 but not 11 or 32, Lebel et al., 2006).

1.2.3 The differential effects of antenatal maternal anxiety on child outcomes

One issue that research has only begun to address is whether different types of antenatal maternal stress have greater effect than others on fetal development. More specifically, the unique and specific effect of maternal antenatal psychopathology, such as depression, as compared to anxiety, is still unknown. Few studies examined both antenatal maternal anxiety and depression in the same sample. As anxiety and depression are quite strongly co-morbid or correlated, marked differences between anxiety and depression predictions might be unlikely, and indeed, comparable associations between anxiety or depression and child outcomes have been reported (e.g. Davis et al., 2011; Werner et al., 2013; O'Donnell et al., 2013).

However, there are also studies reporting conflicting results. For example, Barker and colleagues (2011) reported a broader effect of prenatal maternal depression on child functioning, as compared to maternal anxiety. Specifically, prenatal depressive symptoms predicted both a small increase in children's externalizing problems and decrease in verbal intelligence quotient (IQ), whereas maternal anxiety predicted only an increased risk for child internalizing difficulties. Similarly, Lin and colleagues (2017) reported significant prospective associations between maternal antenatal depression, but not anxiety, and toddler's cognitive development. In contrast, Ibanez and colleagues (2015), found a significant inverse association among maternal antenatal anxiety, but not depression, and children's cognitive performances at 2 and 3 years of age. Likewise, data from the ALSPAC study suggested a stronger effect of maternal anxiety than depression on children's behavioral problems (O'Connor, Heron, & Glover, 2002; O'Connor et al., 2003). Recently, a meta-analysis by Madigan and colleagues (2018) obtained a stronger

effect for maternal antenatal depression versus anxiety on infants' socio-emotional development. O' Connor and colleagues (2005) found that prenatal anxiety symptoms, but not depressive, predicted children's cortisol diurnal pattern at 10 years of age. Similarly, Grant and colleagues (2009) reported maternal prenatal anxiety, rather than depression, predicting 7-month-olds cortisol reactivity.

Methodological issues might account for these conflicting findings. For example, while self-report measures of depressive symptoms during pregnancy often inquire about the past week, anxiety is sometimes evaluated using "trait" measures that assess general functioning. However, there are also possible theoretical explanations for the differential association between prenatal depression versus anxiety and child outcomes. Indeed, while maternal anxiety and depression are often assumed to be part of the same underlying construct of antenatal stress, it is largely unknown the extent to which they are related to different alterations of relevant intra-uterine biological systems during pregnancy, thus possibly affecting fetal development in different ways. Future studies should clarify the prenatal risk phenotype that might be more implicated in fetal/child development, as well as elucidating the specific underlying mechanisms.

1.3 Critical issues

1.3.1 Threats to causality

Many independent prospective studies from different countries around the world have now demonstrated a significant link between antenatal maternal depression and child developmental outcomes in humans, suggesting that this should be a major area for future research. The diversity of outcomes linked to antenatal depression exposure is impressive and, although few studies included long follow-up periods, there is evidence to suggest that antenatal maternal depression exerts persisting effects on offspring's behaviour and physiology (e.g. O'Donnell et al., 2013), thus pointing to antenatal maternal depression as a broad risk factor for child

development. Notwithstanding the substantial evidence base linking antenatal maternal depression to child outcomes, proving that this association is truly causal and that fetal programming is actually occurring is challenging. This is mainly because while the observational designs used in human studies to a certain extent informed us on the temporal order of risk factors (i.e. antenatal maternal depression) and child outcomes, it does not allow us to draw causal conclusions. Inevitably, there are a large variety of confounds, ranging from maternal diet during pregnancy to postnatal environmental factors to shared genetics, that could be linked to both maternal depression and offspring outcomes and cause spurious associations (Rothman, Greenland, & Lash, 2008). Furthermore, as depression during pregnancy is a strong predictor of postnatal depression, continuity of exposure over time might make it difficult to disentangle the risk effect of exposure in utero from later postnatal exposures.

An increasingly adopted approach is to include the assessment of as many potential confounders as possible. However, even then it is not possible to test for all possible confounders, thus unmeasured confounding factors and measurement error in the evaluation of confounding factors may lead to residual confounding that can never be completely ruled out in observational studies (Rice et al., in press; Gage et al., 2016). These kinds of studies have shown that the association between antenatal maternal depression and child outcomes remains consistent even after controlling for many confounding factors, such as prenatal smoking and alcohol consumption, socioeconomic status or postnatal maternal symptomatology (e.g. O'Donnell et al., 2013; O'Connor et al., 2013). Moreover, studies showing a link between antenatal depression and neonatal outcomes evaluated soon after birth (e.g. Davis et al., 2011) or adoption-after-birth studies (e.g. Laurent et al., 2013), although limited, have shown the true effects of antenatal maternal depression, independent from postnatal effects (but not from genetic confounds).

A number of studies have attempted to control for shared genetic background between mother and child that could underlie both maternal symptoms and child behaviour, by using different approaches, including genetically informed designs. These studies have begun to provide initial evidence suggesting that the link between prenatal maternal depression and child development is at least partially independent from genetics. For example, Rice and colleagues employed an in vitro fertilization prenatal cross fostering design, comparing mother-child dyads who were genetically related to those who were not, in order to control for maternal heritable factors (Rice et al., 2010). They found a significant association between maternal stress and child conduct problems, but not on child ADHD or anxiety, in unrelated dyads after controlling for postnatal symptoms, consistent with it being a true prenatal effect. Other studies compare maternal prenatal exposure with paternal prenatal exposures (Smith, 2008), so that if a true intrauterine effect underlies the link between exposure and child outcomes, a stronger association with maternal exposure, rather than paternal exposure, would be expected. Conversely, similar associations among child outcomes and either maternal or paternal exposures, would indicate that alternative factors, including genetic and environmental factors are likely to underlie the observed associations. Using this approach, a stronger association between maternal depression, as compared to paternal depression, was found for child attention problems in the ALSPAC cohort, providing some support for a direct intrauterine mechanism underlying the association; however, such an effect was not observed in the Generation R cohort (Van Batenburgh-Eddes et al., 2013). Alternative designs, such as sibling comparison designs or twin designs, where unexposed siblings or twins are used as “control” matched to exposed individuals, have not yet been widely employed in this field (e.g. Knopik et al., 2016).

Studies on the effects of prenatal exposure to natural disasters, which are presumably randomly distributed with respect to genetics (e.g. King et al., 2012; Yehuda et al., 2005; Huiznik et al., 2007), provide further evidence that the effects of

prenatal stress are not just due to genetic vulnerabilities. Moreover, studies examining biological mechanisms possibly underlying the association between maternal antenatal depression and child outcomes (e.g. Oberlander et al., 2008; Stroud et al., 2016) are starting to uncover intra-uterine mechanisms involved in fetal programming, which lends further credibility to the hypothesis that the association represents a true exposure effect.

Lastly, research on the effectiveness of interventions specifically designed to reduce maternal antenatal depression on child outcomes could help to establish whether the link between prenatal depression and offspring outcomes is truly causal. As limited knowledge exists to date concerning the potential effects of antidepressants on the developing fetus (reviewed in El Marroun et al., 2014) and there is limited acceptance of pharmacological treatments during pregnancy (Freeman, 2007), it is important to elucidate the effects of psychological treatment of antenatal depression on child development. However, to date, there are surprisingly few randomized controlled trials (RCTs) on the effectiveness of psychological therapy for maternal prenatal depression on infant outcomes. Rahman and colleagues (2008) examined the impact of cognitive-behavioral therapy aimed at reducing maternal depression both prenatally and postnatally on child development. While a significant impact of intervention on parenting practices was found, the focus on both the ante- and postnatal periods does not allow to disentangle the specific effects of prenatal treatment of depression. Two different studies examined the impact of cognitive-behavioral therapy for antenatal depression on infant outcomes, respectively, at 2 months (Netsi et al., 2015) and 9 months (Milgrom et al., 2015) after delivery in two independent pilot RCTs. Although differences between treatments groups on infant outcomes did not reach the statistical significance in the study by Netsi and colleagues (2015), an improvement in depression scores during pregnancy was related to “easier” infant temperament and shorter sleep duration in the cognitive-behavioral therapy group but not in the control group. Similarly, Milgrom and colleagues (2015) reported

better infant outcomes in the intervention group, as compared to controls, concerning infant self-regulation, problem solving and negative affectivity.

Beside the scientific significance of testing whether the observed associations between maternal antenatal depression and offspring outcomes are causal, these studies have important clinical implications for early intervention with at risk mother-child dyads. Interventions aimed at reducing prenatal risk factors, such as depressive symptoms, will be effective in promoting child development only if there is a true causal link between prenatal exposure and child outcomes. Thus, more research is needed to inform treatment development regarding the timing and target of interventions so that pregnant women and health practitioners can be provided with accurate and clear guidance and effective support.

1.3.2 Gestational windows of vulnerability

While timing of exposure appears to be important in fetal programming, little is known about the most sensitive time in gestation for all the effects previously described. Effects of antenatal maternal stress on child outcomes have been reported throughout gestation and different studies have shown different periods of vulnerability to the influence of prenatal depression, or more general stress. For example, studies on the risk for schizophrenia indicated that the most sensitive period for prenatal stress exposure was the first trimester (e.g. Khashan et al., 2008). Maternal stress both during the first and second trimesters of pregnancy have been found to affect cognitive development (e.g. Laplante et al., 2004; Velders et al., 2011). Strongest effect of antenatal maternal stress on birth outcomes (e.g. Cherak et al., 2018), HPA axis reactivity (e.g. Davis et al., 2011; Vedhara et al., 2012; Yehuda et al., 2005) and offspring's emotional problems (reviewed in Rice, Jones, & Thapar, 2007) have tended to be reported in late pregnancy as compared to earlier exposure.

These differences in timing, if they are reliable, are likely to depend on the outcome examined and the stage of development of the relevant brain circuit or

function. It is well-established that different organ systems develop along different time courses. Periods of greater development and rapid growth are considered windows of increased sensitivity to environmental stimuli and insults (Drake & Walker, 2004; Seckl & Meaney, 2004). Thus, it is possible that environmental signals, such as changes in nutrition, hormonal balance etc., at a particular developmental stage may interfere with the development of some organs but not others (Grossman et al., 2003).

Fetal magnetic resonance imaging has now shown that the fetal brain undergoes rapid growth and development across pregnancy, changing approximately 17-fold its relative size from mid to late gestation (Huang et al., 2006; 2010), making the fetal brain particularly vulnerable to environmental insults. Brain regions develop at different times and the processes involved followed a well-defined discrete time course which include neurogenesis, neuronal migration and differentiation, synaptogenesis, the development of non-neuronal components (glia, myelination, cerebrovasculature) and apoptosis (Grossman et al., 2003). Despite the fact that these processes are largely driven by genetic influences, especially early in development, there are critical periods of vulnerability for each process during which they are more sensitive to environmental perturbation, so that even a minor environmental deviation could disrupt these developmental processes and have substantial effects on the outcome (reviewed by Rice & Barone, 2000). Thus, for example, severe stress exposure early in gestation might increase the risk for schizophrenia (Kashan et al., 2008) because it alters the processes of neuronal migration, which is also hypothesized to be compromised in schizophrenia. In contrast, late pregnancy is characterized by rapid growth and development, including synaptic migration and the beginning of synaptic differentiation. Alterations in these processes may impact upon the risk for later psychopathology (Rice et al., 2007).

However, the major issue for research into the timing effects of prenatal exposure on child outcomes is that it is obviously not ethically possible to experimentally introduce a stressor at a specific gestational point and the usual study

designs do not us allow to determine the onset and offset of the prenatal stress experience. Thus, there is limited leverage for testing a timing effect and it is hard to disentangle whether the observed associations are specific for the selected gestational windows or might be the result of a continuous prenatal exposure combined with measurement error. For example, maternal reports of anxiety and depression are characterized by their chronicity and by high levels of within-individual stability throughout pregnancy (Dipietro, Costigan, & Sipsma, 2008). Thus, the prenatal stress exposure it is likely to be relatively continuous over gestation rather than discrete. A special case concerns studies that capitalized on acute stress events, such as natural disaster, in order to study timing effects (e.g. King et al., 2012; Yehuda et al., 2005). However, duration and timing of prenatal stress exposure is still likely to be confounded in these studies with women experiencing the event earlier in pregnancy being affected for a greater percentage of gestation than those exposed later.

Additionally, while findings suggest that both males and females are susceptible to the organizational influences of antenatal stress, it has been shown that the patterns of effects may differ depending on the gestational time windows and type of outcomes examined (e.g. De Brujin et al., 2009), thus suggesting that sex differences in the processes underpinning the effects of antenatal stress on child development might exist (Glover & Hill, 2012).

1.3.3 Postnatal environmental moderation

It is widely recognized that postnatal experiences, and particularly those involving mother-child interaction, play a crucial role in shaping child developmental trajectories (Grossman et al., 2003; Tottenham, 2017), inducing neurobiological, epigenetic, and behavioral adaptations (Monk, Spicer, & Champagne, 2012; Maccari et al., 2014). In particular, maternal sensitivity, defined as the caregiver's ability to detect and respond in a timely and accurate manner to infants' cues (Ainsworth et al., 1978), is considered one of the most powerful forces on early child development

(Tottenham 2017), exerting widespread and long-lasting effects on several domains, such as attachment (e.g. Verhage et al., 2016), physiological regulation (e.g. Conradt et al., 2016), social and emotional development (Kok et al., 2013; Leerkes, Blankson, & O'Brien, 2009; Raby et al., 2015), academic achievement (e.g. Raby et al., 2015), and physical health (e.g. Anderson et al., 2011). Thus, research into fetal programming of later outcomes needs to account not only for prenatal effects but also for postnatal factors, related for example to postnatal symptomatology and postnatal maternal care.

As previously mentioned, antenatal depression is one of the strongest predictors of postnatal depression (e.g. Heron et al., 2004) and exposure to maternal depression during the first years of life has been shown to be related to lower maternal sensitivity (Bernard et al., 2018) and to adversely impact child behaviour (e.g. Ashman, Dawson, & Panagiotides, 2008; Bureau, Easterbrooks, & Lyons-Ruth, 2009), stress-related physiology (e.g. Murray et al., 2010) and brain development (e.g. Lupien et al., 2011). A recent study reported substantial continuity in maternal anxiety and depressive symptoms, as well as in family disharmony, from pregnancy to 18 months after delivery (Bekkhus et al., 2011) suggesting that prenatal influences are strongly associated with similar postnatal experiences. To the extent that exposure to environmental adversity continues from prenatal to postnatal life, it might confound fetal programming effects. Thus, on the one hand, continued exposure to adversity might occur, with antenatal depressive symptoms persisting or recurring after birth and influencing the quality of maternal care and in turn, infant's development. On the other hand, it is also possible that the early postnatal environment, such as the quality of maternal care, moderates the link between in utero exposure to maternal depression and child outcomes.

Despite several studies having accounted for the effects of postnatal symptomatology (e.g. O' Donnell et al., 2013), other postnatal environmental factors such as the quality of maternal caregiving has been largely neglected. First of all,

whether poor maternal parenting in the early postnatal period completely or partially mediates the link between prenatal depression and child outcomes has only been rarely addressed (Monk et al., 2012). Endendijk and colleagues (2005) found no association between prenatal maternal distress and maternal sensitivity, thus precluding testing mediation by maternal care in the link between antenatal distress and child behavioral problems. Similarly, maternal antenatal depressive symptoms were not found to be associated with maternal parenting quality (Hayes, Goodman, & Carlson, 2013) and mother-infant attachment (Tharner et al., 2012) in two studies. Thus, to date, no empirical support for a mediation role of maternal parenting in the link between antenatal depression and child outcomes has been found, although more research is needed to explicitly test this hypothesis.

In addition, little is known about the moderating role of early postnatal care in the link between maternal prenatal stress or depression and child outcomes. Considerable evidence from animal models provides evidence that the effects of prenatal stress may be moderated and even reversed by positive postnatal rearing (e.g. Maccari et al., 1995). Meaney and his group have clearly demonstrated how variations in maternal care can have long lasting influences on offspring behaviour and physiology. For example, rodent models showed that even brief periods of postnatal handling or mother licking and grooming cause long-lasting decreases in offspring's anxious behaviors and stress reactivity (reviewed in Meaney, 2001). It has been shown that postnatal handling or increased maternal care can reverse prenatal stress-induced alterations in spatial memory performance and stress reactivity (Brabham et al., 2000) or hippocampal neurogenesis (Lemaire et al., 2006). In addition, adoption after birth was found to completely reverse the effects of prenatal stress on HPA axis function (Maccari et al., 1995), while prolonged maternal separation was associated with a significant increase in HPA axis reactivity to acute stress in prenatally-stressed rats (Plotsky & Meaney, 1993). Although many of these mechanisms are likely to occur in humans too, several differences between humans and animal models make

difficult to generalize findings. What animal research highlights is that the period of sensitivity or programming extends well beyond the prenatal period, thus suggesting that the interaction between prenatal and postnatal environmental factors in influencing developmental trajectories should be a major area of inquiry in humans too.

Initial findings in humans have suggested an interplay between prenatal and postnatal environment. For example, socio-economic status was found to moderate the effects of low birth weight on behavioral problems in childhood and adolescence (Kelly et al., 2001; Bohnert & Breslau, 2008). Similarly, positive maternal care eliminated the association between low birth weight and both child ADHD symptoms (Tully et al., 2004) and hippocampal volume in adulthood (Buss et al., 2007). While these preliminary findings are interesting, it is noteworthy that birth weight is only a proxy of intrauterine conditions.

Similarly, a small set of studies has evaluated the impact of tactile stimulation, such as maternal stroking, over the first weeks of life in the association between prenatal anxiety or depression and infant outcomes. It was shown that maternal stroking moderated the effects of prenatal stress on children's physiological and behavioural reactivity at 7 months (Sharp et al., 2012), internalizing symptoms at 2.5 years (Sharp et al., 2015) and internalizing and externalizing behaviors at 3.5 years (Pickles et al., 2017), suggesting an effect of early maternal behaviors strikingly similar to that reported in animal models.

A limited number of studies examined the influence of maternal care in moderating the effects of prenatal maternal stress on child development. The quality of maternal care has been found to moderate the effects of prenatal maternal anxiety (Grant et al., 2010) and cortisol (Bergman et al., 2010) on infant cognitive development. In the Queensland Flood Study, maternal structuring, defined as the caregiver's ability to scaffold the child's activity, but not sensitivity, interacted with antenatal exposure to the natural disaster to predict language development at 30

months of age (Austin et al., 2017). Furthermore, the quality of early maternal caregiving moderated the influence of self-reported stressful life events during pregnancy on child fearfulness (Bergman et al., 2008), the influence of prenatal psychiatric diagnosis on 4-month-olds' baseline cortisol levels (Kaplan et al., 2009) and the effects of prenatal anxiety on 7-month-old behavioural reactivity to the still-face procedure (Grant et al., 2010b). Lastly, in one study maternal sensitivity appeared to influence the link between maternal depression/anxiety during pregnancy and internalizing problems in 2-5 year olds girls, but not boys (Endendijk et al., 2005). Nevertheless, no effects of maternal care in the link between prenatal stress and child outcomes, such as cortisol reactivity (e.g. Grant et al., 2009), heart rate variability (Kaplan et al., 2009) and impulse control (Graham et al., 2017), have also been reported.

The limited number of studies and considerable methodological variations among them, concerning the assessment of prenatal stress (i.e. self-reported stress symptoms, psychiatric diagnosis, natural disaster or stress hormones levels), maternal care (i.e. maternal sensitivity, structuring or mother-infant attachment) and age of the child does not allow us to draw clear conclusions. However, taken together, data suggest that the consequences of prenatal stress exposure on child development may be at least partially dependent on the quality of the postnatal environment, in line with the PAR model.

Recently, it has been proposed that prenatal experiences might actually affect infant susceptibility to the influences of their postnatal environment in a process that has been described as "prenatal programming of postnatal plasticity" (Pluess & Belsky, 2011) or "meta-plasticity" (O'Donnell & Meaney, 2017). In this sense, antenatal adversity would not influence any specific outcome, but rather the degree of developmental plasticity or susceptibility to subsequent positive or negative environmental influences. However, this hypothesis is only beginning to be addressed. In the study noted above by Grant and colleagues (2010a), a positive association

between maternal sensitivity and cognitive development was found among infants prenatally exposed to maternal anxiety but not among controls. In a subsequent study the authors (Grant et al., 2015) found that the effects of socioeconomic status on long-term memory were greater among children prenatally treated with synthetic glucocorticoid treatment. In particular, memory impairments were reported only in those children who experienced both fetal glucocorticoid exposure and postnatal sociodemographic adversity (Grant et al., 2015). However, antenatal glucocorticoid exposure was not associated with enhanced memory performance in the context of low sociodemographic risk, as a developmental plasticity effect would predict (Belsky & Pluess, 2009).

1.3.4 Genetic moderation

As noted already, beside postnatal environmental factors, genetic factors potentially confound the association between prenatal maternal stress and offspring behaviour. However, as previously mentioned, studies using genetically informative designs or other controls (e.g. paternal versus maternal stress measures) provide some evidence to suggest that effects of prenatal maternal stress on child outcomes exist independently of genetic factors. Another important role for genetics, however, might be in making some individuals particularly vulnerable to the influence of prenatal environment (Pluess & Belsky, 2011). There is now increasing evidence pointing to an interplay between genes and intrauterine exposure to maternal stress in influencing offspring's neurodevelopment. For example, it has been found that the polymorphism in the serotonin transporter promoter gene (5-HTTLPR), which has been found to moderate the effect of adverse early environment on a range of outcomes (e.g. Barry, Kochanska, & Philibert, 2008), also moderates the effects of prenatal anxiety on 6-month-olds negative emotionality (Pluess et al., 2011). In particular, infants carrying the short allele, which has been regarded as the "plasticity gene" (Jay Belsky & Pluess, 2009), were more affected by maternal anxiety during pregnancy, showing

more negative emotionality. Similarly, the monoamine oxidase functional A (MAOA) functional polymorphism moderated the impact of maternal stressful life events during pregnancy on negative emotionality in response to the NBAS when measured a few weeks after birth (Hill et al., 2013). Other studies reported that dopamine gene variants, such as the polymorphism for receptor D4 (DRD4) gene, interacted with maternal prenatal stress to predict offspring aggressive and antisocial behaviour in childhood (Zohsel et al., 2014) and adulthood (Buchmann et al., 2014) and with maternal smoking during pregnancy in influencing boys' externalizing behaviors (O'Brien et al., 2013).

In summary, while there is evidence that the associations between prenatal adversity and offspring neurodevelopment is at least partially independent from genetics (e.g. Rice et al., 2010) or postnatal (e.g. Davis et al., 2011) influences, initial evidence suggests that gene x prenatal environment interactions might affect physiological and behavioural outcomes in later life. In light of the significant influences of genetic and postnatal environmental factors in determining individual developmental trajectories, there is a need for prenatal programming models to account for this complexity by including genetic and postnatal environmental pathways, as well as their interactions.

1.4 Biological mechanisms underlying fetal programming

The data reviewed above shows that there is consistent evidence for an association between maternal antenatal stress, and more specifically depressive symptoms, and a wide range of physiological and behavioral alterations in offspring. In addition, increasing evidence suggest that the impact of maternal antenatal stress on child development depends on timing of exposure and gender, and might be moderated by offspring's genotype and postnatal environmental influences (Figure 1.1).

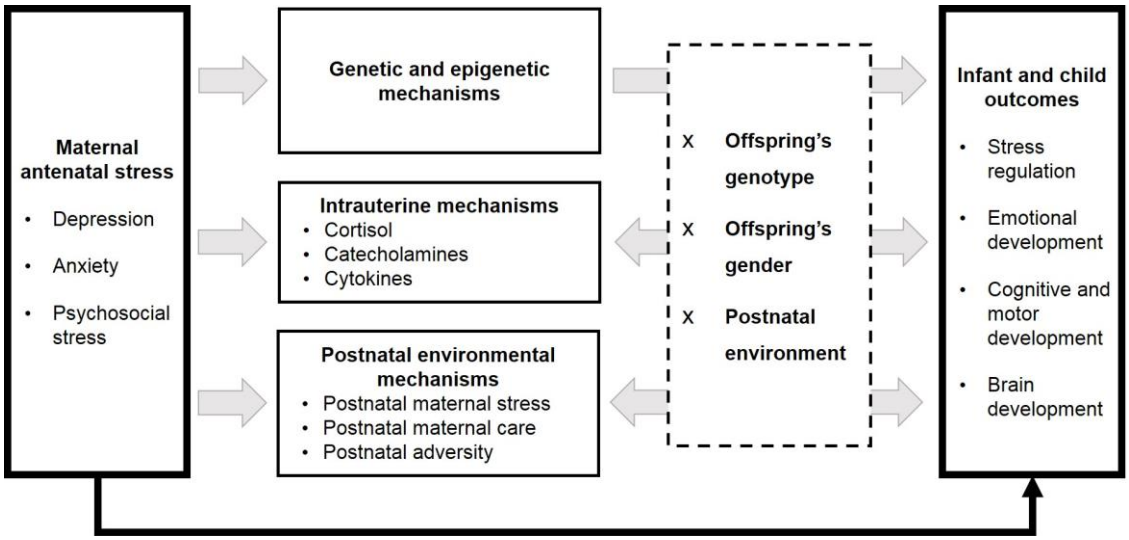

Figure 1.1 - A schematic representation of the principal proposed pathways linking maternal antenatal stress with offspring's neurodevelopmental outcomes.The wider line depict the association between antenatal maternal symptoms of depression, anxiety or stress and child outcomes for which there is strong evidence. Thin boxes indicate possible pathways underlying the well-established association. While for schematic purposes pathways are depicted in separate boxes, they are likely to interact at different stages. Genetic and epigenetic mechanisms will not be the focus of the current dissertation, however, they represent an important potential pathway linking antenatal maternal stress with offspring's neurodevelopmental outcomes. The model also highlights factors that can moderate the impact of antenatal maternal stress on child outcomes at different stages (in the dashed thin box). In the current dissertation the moderating role of postnatal maternal care will be investigated, although all these factors merit greater emphasis in future research.

Studies using genetically informed designs or assessing infants soon after birth indicate that some of these associations may be independent from genetic or postnatal environmental influences, suggesting that intrauterine mechanisms of fetal programming are at work. Despite great effort directed toward elucidating these mechanisms, it is still largely unknown how maternal antenatal distress is actually "transferred" to the fetus to influence fetal development. Stress-induced increases in maternal cortisol levels have traditionally been considered the candidate mediator of maternal stress transfer to the fetus (e.g. Cottrell & Seckl, 2009). However, other potential mediators have been proposed, including catecholamines, cytokines, serotonin and tryptophan, maternal microbiota, as well as placental and epigenetic mechanisms (Rakers et al., 2017).

The current dissertation focuses on the examination of three intrauterine mechanisms possibly underlying the link between antenatal maternal depression and altered outcomes in offspring, involving the major stress responsive systems, namely the HPA axis, the SNS and the IRS, as depicted in Figure 1.1. While these mechanisms will be reviewed separately, they are not likely to be alternative to each other; rather, it can be hypothesized that maternal-stress transfer arises from the combined effect of several different mechanisms acting together in a synergistic way. In particular, under stress conditions, a coordinated physiological response is activated, involving several levels of the central nervous system, including the autonomic, neuroendocrine, and immune system components (Lupien et al., 2009). All these systems are affected by stress and, in turn, affect each other within an efficient and evolutionarily-conserved stress response system. In addition, they are also implicated in the pathophysiological changes that occur in response to chronic stress (Lupien et al., 2009).

The functioning of all these systems is substantially altered during pregnancy (e.g. Sherer, Posillico, & Schwarz, 2017; Christian, 2012). These adaptations are thought to support fetal growth and development, while preventing both the mother and fetus from excessive exposure to stress hormones and stress-related physiological alterations (Christian, 2012a). Thus, individual differences in physiological adaptations during pregnancy may have important consequences for both maternal health and fetal development.

Before discussing in details the possible mechanisms of fetal programming and the available evidence, a brief overview of the functioning of the stress and inflammatory response systems and of the changes undergoing during the perinatal period is provided, as well as available evidence of an association with depressive symptomatology.

1.4.1 The HPA axis

The hypothalamus–pituitary-adrenal (HPA) axis is an integrated neural and endocrine system that is involved in several physiological processes (Michael & Papageorghiou, 2008) and, along with the sympathetic adrenomedullary (SAM) system, is a key component of the stress response systems in mammals.

Under stress conditions, whether psychological or physical, several brain areas are activated to recruit the hypothalamus and more specifically, the paraventricular nucleus (PVN) which, in turn, activates the hormonal cascade of the HPA axis (McGowan & Matthews, 2018), as shown in Figure 1.2. In particular, the PVN releases corticotrophin-releasing hormone (CRH), arginine-vasopressin (AVP) and oxytocin (Sawchenko et al., 1996). CRH and AVP stimulates the secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary and the subsequent release of glucocorticoids from the adrenal cortex into the bloodstream (Godoy et al., 2018).

The main glucocorticoid in humans is cortisol (corticosterone in rodents). Once released, cortisol acts in the brain by binding to glucocorticoid (GR) and mineralocorticoid (MR) receptors, and exerts negative-feedback control over the activity of the HPA axis, in order to prevent detrimental consequences related to a prolonged and excessive stress response (de Kloet, 1991; Joëls & Baram, 2009).

Glucocorticoids exert their effects on neurons expressing GRs and MRs through genomic and non-genomic mechanisms (de Kloet & Sarabdjitsingh, 2008; Groeneweg et al., 2012; Joëls, Sarabdjitsingh, & Karst, 2012). As compared to GRs, MRs show higher affinity for glucocorticoids but are expressed in fewer areas of the brain, while GRs are abundant and widely expressed throughout the brain (De Kloet et al., 2005). As a result, under basal conditions, high affinity MRs are occupied, while GRs are largely free. Under stress conditions or during the peak phase of the diurnal cycle, GRs also are partially occupied (Kitchener et al., 2004). Notably, it has been shown that GR gene expression, and consequently HPA axis function, can be

modulated by environmental experiences through epigenetic mechanisms (Buschdorf & Meaney, 2011) both in animals (Weaver et al., 2004) and in humans (Palma-Gudiel et al., 2015). In addition, GRs and MRs have been found to be implicated in regulation of cortisol levels throughout the day, as shown in clinical pharmacological studies (e.g. Otte et al., 2010).

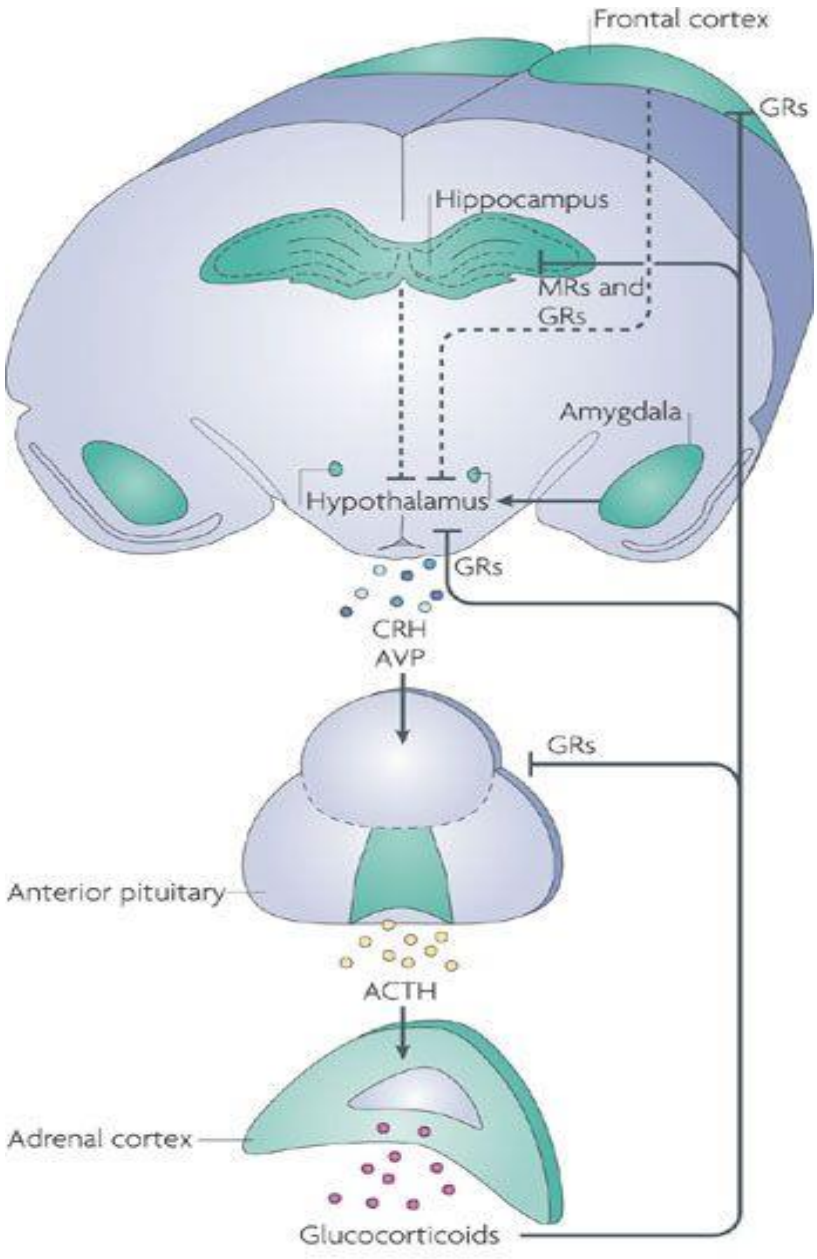

Figure 1.2 -Schematic representation of the Hypothalamic-Pituitary-Adrenal (HPA) axis functioning (from Lupien et al., 2009)

1.4.1.1 Cortisol

Cortisol is a steroid hormone which constitutes the end product of the HPA axis in humans. Cortisol plays an essential function in maintaining health and well-being and supporting several physiological processes, including immunity, growth, reproductive function as well as cognitive and affective processes (de Kloet, Oitzl, & Joëls, 1999). In addition, cortisol exerts a central role in preparing the organism to successfully respond to stress by mobilising energetic resources, shutting down nonessential physiological systems, and coordinating behavioural responses (Johnson et al., 1992). The acute cortisol stress response is characterized by peak levels 10-20 minutes after the stressor, followed by a recovery to baseline levels (Figure 1.3B). Although cortisol stress response is adaptive in the short term, prolonged or extreme HPA axis activation can have detrimental effects on the organism.

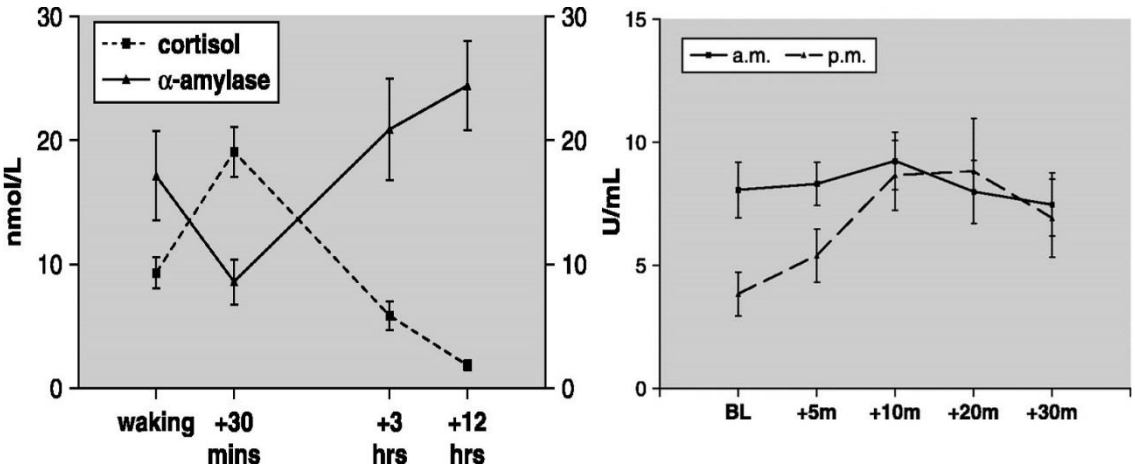

Figure 1.3 - A) Salivary cortisol and alpha amylase diurnal concentrations; B) Salivary cortisol acute stress response (adapted from O' Donnell et al., 2009)

Cortisol follows a distinctive circadian rhythm; as shown in Figure 1.3A, the lowest levels in late evening are followed by an increase towards the end of the sleep-cycle which peaks approximately 30 minutes after awakening and then gradually declines throughout the day. The naturally occurring increase of about 50–75% in cortisol levels as a response to awakening (Pruessner et al., 1997) has been termed

Cortisol Awakening Response (CAR). The CAR is a distinct component of cortisol diurnal rhythm which has been increasingly studied as it is thought to represent a useful marker of HPA axis function (Clow et al., 2010).

1.4.1.2 The HPA axis during the perinatal period

Regulation of the maternal HPA axis changes dramatically during pregnancy and the early postpartum period. In particular, plasma concentrations of CRH, ACTH and cortisol increase progressively across gestation, resulting in gradual hypertrophy of the pituitary and adrenal glands (Mastorakos & Ilias, 2003). Levels of salivary cortisol (reflecting the unbound fraction of the hormone, Obel et al., 2005) begin to rise steadily from the 25th week of gestation and reach around three-fold the non-pregnant levels in the third trimester (Jung et al., 2011; Allolio et al., 1990). As shown in Figure 1.4, the substantial changes in the HPA axis during pregnancy are mainly driven by the growth and development of a new organ, the placenta. During the second and third trimester of pregnancy, large quantities of CRH are also produced by the placenta (Petraglia et al., 1993), with circulating levels of CRH, as detected in plasma, reaching up to 1000-fold increase by the end of gestation (Lindsay & Nieman, 2005). Plasma CRH is considered a marker of the 'placental clock' which sets the timing of parturition (McLean & Smith, 2001). Notably, placental CRH (pCRH) and hypothalamic CRH (hCRH) are structurally and biochemically similar (Petraglia et al., 1996) and pCRH stimulates the secretion of ACTH from maternal anterior pituitary and, in consequence, production of cortisol from the adrenal glands. However, differently from the negative feedback effect at the hypothalamic level, maternal cortisol exerts a positive effect on pCRH synthesis creating a positive "HPA-axis-placental feed-forward drive", leading to dramatic elevations in ACTH, pCRH and cortisol across gestation (Mastorakos & Ilias, 2003; O'Keane et al., 2011).

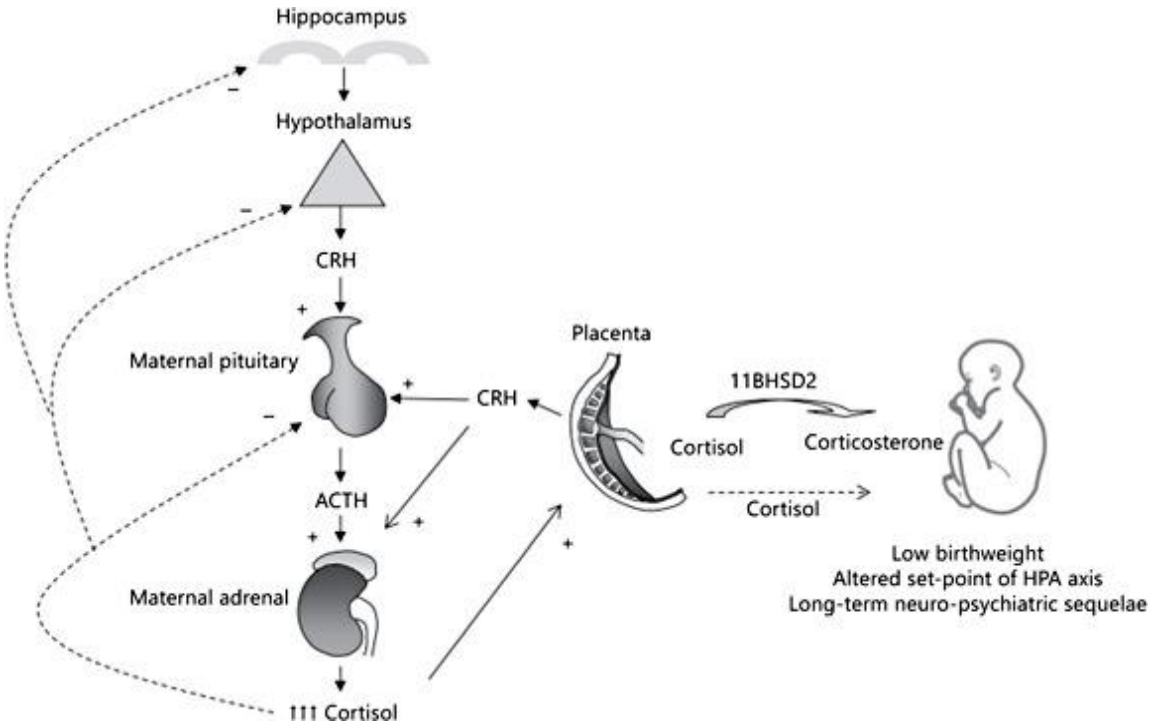

Figure 1.4 - Changes in the maternal HPA axis during pregnancy and possible effects of fetal cortisol overexposure. Placental CRH (pCRH) stimulates both the maternal pituitary and adrenal, leading to increased cortisol production. Increased cortisol levels, in turn, further stimulate pCRH production. Placental enzyme 11β-HSD2 partially inhibit cortisol passage through the placental barrier (from Duthie & Reynolds, 2013)

Despite all these functional changes, the diurnal cortisol secretion pattern, including the CAR, is largely maintained across gestation, with a steep increase after awakening, peaking approximately 30 minutes after awakening, and gradually decreasing throughout the day (Allolio et al., 1990; De Weerth & Buitelaar, 2005; Kivlighan et al., 2008; Hellgren et al., 2013).However, as pregnancy progresses, increased circulating levels of cortisol downregulate the secretion of hCRH, thus leading to a dampened responsiveness of the HPA axis to both psychological and physical stressors (Kammerer et al., 2002; De Weerth & Buitelaar, 2005). This physiological change is considered to be adaptive in that it prevents potential detrimental consequences of foetal exposure to heightened levels of maternal glucocorticoids and promotes maternal anabolic adaptations that are necessary for a successful pregnancy (Brunton, 2010).

The process of parturition is associated with a substantial increase in maternal cortisol. Soon after delivery, the sharp drop in pCRH levels following the expulsion of the placenta exposes the maternal HPA axis to severe perturbations. While plasma CRH, cortisol and ACTH levels decline after birth, returning to pre-pregnancy values within 1-4 days postpartum (Allollio et al., 1990), the HPA axis stress responsiveness is still relatively suppressed during the early postpartum period and it takes approximately 12 weeks for the HPA axis to return to the pre-pregnancy level of function (Magiakou et al., 1996).

Recently, it has been suggested that some of the pregnancy-related adaptations in the HPA axis depend on fetal sex. Higher cortisol levels in women carrying female foetuses in the third trimester have been reported (DiPietro et al., 2011; Bleker et al., 2017), while the opposite pattern was found in the second trimester (DiPietro et al., 2011). In addition, Giesbrecht and colleagues (2015) reported a flatter cortisol diurnal patter in women carrying a female fetus, as compared to a male fetus. Despite being preliminary, these findings highlight the complex bi-directional communication between mother and fetus, suggesting a reciprocal regulation of stress physiology, which deserves further investigation. In addition, cortisol during pregnancy can be affected by several biological and lifestyle factors so that higher cortisol levels have been associated with being primiparous, (e.g. Bleker et al., 2016), non-smoking (Dušková et al., 2014), lower maternal age (Bleker et al., 2016), lower pre-pregnancy maternal body mass index (BMI) (e.g. Stirrat et al., 2016), higher C-Reactive Protein (CRP) levels (Bleker et al., 2016) and self-reported sleep (e.g. Crowley et al., 2016), thus highlighting the multi-faceted nature of cortisol regulation.

Lastly, findings of an association between cortisol levels and several measures of psychosocial stress during pregnancy are inconsistent. While some studies found modest associations (e.g. Evans, Myers, & Monk, 2008; Giesbrecht et al., 2012; Simon et al., 2016; Heuvel et al., 2018; Kivlighan et al., 2008; O'Connor et al., 2014; Obel et al., 2005), many others reported non-significant findings (e.g. Harville et al.,

2009; Baibazarova et al., 2013; Salacz et al., 2012; Shea et al., 2007; Voegtline et al., 2013; Bleker et al., 2017; Himes & Simhan, 2011), thus leading to hypothesize that biological and environmental factors during pregnancy might be more closely related to maternal cortisol secretion, as compared to psychological factors (Bleker et al., 2017), and suggesting a potential discrepancy between self-reported and endocrine stress measures.

These findings call into question the traditional view of cortisol as the primary candidate mediating the effects of antenatal stress on foetal development, which are based on observations of subjective stress. However, the discrepancies in the literature might be related to several methodological variations in cortisol assessments (e.g., single assessments, CAR, morning and evening cortisol, as well as home versus laboratory/clinic collection), assay methods (e.g., saliva, plasma, urine) and gestational timing. Concerning this latter point, it is noteworthy that most studies are limited to the third trimester of gestation (e.g. Davis et al., 2007; Hellgren et al., 2013; Kivlighan et al., 2008; Simon et al., 2016). As cortisol significantly increase over the course of pregnancy, it is unclear whether the potential association between maternal stress and cortisol levels might still be detected with advancing gestation. For example, Obel et al. (2005) found paradoxical associations between psychological distress and cortisol levels in early pregnancy as compared to late pregnancy, thus suggesting that the stage of pregnancy can play a role in the link.

Lastly, the available literature suffers from considerable heterogeneity in the assessment of maternal “stress” experience. While assessments are mainly based on self-report questionnaires, a wide range of constructs have been examined in different studies including perceived stress, state-trait anxiety, pregnancy-specific anxiety and hassles, depressive symptoms, coping style, life events and social support. In the following section, the available evidence for a specific association between maternal depressive symptoms and HPA axis functioning during the perinatal period is reviewed, which leads to the suggestion that indices of cortisol diurnal secretion, rather

than absolute values, might provide a more reliable measure during pregnancy. Next, we will examine potential mechanisms involving prenatal programming by the HPA axis.

1.4.1.3 The HPA axis and perinatal depressive symptoms

A dysregulation of the HPA axis in major depressive disorders (MDD) is a well-established finding in psychiatry (Ising et al., 2007; Holsboer, 2000). According to meta-analytical evidence, both morning and evening salivary cortisol levels are increased in patients with MDD as compared to controls (Knorr et al., 2010). In addition, a recent meta-analysis showed blunted cortisol stress responsiveness in women with MDD, while an increased cortisol reactivity in depressed men (Zorn et al., 2017). A flatter diurnal cortisol rhythm has also been reported in women with MDD as well reduced suppression of the cortisol response by dexamethasone (Jarcho et al., 2013), indicating reduced glucocorticoid receptor sensitivity in the HPA axis negative feedback, commonly referred to as “glucocorticoid resistance”. These findings suggest a dysregulation of the HPA axis at multiple levels. In addition, it has been proposed that distinct clinical depressive syndromes, namely melancholic and atypical depression, might relate to different profiles of HPA axis functioning (Gold & Chrousos, 2002). Specifically, atypical depression, characterized by mood reactivity, weight gain, hypersomnia etc., have been linked to HPA axis hypo-responsiveness (Levitan et al., 2002), with lower cortisol levels at awakening and flattened diurnal cortisol patterns (Lamers et al., 2013). In contrast, melancholic depression, characterized by depressed mood, loss of pleasure, insomnia, reduced appetite and/or weight loss, has been consistently associated with HPA axis hyperactivation (Lamers et al., 2013).

Given that dysfunction of the HPA axis play a central role in the pathophysiology of MDD, it has been proposed that the profound alterations of the HPA axis during pregnancy and in the early postpartum might contribute in influencing the risk for mood disorders over the perinatal period (e.g. Glynn, Davis, & Sandman,

2013; Kammerer, Taylor, & Glover, 2006; Mastorakos & Illias, 2000). For example, the abrupt cortisol withdrawal occurring soon after delivery may trigger postnatal depressive symptoms (Bloch, Daly, & Rubinow, 2003). However, this hypothesis has not yet received strong empirical support.

Few studies have examined the association between cortisol levels and depression during the first trimester of pregnancy and those generally report no association (Glynn & Sandman, 2014; Goedhart et al., 2010; Luiza, Gallaher, & Powers, 2015; Pluess et al., 2010; Heuvel et al., 2018). Evidence for an association between cortisol levels and depressive symptoms across the second trimester is mixed. Higher urinary cortisol levels have been reported in women with elevated depressive symptoms (Diego et al., 2009). In addition, O' Keane and colleagues (2011) reported higher evening, but not morning, cortisol concentrations in women with depression, while O' Connor and colleagues (O'Connor et al., 2014) reported lower cortisol levels at awakening and a flatter diurnal pattern (but no differences in CAR), resulting in overall higher cortisol levels across the day, in clinically depressed women, with a stronger effect for diagnostic data than self-reported symptoms. Furthermore, a number of studies failed to detect any significant association between second-trimester cortisol and depression (Braithwaite, Murphy, & Ramchandani, 2016; Field et al., 2009; Davis et al., 2007; Glynn and Sandman, 2014; Shea et al., 2007, Heuvel et al., 2018). A greater number of studies explored the association between third-trimester cortisol and depressive symptomatology. Higher urinary and serum cortisol levels (Diego et al., 2009; Lommatzsch et al., 2006), as well as higher morning and afternoon salivary cortisol concentrations (Bjelanovic et al., 2015) have been reported in women with antenatal depression in this stage of pregnancy. O'Connor and colleagues (2014) reported a pattern of association (i.e. lower morning levels and flatter diurnal decline in depressed women) similar to the one detected in the second trimester. Lastly, two studies found significantly higher morning cortisol levels in women with a comorbid diagnosis of depression and anxiety, as compared to controls,

but not in women with only one diagnosis (Monk et al., 2011; Evans et al., 2008) and a considerable number of reports failed to detect any significant association (Braithwaite et al. 2016; Cheng & Pickler, 2010; Davis et al. 2011; Deligiannidis et al., 2016; Field et al. 2009; Glynn and Sandman 2014; Hellgren et al. 2013; Iliadis et al., 2015; Pedersen et al., 1993; Peer et al., 2013; Pluess et al. 2010; Salacz et al. 2012, Heuvel et al., 2018). Substantial methodological variations limit possible comparisons across studies. However, a recent systematic review selected “higher quality” studies based on the “Systematic Assessment of Quality in Observational Research” (Ross et al., 2011) and concluded that no significant evidence exists for an association between antenatal maternal cortisol levels and depression (Seth, Lewis, & Galbally, 2016). These findings are striking as compared to well-established meta-analytical evidence regarding the association between MDD and HPA axis dysregulation in non-pregnant populations (e.g. Knorr et al., 2010, Helhammer et al., 2009) and might suggest that the dramatic HPA-axis resetting occurring during pregnancy might overcome our ability to detect significant hormone-behaviour associations.

Likewise, evidence for an association between maternal depressive symptoms and cortisol levels in the early postpartum period is inconsistent (Seth et al., 2016). Early studies reported higher cortisol levels in women displaying higher depressive symptoms in the first week postpartum (Handley, 1980; Ehlert et al., 1990; Okano & Nomura, 1992; Taylor, Littlewood, & Adams, 1994), although Harris and colleagues (1996) reported lower cortisol concentrations in depressed women following delivery. More recent studies, reported lower cortisol levels in women at risk for depression respectively at 4-6 weeks postpartum (Groer & Morgan, 2007) and within 12 months postpartum (Parry et al., 2003). In addition, some studies provided evidence for an altered cortisol diurnal pattern in postnatally depressed women. Specifically, Taylor and colleagues (2009) showed a dampened CAR in depressed women 4-6 weeks postpartum and De Rezende and colleagues (2016) reported a suppression of the CAR jointly with higher cortisol concentrations 12 hours after waking, suggesting a

reduction in cortisol diurnal variation, in the group of depressed women compared to controls 6 months postpartum. Lastly, Corwin and colleagues (2015) reported higher diurnal cortisol, as indexed by Area Under the Curve with respect to the ground (AUCg, Pruessner et al., 2003), in women with depressive symptoms in the early postpartum (14 days after delivery), but not in the following 6 months or in the first week after delivery. In contrast, numerous studies failed to detect any significant association between cortisol levels and postnatal depression (e.g. Groer, Jevitt, & Ji, 2015; Shimizu et al., 2015; Diego et al., 2009; Harris et al., 1994; Figueiredo & Costa, 2009; Tsubouchi et al., 2011; Feksi et al., 1984; Abou-Saleh et al., 1998; Susman et al., 1999; Davis et al., 2007; O'Keane et al., 2011; Cheng & Pickler, 2010; Kuevi et al., 1983, Brimsmead et al., 1985).

There are several possible methodological reasons for inconsistencies in the direction and effect of the association between depression and cortisol during the perinatal period. First of all, there is the issue of the comparatively low statistical power of the studies, due to limited samples size and the inclusion of small number of depressed participants. Secondly, many studies have used relatively poor quality cortisol measurements, and in particular, limited sampling occasions which do not allow the capture of relevant information about the cortisol diurnal pattern. In addition, studies vary widely in the substrates and methods for cortisol assay which limits possible comparisons across studies. Third, extant studies inconsistently include control variables known to affect cortisol, such as sleep, food intake, physical activity, medication (Hellhammer, Wüst, & Kudielka, 2009), together with pregnancy-related factors (i.e., gestational age, parity, pre-pregnancy BMI or foetal sex, e.g. DiPietro et al., 2011; Kivighlan et al., 2008), which may further confound results. Indeed, recent results from Bleker and colleagues (2017) on a very large sample of pregnant women (N=3039) suggested that maternal psychosocial factors were not associated with maternal cortisol levels during pregnancy, once the effects of biological and lifestyle factors were taken into account. Fourth, differences in the assessment of maternal

depression (i.e. self-reported measures or diagnostic interview) might influence the results.

However, it is also possible that due to the substantial physiological alterations occurring during pregnancy, involving in particular the HPA axis, but not limited to it, well-established research findings indicating elevated cortisol as a biomarker of depression (e.g. Knorr et al., 2010), may not extend to the perinatal period. For example, it may be argued that observed cortisol values during pregnancy primarily reflect maternal and fetal development (Davis et al., 2011). In addition, as maternal HPA axis stress reactivity is gradually attenuated as pregnancy progresses (Kammerer et al., 2002), it has been suggested that the link between cortisol and measures of depression might become weaker as pregnancy advances (O'Connor et al., 2014). However, as previously described, generally no significant associations have been reported in the first trimester of pregnancy, thus, the proposed mechanism of shift toward a weaker association between distress and cortisol in late gestation seems to be insufficient to fully explain the picture.

As most studies have been conducted on low risk samples, it might be hypothesized that "mild" conditions of distress do not significantly affect the regulation of the HPA axis, while above a threshold of significant psychological distress, biological and psychological stress measures might become coupled, so that a significant change in one may be related to changes in the other. For example, salivary cortisol levels at 36 weeks gestation were not found to be elevated in a sample of clinically depressed or anxious women compared to controls, while women with a comorbid diagnosis of depression and anxiety displayed higher levels than controls or either group with a single diagnosis (Evans et al. 2008). Nevertheless, non-significant associations between antenatal cortisol and depression have been reported also in several clinical samples (e.g. Shea et al., 2007; Hellgren et al., 2013; Hellgren et al., 2016; Rouse & Goodman, 2014; Katz et al., 2012).

Lastly, it might be simply that cortisol is not the most accurate marker of subjective stress experiences, particularly during pregnancy. Indeed, it is affected by many other factors other than stress (Kudielka, Hellhammer, & Wüst, 2009) and a poor or null covariance of perceived stress and cortisol has often been reported even outside the perinatal period (Helhammer et al., 2009), possibly because of the complex interplay of neurobiological events that link psychological stress to HPA axis activation, but also because of methodological difficulties in the assessment of perceived stress through self-report questionnaires (Helhammer et al., 2009, Harville et al., 2009).

These findings are striking bearing in mind that cortisol has been traditionally considered the primary mediator of the effects of antenatal stress on foetal development. Nonetheless, it might be hypothesized that the programming influences of prenatal stress, such as maternal depression, on offspring development outcomes is directly mediated by cortisol levels only in cases of very severe stress or that alternative mechanisms involving for example placental permeability to cortisol might be involved in fetal programming (e.g. O'Donnell 2011; Glover, O'Connor, & O'Donnell, 2010). In what follows, the possible mechanisms of fetal programming by maternal cortisol are discussed and the available evidence for the effects of maternal cortisol during pregnancy on child outcomes is reviewed.

Lastly, it is possible that maternal depression might be related to a dysregulation of other biological systems, such as the SNS or immune system, independent of, though related to, the HPA axis, and that these alterations might result in fetal programming effects. These latter possibilities will be examined in section 1.4.2 and 1.4.3.

1.4.1.4 Prenatal programming by HPA axis

Glucocorticoids, such as cortisol, play an essential role in supporting fetal growth, organ maturation and brain development during pregnancy (Lupien et al.,

2009; Waffarn & Davis, 2012). However, it has been shown that prenatal exposure to excessive glucocorticoids levels, both synthetic and endogenous, during sensitive developmental periods can have detrimental effects on fetal growth and brain development, especially in regions rich in cortisol receptors (e.g. Coe & Lubach, 2005; Khalife et al., 2013; Davis et al., 2009; Davis et al.,, 2013; Giesbrecht et al., 2016; Lewinn et al., 2009). In particular, glucocorticoid overexposure can interfere with synaptogenesis processes and neurotransmitter function in the developing fetal brain, as well as adversely affect glucocorticoid receptor expression and the development of the stress response systems (Seckl & Meaney, 2004; McGowan & Matthews, 2018). A study by Salaria and colleagues (2006) employing a microarray analyses, showed that chronic cortisol exposure is able to regulate a huge number of genes involved in cell growth and intracellular signaling within fetal brain tissue, highlighting the widespread effect of cortisol exposure on the fetal brain.

A body of studies has investigated the effects of prenatal exposure to synthetic glucocorticoids, which are often administered to accelerate fetal lung development in woman at risk for premature delivery and, differently from endogenous glucocorticoids are not metabolized by the placenta. These studies document a range of associated adverse child outcomes, including lower birth weight and smaller head circumference at birth (Davis et al., 2009; Piazze et al., 2005), altered stress reactivity (Davis et al., 2004; 2006; 2011), inattention and poor cognitive performance (Khalife et al., 2013). Thus, these data provide further evidence for the role of early exposure to glucocorticoids in shaping brain development.

Under normal conditions, the fetus is largely protected from exposure to elevated maternal cortisol levels through the activity of the placental enzyme 11b-hydroxysteroid-dehydrogenase type 2 (11β-HSD2), which converts cortisol to the inactive cortisone (Figure 1.4, Benediktsson et al., 1997). However, it has been shown that a proportion of maternal cortisol is still able to cross the placenta (Gitau et al., 1998; 2001), reaching fetal blood at approximately 10-20% of maternal levels (Murphy

& Clifton, 2003) and directly influencing fetal brain development (Seckl & Meaney 2004). Additionally, gene expression of the placental enzyme 11βHSD2 has been shown to increase over pregnancy, in parallel with increased cortisol levels, to protect the foetus from excessive cortisol exposure (Schoof et al., 2001; Seth et al., 2015).

At least four mechanisms through which maternal cortisol levels could influence fetal development have been proposed and will be described separately, although it is likely that a combination of these mechanisms might underline the effect of prenatal stress on fetal development. First, the traditional model of fetal programming by exposure to excessive maternal glucocorticoids posits that mood-related increases in maternal cortisol levels might lead to cortisol crossing the placental barrier and directly affecting fetal development (Seckl & Holmes, 2007). However, while this model has proved to be generally consistent in animal models (Seckl & Meaney, 2004), evidence both for an association between maternal antenatal cortisol levels and offspring outcomes (Zijlmans et al., 2015) and for an association between maternal antenatal depression and cortisol levels (Seth et al., 2016) in humans is weak. Secondly, individual variability in cortisol placental permeability has been shown. In particular, in animals it has been reported that maternal diet (Langley-Evans et al., 1996), and stress (Welberg, 2005; Mairesse et al., 2007; Lucassen et al., 2009) might affect the placental barrier. In humans, preliminary evidence indicates that maternal medical conditions, such as pre-eclampsia (McCalla et al., 1998) or asthma (Murphy et al., 2003), infection (Johnstone et al., 2005), inflammation (Kossintseva et al., 2006), norepinephrine levels (Sarkar et al., 2001), licorice consumption (Räikkönen et al., 2010), maternal anxiety (O'Donnell et al., 2012) and depression (Seth et al., 2015), might be related to a reduced expression and activity of the placental 11β-HSD2, allowing more cortisol to transfer through the placenta). This latter mechanism would explain how overexposure to glucocorticoids might occur even in absence of distress-linked higher than typical maternal cortisol levels. Third, maternal cortisol might affect fetal development by placental secretion of CRH (Petraglia et al., 1987).

PCRH enters the fetal circulation through the umbilical vein and stimulates the fetal HPA axis to produce ACTH and cortisol, which, in turn, further stimulates pCRH secretion in a fetal positive feedback loop (Majzoub & Karalis, 1999). At the same time, maternal cortisol stimulates the secretion of pCRH (Cheng et al., 2000), resulting in increasing concentrations of maternal cortisol and pCRH within the maternal positive feedback loop. Lastly, elevated cortisol may affect utero-placental blood flow with indirect negative effects on offspring growth and brain development (Mina et al., 2015).

1.4.1.5 Maternal cortisol and child outcomes

Despite the relative lack of association between maternal psychological stress and cortisol levels in pregnancy, several studies have investigated the relationship between maternal HPA axis functioning during pregnancy and offspring's outcomes and these have yielded mixed findings (reviewed in Zijlmans, Riksen-Walraven, & de Weerth, 2015). Most of these studies collected maternal salivary samples to assess free cortisol levels during pregnancy and the most robust protocol included multiple samples collected over two days in order to calculate the average total cortisol output across the day as well as the diurnal rhythm of cortisol secretion (Harville et al., 2009). A small number of studies measured amniotic fluid cortisol, which is thought to represent a marker of fetal cortisol exposure (Sarkar et al., 2007), and only a handful of reports assessed maternal urine or serum cortisol levels.

Generally, higher maternal cortisol levels, in particular a higher CAR (Bolten et al., 2011), higher morning cortisol values (Goedhart et al., 2010) and a flatter diurnal cortisol slope (D'Anna-Hernandez et al., 2012) have been related to lower birth weight. Significant associations between maternal cortisol and body length at birth were found only in late pregnancy (Bolten et al., 2011), while no associations were found with head circumference at birth (Bolten et al., 2011), as well as BMI at 5 years of age (Van Dijk et al., 2012). In contrast, a recent study reported maternal cortisol in late gestation

to predict infant body fat increase over the first 6 months of life (Entringer et al., 2017), suggesting a potential role of antenatal cortisol in programming later obesity risk.

Findings of an association between prenatal maternal cortisol and gestational age at birth are mixed, as either negative (e.g. Diego et al., 2009; Erickson et al., 2001; Mercer et al., 2010) or no associations (D'Anna-Hernandez et al., 2012; Ruiz et al., 2001) have been reported. Baibarazova and colleagues (2013) reported higher amniotic fluid cortisol, but not maternal cortisol, to be associated with shorter gestational age and lower birth weight.

Evidence for a link between maternal cortisol concentrations during pregnancy and infants' mental and motor development are inconclusive. Higher maternal cortisol in late, but not early, pregnancy predicted lower infant mental development in four studies at 3 months of age (Buitelaar et al., 2003; Huizink et al., 2003), 17 months (Bergman, Sarkar, et al., 2010) and 7 year olds respectively (LeWinn et al., 2009), but not in two studies at 8 months (Buitelaar et al., 2003; Huizink et al., 2003). Similarly, higher maternal basal cortisol and reduced cortisol response during mid- and late pregnancy predicted learning and memory performance in 5-month-old infants but not at 3 months of age (Thompson et al., 2017). In contrast, Davis and Sandman (2010) reported higher levels of cortisol, both in early and late pregnancy, to predict accelerated mental development during the first year of life, resulting in better cognitive outcomes at 12 months of age. Furthermore, in a recent study, the authors showed that higher maternal cortisol during the third trimester predicted better child cognitive functioning between 6 and 9 years of age (Davis et al., 2017). In addition, maternal cortisol in late pregnancy, but not in early gestation, was negatively related to infants' motor development in a few studies (Buitelaar et al., 2003; Huizink et al., 2003), though not all (Davis and Sandman, 2010).

Similarly, little evidence exists for a link between maternal cortisol in early and mid-gestation and children's temperament (Davis et al., 2007; Gutteling et al., 2005; Buitelaar et al., 2003), while mixed findings have been reported concerning an

association between maternal cortisol measured during late gestation and offspring's temperament. One study reported a link between higher maternal cortisol in late gestation and greater child negative reactivity at 8 weeks of age (Davis et al., 2007) and another report showed high maternal cortisol levels at waking to relate to infants' higher 'emotion' and 'activity' scores at week 7, but not in week 18 (De Weerth, Van Hees, & Buitelaar, 2003). However, a number of studies failed to detect any significant associations (Buitelaar et al., 2003; Gutteling et al., 2005; Rothenberg et al., 2011; Braithwaite et al., 2017).

A few papers examined the association between prenatal cortisol and children's behavioral problems. Higher cortisol levels during early and mid-pregnancy were related to higher levels of child anxiety, according to maternal reports in one study (Davis & Sandman, 2012), and more affective problems in 7-year-old girls but not boys in another (Buss et al., 2012). In contrast, no significant association was found between maternal prenatal cortisol in late gestation and children behavioral problems in two studies (Gutteling et al., 2005; Buss et al., 2012).

Some evidence exists for an association between antenatal maternal cortisol levels and children's stress reactivity. Higher levels of maternal cortisol during pregnancy have been related to children's greater cortisol response to the heel stick at birth (Davis et al., 2011), to the inoculation at four years of age (Gutteling, De Weerth, & Buitelaar, 2004) and to the first day of school at five years of age (Gutteling et al., 2005). In contrast, a few studies failed to detect any significant associations between prenatal maternal cortisol and infant cortisol responses during the first year of life (Tollenaar et al., 2011; Braithwaite et al., 2017). A recent study by Giesbrecht and colleagues (2017) revealed sex differences in the interactive effects of maternal prenatal cortisol and distress on 3-month-olds cortisol reactivity to a blood draw. Specifically, a dampened infant cortisol reactivity was associated with exposure to high maternal distress and flattened patterns of diurnal maternal cortisol in females, and with prenatal exposure to a steeper maternal CAR and daytime slope in males. In

addition, higher maternal cortisol concentrations were found to predict infant's slower behavioral recovery after the heel-stick soon after birth (Davis et al., 2011) and more negative behavioral response at bathing sessions during the first 20 weeks of life (De Weerth et al., 2003).

Lastly, preliminary evidence exists for a role of antenatal cortisol levels in influencing fetal brain development. Specifically, maternal prenatal cortisol was significantly negatively associated with ultrasound parameters of fetal brain growth (Li et al., 2012). Buss and colleagues (2012) reported that higher maternal cortisol levels in early, but not late, gestation, predicted larger right amygdala volume in 7-year-old girls, but not in boys, and this, in turn was associated with more affective problems. Lastly, Davis and colleagues (2017) showed that higher maternal cortisol concentrations in late pregnancy were associated with greater child cortical thickness in frontal regions at 6-9 years of age, suggesting that elevations in maternal cortisol in a normative sample might promote fetal brain development.

To summarize, despite maternal cortisol has been the most studied mediator of the effects of maternal antenatal distress on infant outcomes, empirical evidence of a link between maternal cortisol concentrations and a wide range of child outcomes is not straightforward and, according to a recent review, approximately 76% of studies failed to detect a significant association (Zijlmans et al., 2015). Thus, it is increasingly clear that cortisol alone cannot fully explain all the programming effects of maternal antenatal stress, especially during early gestation, and that alternative mechanisms, involving, for example, other stress-related alterations or inflammatory processes might play a role.

1.4.2 The Sympathetic-Nervous-System (SNS)

The Autonomic Nervous System (ANS) is a very complex, multifaceted neural network that is involved in regulating a wide range of physiological processes, including the response to stress. In particular, the Sympathetic Nervous System

(SNS), one branch of the ANS, and the adrenal medulla together constitute the sympathetic adrenomedullary (SAM) system which, besides the HPA-axis, is the second key component of the Stress Response System[1]. The SAM is activated within seconds by stress and triggers a rapid physiological adaptation in order to prepare the organism to face the initial phase of a stressful event (De Kloet et al., 2005). As compared to the hormonal HPA axis response which takes approximately ten minutes to release cortisol and reach its peak concentration in serum, the SAM response represents the first response to stress which is rapid but short-lasting.

As shown in Figure 1.5, the chromaffin cells of the adrenal medulla are innervated by sympathetic preganglionic neurons located in the intermediolateral gray matter of the spinal cord. Activation of the SAM triggers the release of chatecholamines, and in particular epinephrine (E, 80%) and norepinephrine (NE, 20%) from the chromaffin cells of the adrenal medulla into circulation and NE from sympathetic neurons that innervate almost all organs, which mediate a number of behavioral and physiological changes that are part of the short-term “fight or flight” response (Wetherell et al., 2006). Specifically, chatecholamines bind to β-adrenergic receptors on a vast array of target tissues and induces changes in blood vessels, glands, visceral organs and smooth muscles, in order to promote blood supply to the brain and muscles (Tank & Lee Wong, 2015). For example, catecholamines increase heart rate and stroke volume, cause vasodilatation in muscles and constriction of blood vessels in the skin and gut. Although neither E nor NE cross the blood-brain barrier, their peripheral actions are paralleled in the brain by NE produced by the locus coeruleus (Morilak et al., 2005) which serve the function of maintaining alertness and participate in processes that activate the HPA axis.

[1] Although it is acknowledged that the parasympathethic branch of the ANS is involved in stress regulation, a fuller discussion of autonomic nervous system response is beyond the scope of this thesis.

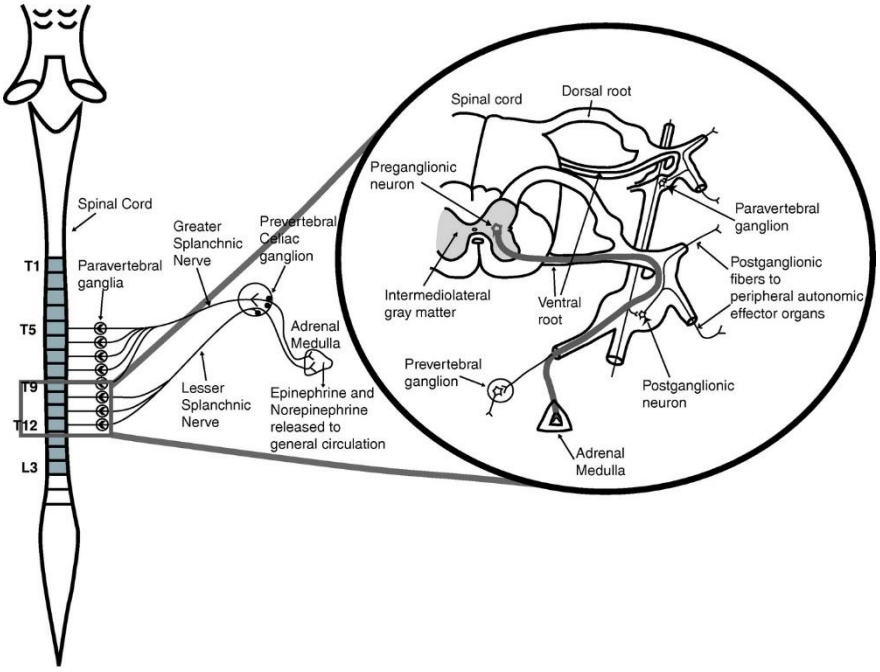

Figure 1.5 - Representation of the anatomy of the SAM system, a component of the SNS. Its cell bodies (preganglionic neurons) are located in the interomediolateral (IML) cell column and exit the spinal cord via the ventral root to form cholinergic direct synapses on the chromaffin cells of the medulla of the adrenal glands. When stimulated, the chromaffin cells (equivalent to postgangiolinc sympathetic neurons) release catecholamines (epinephrine and norepinephrine) in the general circulation that act like hormones affecting organs and tissues via adrenergic receptors and enhance SNS activity (from Gunnar & Quevedo, 2002)

1.4.2.1 Salivary Alpha Amylase (sAA)

The assessment of chatecholamine levels in humans is not straightforward. Repeated blood draws from venous blood are needed to obtain a reliable assessment of chatecholamine levels and SNS activity (Goldstein, Eisenhofer, & Kopin, 2003) This is often perceived as stressful and might be biased by local sympathethic activity (Veith, Best & Halter, 1984). In addition, salivary catecholamine concentrations are consistently lower than blood levels, thus being inadequate to reflect the acute changes in SNS activity (Kennedy et al., 2001).

In the last decade, increasing attention has been directed to salivary enzyme alpha-amylase (sAA) which is thought to be a non-invasive stress marker of SNS activation (reviewed in Nater & Rohleder, 2009). sAA is an enzyme mainly involved in the digestion of starch in the oral cavity and also has bacterial interactive functions

(Scannapieco, Torres, & Levine, 1993). Since sAA secretion is regulated by the ANS, and particularly the SNS which controls salivary glands via beta-adrenergic receptors (Baum, 1987), sAA levels have been proposed to reflect stress-related changes in SAM activity (Chatterton et al., 1996; van Stegeren et al., 2006) and in a series of studies have been shown to be highly sensitive to stress (reviewed in Nater & Roheleder, 2009).

Early human studies showed that immersing participants in cold water (Speirs et al., 1974) or intense physical exercise (Gilman et al., 1979) activates an SNS response and associated sAA increase. In addition, beta-adrenergic blockers were found to decrease sAA concentrations (e.g. Nederfors & Dahlöf, 1996). More recently, several studies have shown increased levels of sAA in response to physical and psychological stress conditions (e.g. academic examinations, the trier social stress test, the cold pressor task; reviewed in Nater and Rohleder, 2009). However, mixed findings have been reported concerning an association between sAA concentrations and plasma catecholamines (e.g. Ehlert et al., 2006; Rohleder et al., 2004; Thoma et al., 2012). In particular, while in all these studies a stress-induced increase in both sAA and catecholamines levels paralleling each other was found, significant correlations between individual time points of sAA and catecholamines or averaged total measures (such as the AUC) have not always been found (Rohlder et al., 2004; Wetherell et al., 2006). Thus, it has been questioned whether a strong association between plasma catecholamines and sAA in response to stress exists. Nevertheless, sAA levels in response to stress were found to be associated with some indices of SNS activity such as left ventricular ejection time (Bosch et al., 2003), ratio between low frequency power and high frequency power of heart rate variability, thought to reflect sympathetic tone, (Nater et al., 2006) and skin conductance (El-Sheikh et al., 2008). In addition, a recent study using multilevel models showed strong increases in sAA in response to stress that were mediated via NE and E (Ditzen, Ehlert, & Nater, 2014).

SAA levels in response to acute stress peak between 5 and 10 min after the onset of a stressor and return to baseline approximately 10 minutes after the stressor, consistent with it being a marker of the rapid SNS response (Granger et al., 2007; Nater et al., 2006). In addition, sAA stress responses have been reported to be either largely uncorrelated (Granger et al., 2007) or associated at different time lags with cortisol responses (Engert et al., 2011). This has led to regard sAA as a non-redundant stress marker that allows to complement a more comprehensive assessment of the stress response.

As shown in Figure 1.3A, sAA is characterized by a marked diurnal profile. In particular, sAA levels show a significant decrease in the first 30 minutes after awakening and a steady increase towards the afternoon and evening (Marchand et al., 2016; Nater et al., 2007). While sAA daily pattern is independent of the cortisol diurnal rhythm (Nater et al., 2007), the opposite diurnal pattern between the two markers has been suggest reflecting *"a circadian compensatory drive related to allostatic mechanisms"* (Marchand et al., 2016). The sAA diurnal pattern has been shown to be influenced by age (Nater et al., 2007) and sex (Marchand et al., 2016), but not by factors such as physical activity, smoking, eating or drinking and BMI (Nater et al., 2007). Thus it is considered a "relatively robust" stress marker (Wingenfeld et al., 2010). In addition, preliminary evidence suggests that psychosocial factors might influence sAA levels. For example, strong correlations between sAA concentrations and state anxiety measures during stress task have been reported (Noto et al., 2005; Takai et al., 2004).

1.4.2.2 The SNS system during the perinatal period

As previously mentioned, pregnancy is accompanied by substantial changes in maternal stress response systems that serve the function of sustaining pregnancy and supporting both maternal and fetal energetic requests. In particular, during this phase the ANS shows a shift toward greater sympathetic and reduced parasympathetic

modulation (DiPietro, Costigan, & Gurewitsch, 2005), paralleled by significant increases in blood volume and heart rate and a reduction in vascular resistance in order to support the increased cardiac output (Silversides & Colman, 2007). Changes in ANS parameters have been shown to begin early in gestation and increase linearly as pregnancy progresses (Di Pietro et al., 2005). In addition, there is a progressive decrease in blood pressure which reaches the lowest values at mid-gestation, followed by an increase to pre-pregnancy levels near delivery. These substantial changes are crucial to support fetal development and a lack of such adaptation has been associated with adverse outcomes. For example, higher blood pressure in early pregnancy predicts greater risk of preeclampsia later in pregnancy, as well as lower birth weight (reviewed in Christian et al., 2012).

Evidence concerning pregnancy-related changes in plasma NE and E are mixed. Either no changes (Barron et al., 1986; Lederman et al.,1989), as well as increasing levels (Elenkov et al., 2001) or decreasing levels (Nisell, Hjemdahl, & Linde, 1985) as gestation advances have been reported. Nonetheless, higher levels of plasma catecholamines have been consistently reported among pregnant women with preeclampsia or gestational hypertension (Kaaja et al., 1999; Manyonda et al., 1998). In addition, preliminary evidence suggests that diurnal variations in plasma E and NE are maintained in normal pregnancy (De Weerth & Buitelaar, 2005).

Similarly to the HPA axis, maternal SNS stress reactivity to psychological or physiological stressors is significantly dampened during pregnancy (e.g. de Weerth and Buitelaar, 2005; Entringer et al., 2010; Klinkenberg et al., 2009; Nierop et al., 2006).

To date, little is known about pregnancy-related changes in sAA levels. A number of early small studies suggested that salivary flow rate and sAA levels are not affected by advancing gestation (D'Alessandro et al., 1989; Salvolini et al., 1998). One study examined antenatal sAA response to stress and reported significantly lower sAA levels following a psychosocial stressor in pregnant women, as compared to non-

pregnant women (Nierop et al., 2006). In addition, Giesbrecht and colleagues (2013) reported that sAA diurnal rhythm is preserved during pregnancy and is not influenced by factors such as fetal sex, gestational age, maternal age and BMI. In contrast, previous history of miscarriage was related to an altered sAA pattern, as indicated by a flatter sAA diurnal profile. More recently, in a larger study, Giesbrecht and colleagues (2015) reported fetal sex differences in sAA diurnal pattern, with women carrying a female fetus showing elevated daytime sAA slopes as compared to women with a male fetus.

Despite sAA response appearing to be dampened during pregnancy, preliminary evidence indicates that sAA remains sensitive to the influences of psychosocial stress. In particular, Giesbrecht et al., (2013), reported that higher trait anxiety was associated with increased sAA levels over the day in mid-pregnancy, while chronic fatigue was related to decreased sAA levels. In addition, momentary depression and positive mood were associated with momentary increases in sAA concentrations, providing evidence for the responsivity of this biomarker to emotional arousal even during pregnancy.

1.4.2.3 SNS and perinatal depressive symptoms

An imbalance of the ANS is frequently observed in patients suffering from major depression and is thought to play a role in the pathophysiology of this condition (Moret & Briley, 2011; Schumann, Andrack, & Bär, 2017). Several methods of measuring ANS have been used and generally an increased sympathetic activation has been reported in depressed patients as compared to controls. In particular, increased heart rate (Agelink et al., 2002; Berger et al., 2012), skin conductance (e.g. Schuman et al., 2017), pupil diameters (e.g. Bär et al., 2004), as well as lower heart rate variability (HRV; Licht et al., 2008) has often been found among depressed individuals. In addition, higher levels of catecholamines have been reported in

depressed patients (e.g. Veith et al., 1994) and a number of antidepressants act by inhibiting noradrenaline reuptake (Moret and Briley, 2011).

The role of sAA as a biomarker of SNS activation in major depression has received limited attention. A number of studies reported higher sAA levels measured at different times during the day in adults with unremitted MDD, as compared to healthy controls (Booij et al., 2015; Cubała et al., 2014; Ishitobi et al., 2010; Tanaka et al., 2012). In addition, a large cohort study showed a trend toward an increase in evening sAA concentrations according to psychiatric conditions (Veen et al., 2013) with current MDD patients showing the highest sAA levels, followed by remitted MDD patients and, lastly, healthy adults. In contrast, no differences in sAA levels in response to stress have been reported among women with remitted MDD and never depressed controls in another study (Bagley, Weaver, & Buchanan, 2011).

Despite evidence of a dysregulation of the ANS among depressed non-pregnant individuals, only a limited number of studies have examined the association between depressive symptoms and ANS functioning during the perinatal period. Higher norepinephrine and lower dopamine levels in depressed pregnant women have been found in some studies (Lundy et al., 1999; Field et al., 2004; Diego et al., 2006), while others failed to find any association between catecholamines levels and depression (Field et al., 2006; Shimizu et al., 2015). In addition, increased noradrenergic activity has been reported in women with postpartum blues 6 weeks after delivery (Doornbos et al., 2008).

Moreover, a lack of dampening of ANS stress reactivity, evaluated through HRV, was shown in more anxious women (Braeken et al., 2015), although depressive symptoms were not assessed in this study.

To our knowledge, only two studies evaluated the association between prenatal sAA diurnal levels and depression. Specifically, Braithwaite and colleagues (2015) found higher sAA levels at wakening in women with depressive symptoms from a low risk sample compared with controls without depressive symptoms in late

pregnancy, while Giesbrecht and colleagues (2013) found no association between the average sAA trajectory throughout the day and depressive symptoms in a non-clinical sample. In contrast, chronic anxiety, momentary depressed and positive mood were all positively associated with sAA diurnal levels during pregnancy.

1.4.2.4 Prenatal programming by maternal SNS

Despite the fact that alterations of maternal SNS functioning and, more specifically, increased maternal chatecholamines levels, have been suggested as a possible mechanism underlying the impact of prenatal mood on foetal development (Braithwaite, Murphy, & Ramchandani, 2014; Rakers et al., 2017), to date it has received limited empirical attention. This is possibly related to the evidence that catecholamines are hydrophilic and do not directly cross the placental barrier in physiologically relevant concentrations (Giannakoulopoulos et al., 1999). While a number of early studies in animals and human placental tissues showed a minor placental passage of NE (around 5-10%) from mother to fetus (e.g. Morgan, Sandler, & Panigel, 1972), others do not (e.g. Jones & Robinson, 1975). In addition, it was shown that fetal and maternal NE levels in response to invasive procedures were not correlated, suggesting that no transfer from mother to fetus occurs (Giannakoulopoulos et al., 1999).

While uncertainty exists concerning a potential direct effect of maternal catecholamines levels on fetal development, stress-induced release of catecholamines might affect fetal development indirectly, by affecting placental metabolism (Merlot, Couret, & Otten, 2008). Indeed, it has been shown that increased maternal catecholamines could cause constriction of placental blood vessels and reduce placental blood supply, thus leading to restricted oxygen and nutrients to the foetus (Rakers et al., 2015) and, possibly, contributing to adverse birth outcomes, such as lower birth weight and premature delivery.

While the impact of maternal stress and catecholamine levels during pregnancy on uterine blood flow has been clearly demonstrated in animals (e.g. Rakers et al., 2015), to date, no study has examined the association between maternal antenatal catecholamine levels and fetal or placental hemodynamics in humans. In addition, evidence of an association between maternal psychological distress during pregnancy and altered uterine blood flow, assessed through Doppler ultrasound techniques, is inconclusive (reviewed in Rakers et al., 2017). For example, Helbig and colleagues (2013) found that maternal distress was associated with a decrease in umbilical blood flow in late pregnancy. In contrast, Monk and colleagues (2012) failed to detect a significant association between maternal depression or anxiety and uterine or umbilical blood flow during pregnancy. However, differently from animal studies, all these reports are based on at-rest assessment and none examined uterine blood flow in response to stressful situations. Additionally, foetal oxygen and nutrient restriction have been found to activate the foetal HPA axis (Edwards & McMillen, 2002) and fetal catecholamine release (Gu & Jones, 1986), thus constituting a possible pathway through which maternal distress might affect the development of fetal stress response systems. A preliminary study showed that maternal antenatal depression was associated with a decreased placental expression of monoamine oxidase A (MAO A) (Blakeley et al., 2013), an enzyme that metabolizes a range of monoamines and that is considered responsible of placental catecholamine clearance, thus suggesting an additional pathway for the effects of maternal antenatal mood on fetal development, possibly mediated by catecholamine-related mechanisms. Lastly, catecholamines have been found to down-regulate 11β-HSD2 gene expression in human placental trophoblast (Sarkar et al., 2001). Impairment of the placental glucocorticoid barrier via activation of the SNS could be another possible mechanism increasing fetal exposure to maternal glucocorticoids.

The above mentioned evidence converges to suggest that increases in maternal catecholamine levels, as a result of stress-induced activation of the SNS,

could be an alternative mechanisms by which antenatal depression might impact offspring's outcomes. However, SNS-mediated mechanisms of fetal programming have been very poorly explored in humans. In the following section, we will review existing evidence regarding the link between maternal SNS functioning and offspring outcomes.

1.4.2.5 Maternal SNS functioning and child outcomes

Very few studies have explored the link between variations in maternal SNS activity and changes in fetal physiology and behaviour in humans (Braeken et al., 2013; Monk et al., 2011). Specifically, Braeken and colleagues (2013) showed reduced maternal HRV during the first trimester of pregnancy in women with a history of anxiety disorder and in offspring of these women at 2-4 months of age, which, in turn, was related to greater fearfulness at 9-10 months. In addition, in the anxious group of women, maternal HRV and infant HRV were correlated, unlike the control group. However, it is not possible to establish whether this was a causal association or simply the effect of other underlying processes. In contrast, Monk and colleagues (2011) reported no association between changes in fetal heart rate and women's concurrent cardiorespiratory activity.

Furthermore, in animal studies, it has been consistently demonstrated that excessive levels of catecholamines can impair fetal development, leading to fetal growth retardation or fetal hypoxia (e.g. Bassett & Hanson, 1998; Macko et al., 2016). However, in humans, only one study evaluated the impact of maternal antenatal catecholamines levels on fetal/child outcomes (Holzman et al., 2009), showing that high levels of maternal urinary NE, E and dopamine during mid-gestation predicted a greater risk of preterm birth.

To our knowledge, only two studies explored the association between maternal prenatal sAA levels and pregnancy outcomes. An altered diurnal sAA pattern in women with history of miscarriage compared to women with no such history has been

reported (Giesbrecht et al., 2013). In contrast, Garcia-Blanco and colleagues (2017) reported that, differently from cortisol, maternal sAA levels at the timed of threatened preterm labor did not significantly predict time until delivery.

Similarly, evidence for a link between maternal sAA levels and child outcomes are still scarce. Giesbrecht and colleagues (2015) showed an association between maternal prenatal diurnal levels of sAA and newborns' weight at birth, with women showing a blunted sAA awakening response having lower birth weight infants. Rash and colleagues (2016) employed discriminant function analyses and showed that maternal diurnal sAA levels together with other stress biomarkers and self-report stress measures during pregnancy distinguished among 6-month-olds HPA axis and ANS reactivity profiles. Furthermore, recently, Braithwaithe and colleagues (2017) found that maternal sAA levels in the second or third trimester of gestation predicted 2-month-olds negative emotionality in a sex dependent manner, with higher maternal antenatal diurnal sAA levels being related to lower levels of distress to limits in males but not in females.

1.4.3 Inflammatory response system (IRS)

Infections, injuries and stress conditions activate an inflammatory response of the immune system. Importantly, inflammation, which plays a key role in many human diseases, has been shown to be activated not only by immune-challenges such as infections or injuries, but also by non-immunological environmental and psychological stimuli, such as stress conditions (e.g. Miller et al., 2006). Thus, it is increasingly regarded as a potential pathway linking stress experiences with health and disease.

A full description of the immune system is beyond the scope of the current thesis. In what follows a brief overview of the immune system functioning is provided with the purpose of elucidating the link between antenatal depression and inflammation, as well as possible mechanisms of fetal programming by maternal inflammation.

In order to elucidate the relationship between psychological stressors and the immune system, as well as changes occurring in the immune system during the perinatal period, it is useful to distinguish between natural or innate immunity and specific or adaptive immunity. Natural immunity is a non-specific immune response which is carried out by all-purpose cells that are able to attack several different pathogens quickly (minutes to hours from the challenge). Granulocytes are the main group of these cells and include neutrophils and macrophages, which are responsible for the generalized inflammatory response. Neutrophils and macrophages converge at the site of infection or injury where they release toxic substances and phagocytose both pathogens and damaged tissues. In addition, macrophages release cytokines, communication molecules that have a wide range of effects on the organism, including fever and inflammation, and promote healing. Pro-inflammatory cytokines include Interleukine-1 (IL-1), Interleukine-6 (IL-6), and tumor necrosis factor alpha (TNFα). Other cells involved in natural immunity include the natural killer cells, that are thought to play an important role in the early phases of infections before specific immunity is fully activated, and complement, proteins that up-regulate inflammation and also aid in antibody-mediated immunity.

Specific immunity involves a more specific, although slower, immune response which is mainly mediated by lymphocytes. Lymphocytes are characterized by antigen-specific receptors that respond to only one kind of pathogen. When activated, they give rise to a proliferative response known as clonal proliferation, where they divide to produce a population of antigen-specific cells in a process that can take up to several days before a full defence is mounted. Three types of lymphocytes are involved in specific immunity, namely, T-helper cells, T- cytotoxic cells, and B cells. T-helper cells primarily produce cytokines that amplify the inflammatory response. They can be distinguished into Th1 and Th2 cells. Th1 cells activate a cellular immune response to intracellular pathogens such as viruses, by producing cytokines, including IL-2 and interferon gamma (IFNγ), that selectively activate T-cytotoxic cells and natural killer

cells. Th2 cells are responsible of humoral immune response against extracellular pathogens, like parasites and bacteria, and produce different cytokines, such as IL-4 and IL-10, which selectively activate B cells and mast cells to attack extracellular pathogens. T-cytotoxic cells recognize cells that are infected by viruses or are damaged and destroy them. B cells produce antibodies (i.e. Immunoglobulin (Ig) A, IgE, IgM, IgG and IgD), proteins that serve several functions such as neutralizing toxins or binding to free viruses to prevent their entry into cells.

1.4.3.1 Stress and inflammation

Although initial views posited that the immune system was autonomous, important pathways linking it with many other biological systems, in particular the nervous system and the endocrine system, are now well-established. This means that stressful events that activate a response from the ANS or HPA axis can also elicit responses from the immune system, as shown in Figure 1.6; the coordination of the SNS, HPA-axis and IRS response, in particular to acute stress, is essential to health and well-being (Gunnar & Quevedo, 2007; Kuhlman et al., 2017).

It is now well-established that stress is associated with changes in immune system functioning, as indicated by elevated pro-inflammatory cytokines levels, greater inflammatory response to psychological stressors and to in vitro and in vivo immune challenges (e.g. Dunn & Swiergiel, 2005; Dunn, Swiergiel, & de Beaurepaire, 2005; Yang & Glaser, 2002; Johnson et al., 2002; Pace et al., 2006; Kiecolt-Glaser et al., 2003; Lutgendorf et al., 1999).

There are different stress-induced neuroendocrine pathways through which stress can activate the immune system and alter its functioning. First of all, sympathetic fibres from the brain descend into lymphoid tissues and release several substances that bind to receptors on white blood cells and influence the inflammatory response (Felten & Felten, 1994).

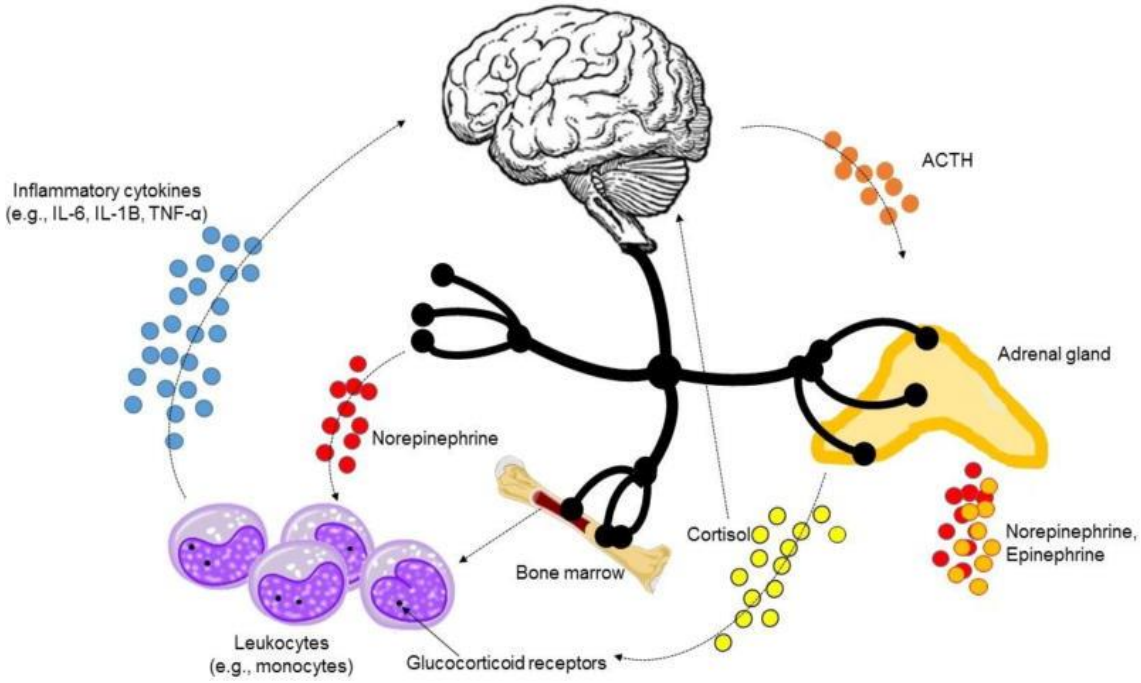

Figure 1.6 - Coordinated HPA axis, SAM system and IRS response to acute stress (from Kuhlman et al., 2017)

All lymphocytes have adrenergic receptors, although density and sensitivity vary among cell types and influence cells responsiveness to stress. For example, natural killer cells have a large number of high-affinity β2-adrenergic receptors, while B cells have high density but lower affinity receptors, and T cells have the lowest density (Kuhlman et al., 2017). Secondly, NE, E and cortisol, secreted respectively by the SAM and HPA axis under stress conditions, bind to specific receptors on white blood cells and have different regulatory influences on their distribution and function (Felten & Felten, 1994). Cortisol has mainly anti-inflammatory effects, although this has been shown to depend also on circumstances and concentrations (Dhabhar, 2002).

Glucocorticoids receptors can be found in immune cells and binding of glucocorticoids to these receptors downregulates cytokine production, thus preventing excessive cytokines elevations (Silverman & Sternberg, 2012). It has been found that prolonged or chronic stress in humans can lead to insensitivity of the glucocorticoids receptors in immunes cells that become unresponsive to the inhibitory signals of

glucocorticoids (Cohen et al., 2012). Less is known about the role of the SNS, and catecholamines in particular, in modulating the immune response. However, the SNS appear to exert both anti-inflammatory and pro-inflammatory influences, by suppressing inflammation in already activated cells, while activating inflammatory responses in non-activated immune cells (Rohleder, 2012). Third, it has been proposed that changes in lifestyle due to stressful experiences (such as alcohol use or sleep changes) can also alter immune system functioning (Segerstrom & Miller, 2004). Lastly, beside these neuroendocrine pathways, recent interest has been directed toward inflammasome, a multiprotein complex that is produced in response to pathogens or stressors, and could represent a key immunological interface between stress and inflammation (for an exhaustive discussion see Iwata, Ota, & Duman, 2013).

While initial models posited that stress generally suppresses the immune response, thus leading to greater incidence of infectious or neoplastic diseases among chronically stressed individuals (Andersen, Kiecolt-Glaser, & Glaser, 1994), it was later proposed that chronic stress might shift the Th1-Th2 balance of the immune response, without necessarily altering the overall system functioning (Cohen et al., 2012). Specifically, it was suggested that chronic stress alters patterns of cytokine secretion, possibly mediated by stress hormones such as cortisol, thus simultaneously suppressing Th1 (cellular) activity and increasing production of Th2 (humoral) cytokines (Chiappelli et al., 1994). This Th1-to-Th2 shift could increase vulnerability to infectious and neoplastic disease, because Th1 is suppressed, as well as vulnerability to autoimmune and allergic diseases, because Th2 is enhanced. This model is able to reconcile stress-related immune changes with stress-related disease outcomes (Cohen et al., 2012).

Segerstrom and Miller (2004) performed a meta-analyses of 293 independent studies for a total of 18,941 participants in order to examine the association between different psychosocial stressors and immune parameters. Results indicate that

stressful events are consistently associated with changes in the immune system and that characteristics of those events influence the kind of changes. In particular, acute stressors were related to an upregulation of natural immunity and downregulation of specific immunity, brief naturalistic stressors (such as exams) elicited a cytokines shift with a suppression of cellular, but not humoral, immunity, while chronic stressors trigger a global immunosuppression.

1.4.3.2 C-Reactive Protein (CRP) and Interleukin-6 (IL-6)

Measurement of concentrations of serum inflammatory markers by immunoassay is increasingly performed to evaluate the IRS functioning. Psychiatry research has focused on the assessment of a number of cytokines, such as IL-6, IL-1β, TNF-α, or Interferon-gamma (IFN-gamma), or ratios of some of these, as well as acute-phase proteins, a class of proteins which is produced by the liver in response to inflammation, such as C-Reactive Protein (CRP). In the current thesis, we will focus on IL-6 and CRP that are consider the most important biomarkers of chronic or systemic low-grade inflammation (Rohleder et al., 2012). Chronic low-grade systemic inflammation has been identified as one of the major pathophysiological mechanisms, being predictive of later morbidity and mortality (Ershler & Keller, 2000). It has been associated with several disease and disorders and, as will be discussed in what follows, IL-6 and CRP circulating levels have been found to be consistently increased in patients with depression (Valkanova, Ebmeier, & Allan, 2013), thus representing a promising avenue for research into inflammatory pathways involved in perinatal depression and prenatal programming.

Interleukine-6 (IL-6) is produced by both Th1 and Th2 cells (Szelényi, 2001). It is primarily regarded as a pro-inflammatory cytokine because it is mainly involved in triggering inflammation (Rohleder et al., 2012); however, it has been shown that, depending on biological conditions, IL-6 can have also anti-inflammatory or immunosuppressive influences (Ohno et al., 2016). It has been shown that infections

or tissue damage trigger an immediate expression of IL-6 which sends out an alarm signal to the entire body and induces synthesis of acute phase proteins such as CRP. In addition, IL-6 is involved in the acquired immune response by stimulating antibody production and effector T-cell development. Furthermore, like other pro-inflammatory cytokines, IL-6 is known to trigger an HPA axis response and contribute to increase cortisol secretion (Steensberg et al., 2003). IL-6 expression is terminated once the source of stress is removed. However, it has been shown that dysregulated persistent IL-6 production might lead to the onset or development of several diseases (Tanaka, Narazaki, & Kishimoto, 2014). Recently, a number of lines of evidence point to IL-6 as a key pro-inflammatory factor involved in the pathogenesis of depression (Walsh et al., 2016; van Dooren et al., 2016; Sjögren et al., 2006). In addition, it has been proposed that elevated IL-6 serum levels might serve as a future diagnostic biomarker (e.g. (Chase et al., 2016), as well as a marker of response to treatment (Manoharan et al., 2016) that deserves further investigation.

C-Reactive Protein (CRP) has attracted increasing interest in recent years. It is an acute phase serum protein that is primarily synthesized in the liver as a part of the acute-phase response of the IRS. CRP is synthesized very rapidly and reaches very high levels during the height of the inflammatory response. Its synthesis is mainly induced by IL-6 and IL-1, although it has been shown that different agents can influence its production, including corticosteroids (Du Clos & Mold, 2004). Accumulating evidence suggests that CRP is a marker of “low-grade” systemic inflammation which strongly predict several health outcomes, including cardiovascular diseases (e.g. Genest, 2010), cancer (e.g. Nakamura et al., 2012), cirrhosis (e.g. Cervoni et al., 2012), and adverse pregnancy outcomes, such as premature ruptures of membranes and preterm birth (e.g. Moghaddam Banaem et al., 2012). Thus, interest in CRP as an easily measurable prognostic biomarker for early detection of risk of morbidity has consistently increased during the last decade. Recently, higher CRP levels have been reported in an increasing number of psychopathological

conditions, such as depression (e.g. Howren, Lamkin, & Suls, 2009), anxiety (e.g. Liukkonen et al., 2011) and psychological distress (e.g. Goldman-Mellor, Brydon, & Steptoe, 2010) across a continuum of severity. Thus, it has been suggested that heightened levels of CRP, in the absence of acute medical conditions, reflect an increased rate of synthesis of CRP induced by low-grade inflammatory processes during periods of chronic stress (Carpenter et al., 2012), that might be measurable well before the clear onset of significant clinical syndromes.

Levels of circulating inflammatory markers have been shown to be impacted by sample collection protocols, including handling, processing and storage procedures, and time of the day of collection (e.g. Skogstrand et al., 2008), as well as by some demographic and health-related factors, such as age, ethnicity (Christian et al., 2013; Gillespie, Porter, & Christian, 2016), body mass index and medications (Christian & Porter, 2014). In contrast, no effects of fetal sex on serum concentrations of maternal cytokines during pregnancy have been reported (Mitchell, Palettas, & Christian, 2017).

Notably, both IL-6 and CRP serum levels have been found to be affected consistently by both acute and chronic stress (Marsland et al., 2017; Segerstrom & Miller, 2004; Steptoe, Hamer, & Chida, 2007). Cross-sectional studies have shown association between psychosocial factors and both increased IL-6 (e.g. Kiecolt-Glaser et al., 2003) and CRP concentrations (e.g. Rohleder et al., 2009) in non-pregnant adults and preliminary evidence suggests that associations also exist during pregnancy (Christian, 2012b; Coussons-read et al., 2012; Coussons-Read et al., 2005).

1.4.3.3 Immune system functioning during the perinatal period

Similarly to the HPA axis and SNS, maternal immune system functioning is substantially altered during normal pregnancy to protect women from pathogens and, at the same time, avoid fetal rejection (La Rocca et al., 2014). While it was initially believed that pregnancy was a state of general immunosuppression, it has been later

proposed that pregnancy is a period of immunomodulation with early and late pregnancy being characterized by greater inflammation or pro-inflammatory to anti-inflammatory activity (Th1>Th2 activity), while mid-pregnancy is characterized by lower inflammation (Th2>Th1 activity) (e.g. La Rocca et al., 2014; Sykes et al.,, 2012). Pregnancy hormones, including progesterone and estradiol, are known to promote Th2 cells activity and are held responsible for the predominance of Th2 activity associated with advancing pregnancy (Sykes et al., 2012). In particular, immune system regulation across gestation is now believed to follow three phases. During the first phase, characterized by a pro-inflammatory state, inflammatory factors, such as cytokines, are produced in the endometrium and released to support implantation and placentation processes (Mor et al., 2011). The second phase corresponds to a period of rapid fetal growth and is characterized by an anti-inflammatory state, often associated with greater maternal well-being (Mor et al., 2011). During this period, the placenta is thought to play an important role by supporting the shift from Th1 (cell-mediated) to Th2 (humoral-mediated) immune responses (Kumpel & Manoussaka, 2012). Paralleling these changes, pregnancy-related improvement or remission of Th1 autoimmune diseases (e.g. rheumatoid arthritis) and worsening of diseases characterized by Th2 dominance, such as lupus, are often reported (e.g. Straub, Buttgereit, & Cutolo, 2005; Langer-Gould et al., 2002). In addition, the reduced cell-mediated immunity associated with the Th2 bias might, at least partially, explain the increased susceptibility during pregnancy to conditions with an intra-cellular pathogenesis, such as influenza and Listeria monocytogenes (Poole & Claman, 2004). Lastly, the third phase begins before labor, when immune cells migrate into the myometrium and create a pro-inflammatory state (Brewster et al., 2008). The inflammatory response is heightened during labor and continues into the early postpartum period to support healing and involution (Sennstrom et al., 2000). A shift to a predominant Th1 activity has been reported in the postpartum period and has been associated with increased susceptibility to infections during that period (Elenkov et al.,

2001). The Th2 cytokine predominance which exists during pregnancy has been shown to return to non-pregnant Th1:Th2 cell ratio by 4 weeks postpartum (Saito et al., 1999).

Concerning circulating levels of cytokines, both pro-inflammatory and anti-inflammatory cytokines have been shown to be mildly increased during pregnancy (e.g. (Curry et al., 2008; Christian & Porter, 2014). In particular, IL-6 is known to serve multiple functions in pregnancy maintenance (Markert, Morales-Prieto, & Fitzgerald, 2011) and higher than typical elevations in IL-6 concentrations during pregnancy have been associated with many adverse pregnancy outcomes, such as miscarriage (Galazios et al., 2011), preeclampsia (Guven et al., 2009), gestational diabetes (Kuzmicki et al., 2009) and preterm delivery (Coussons-Read et al., 2012).

In addition, attenuated pro-inflammatory cytokine production in response to in vitro and in vivo immune challenges has also been reported in healthy pregnancy, particularly in the third trimester, and it is thought to prevent fetal rejection and protect the fetus from excessive maternal inflammation (Christian, 2012b; Gillespie et al., 2016).

1.4.3.4 IRS functioning and perinatal depression

A role for inflammation and related cytokines in MDD was first suggested in the 1980's and since then has been well-documented (reviewed in Miller & Raison, 2016). Depressed non-pregnant adults show the key features of an inflammatory response, including an increased expression of pro-inflammatory cytokines and their receptors and elevations in acute-phase reactants both in blood and cerebrospinal fluid (Miller, Maletic, & Raison, 2009). In addition, the expression of a wide range of immune genes and proteins was found to be upregulated in post-mortem brains of depressed individuals who committed suicide (Bufalino et al., 2013). Several meta-analyses demonstrated significant association between depression and elevations in circulating levels of pro-inflammatory cytokines, namely IL-1 β, IL-6 and tumour necrosis factor-

alpha (TNF-α), and acute phase proteins, such as C-reactive protein (CRP), suggesting that these might represent the most reliable biomarkers of inflammation in patients with depression (e.g. Dowlati et al., 2010; Howren et al., 2009; Liu, Ho, & Mak, 2012; Valkanova et al., 2013). Although the association is likely to be bidirectional, findings support causality as well. It has been demonstrated that therapeutic administration of pro-inflammatory cytokines causes non-depressed individuals to experience depressive symptoms (Bonaccorso et al., 2002; Capuron et al., 2002). Similarly, blockade of cytokines reduces depressive symptoms in patients with MDD or patients with medical diseases (Abbott et al., 2015; Köhler et al., 2014; Miller & Raison, 2015; Raison et al., 2013). Lastly, polymorphisms in inflammatory cytokine genes have been implicated in depression and its response to treatment (Bufalino et al., 2013). Consistent with this body of literature, large community studies showed that increased inflammation, as indicated by heightened CRP or IL-6 levels, predict the later development of depression (Au et al., 2015; Gimeno et al., 2009).

Based on this evidence, it has been hypothesized that dramatic changes in both the stress and immune system during the perinatal period might play a role in the development of depression during the perinatal period (e.g. Corwin & Pajer, 2008). However, this is still a relatively new and unexplored area of inquiry. A number of studies over the last two decades have attempted to clarify the relationships among IRS functioning and perinatal depressive symptoms. Nevertheless, heterogeneity in both mood and inflammation assessment do not yet allow any firm conclusions to be drawn (see Osborne & Monk, 2013 for a review). Several studies reported higher levels of pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α, or CRP, IFN-gamma, in depressed pregnant women regardless of gestational timing (Azar & Mercer, 2013; Cassidy-Bushrow et al., 2012; Christian et al., 2009; Coussons-Read, Okun, & Nettles, 2007; Haeri, Baker, & Ruano, 2013; Roomruangwong, Kanchanatawan, & Sirivichayakul, 2017; Scrandis et al., 2008). Furthermore, Christian and colleagues (2010) showed that depressive symptoms predict exaggerated inflammatory

responses to influenza virus vaccination during pregnancy, suggesting a sensitization of the inflammatory response related to depression during the antenatal period. However, negative associations between depressive symptoms and a number of inflammatory markers levels have also been reported both in early (Latendresse, Ruiz, & Wong, 2013), mid- (Shelton, Schminkey, & Groer, 2015) and late (Edvinsson et al., 2017) pregnancy, suggesting that depression during pregnancy might be different from depression outside the perinatal period. In addition, a handful of studies did not find any evidence of significant association between depressive symptoms and a number of inflammatory markers during pregnancy (e.g. Blackmore et al., 2014; 2011, Karlsson et al., 2016; Simpson et al., 2016; Walsh et al., 2016).

Inflammation is also thought to play a role in the development of postpartum depression. In particular, an excessive inflammatory response to labor and delivery, both alone or in interaction with a dysregulation of the HPA axis, has been proposed to predispose some women to experience depressive symptoms in the postnatal period (e.g. Corwin & Pajer, 2008). For example, Kendall-Tackett (2007) suggests that many previously identified risk factors for postpartum depression, such as stress, sleep disturbances, pain, history of depression or trauma, are all characterized by having inflammation as the underlying mechanism. Increased serum concentrations of inflammatory markers in women experiencing postpartum depression have been reported in a number of studies (e.g. Boufidou et al., 2009; Liu et al., 2016; Maes et al., 2000b), beginning soon after delivery throughout the first 6 months postpartum. Nevertheless, contradictory findings have also been reported. Few studies showed negative associations between postnatal depression and levels of inflammatory markers associations (e.g. Scrandis et al., 2008; Corwin et al., 2015), while a consistent number of reports did not detect any significant associations (Groer & Davis, 2006; Blackmore et al., 2014; Corwin et al., 2015; Skalkidou et al., 2009; Groer et al., 2015; Cheng & Pickler, 2014). Corwin and colleagues (2008) reported increased IL-1β levels 2 weeks after delivery in women with symptoms of depression, but not 1

or 4 weeks postpartum. Groer and Morgan (2007) reported a lower Th1/Th2 ratio in postnatally depressed women, suggesting some impairment in cellular immunity. In addition, they showed that this was not true for women who were breastfeeding, as compare to formula-feeding mothers, suggesting that breastfeeding, and related physiological changes, may exert something of a protective role (Groer & Davis, 2006).

1.4.3.5 Prenatal programming by maternal inflammation

Animal studies have documented that cytokines are present in the fetal brain from early gestation (e.g. Meyer et al., 2006) and that increases in specific cytokines levels, such as TNF-α and IL-1β, are related to important developmental events in fetal brain (Dziegielewska et al., 2000). An early study in humans suggested a role of cytokines in fetal brain development due to cytokine and chemokine expression in forebrain cells of human fetuses from 5 weeks of gestation (Mousa et al., 1999). In addition, in vitro studies shown that cytokines and chemokines can be produced by human fetal microglia and astrocytes (Lee, Nagai, & Kim, 2002; Rezaie et al., 2004), with an increased production in response to infections (Cheeran et al., 2001; Lokensgard et al., 2001), and that glial cells in fetal brain receive and respond to signals from inflammatory cytokines (Hanisch, 2002). Thus, it has been hypothesized that cytokines could play a critical role in fetal neural, synaptic and glia cell development (Deverman & Patterson, 2009), although further research is needed in humans.

While an increase in pro-inflammatory cytokines as gestation advances is normal and, to a certain extent might promote fetal brain development, it has also been shown that excessive inflammation and a shift toward a Th1 predominance can represent a threat to successful pregnancy. Heightened levels of pro-inflammatory cytokines in maternal serum or amniotic fluid have been causally linked with greater risk of preterm delivery (e.g. Hagberg, Mallard, & Jacobsson, 2005) or pre-eclampsia

(e.g. Kurki et al., 2000). In addition, a lack of attenuation of inflammatory responses to in vitro challenges has been found among women who later miscarried or gave birth to small for gestational-age babies (Marzi et al., 1996) or women with a history of recurrent spontaneous abortions (Makhseed et al., 2000).

Recently, it has been proposed that maternal inflammation, through release of cytokines, might play a significant role in “transferring” maternal distress to the fetus (Entringer et al., 2010; Ratnayake et al., 2013). However, the actual mechanisms by which this might occur are still mostly unknown. Both direct mechanisms via placental transfer into the fetal compartment or indirect effects mediated by placental inflammation have been hypothesized and require further exploration (reviewed in Rakers et al., 2017). Recently, Ross and colleagues (2016) reported significant associations between maternal pro-inflammatory markers during pregnancy and cord blood inflammation at birth, suggesting that maternal inflammation can be translated to fetal circulation, although the mediating mechanisms are still to be elucidated. Studies have shown that heightened levels of circulating maternal cytokines can cross the placenta to reach the fetus and influence its development. For example, rodent models revealed that the administration of cytokines, such as IL6, leads to significant levels of those markers in the amniotic fluid and fetal tissue (Dahlgren et al., 2006; Ponzio et al., 2007). Similarly, it has been shown that placental bidirectional transfer of IL-6 occurs in human healthy pregnancy (Zaretsky et al., 2004), indicating that some specific cytokines, at least, can directly cross the placenta and get into fetal circulation. Nonetheless, different ex vivo perfusion studies in human placentas did not find evidence of placental transfer of cytokines (e.g. Aaltonen et al., 2005).

Another possible mechanism for the adverse effect of maternal inflammation on fetal development involves the production of cytokines from the placenta itself, which would explain why these substances are found in the amniotic fluid and fetal circulation (Ratnayake et al., 2013). Interestingly, maternal IRS activation and related cytokine secretion has been found to induce placental production of pro-inflammatory

cytokines (Hsiao & Patterson, 2012), although it is still unclear whether maternal antenatal distress can trigger placental cytokine production. It has also been proposed that an altered balance between pro- and anti-inflammatory cytokines, rather that excessive production and release of specific cytokines into the fetal compartment, might adversely impact normal brain development (Meyer et al., 2006; Deverman & Patterson, 2009), although this hypothesis requires exploration in humans.

The effects of maternal cytokines on fetal development is thought to be mediated by an effect on fetal development of glial cells or stress response systems (Ratnayake et al., 2013). Animal studies have shown that prenatal infection or inflammation can alter the function of glial cells with a permanent adverse impact on the fetal brain (Perry, Nicoll, & Holmes, 2010). Furthermore, once increased levels of maternal cytokines have entered into the fetal circulation, they can activate the fetal HPA axis to elicit a stress response, which can subsequently influence HPA axis function in later life (Chrousos & Kino, 2005). For example, Samuelsson and colleagues (2004) showed that peripheral injection of IL-6 in pregnant rats affects offspring cardiovascular and HPA regulation, increasing basal activity and reactivity to acute stress. Consistent with animal data, preliminary studies on very preterm babies have suggested a link between prenatal exposure to inflammation and altered infants HPA activity (e.g. Gover et al., 2013), indicating that exposure to inflammatory signals in the intrauterine environment could influence the development of the HPA axis, leading to long-term functional changes. Lastly, it has been shown that maternal pro-inflammatory cytokines, specifically IL-1β, IL-6 and TNF-α, can reduce the activity of the placental 11-βHSD2, leading to more than 75% of the enzyme activity being suppressed (Kossintseva et al., 2006). Thus, fetal glucocorticoid overexposure due to maternal inflammation might constitute an additional mechanism of fetal programming.

1.4.3.6 Maternal inflammation and child outcomes.

The effects of maternal inflammation during pregnancy on offspring neurodevelopment remains relatively unknown. Strong evidence indicates that prenatal exposure to IRS-activating infections influences several outcomes in later life, including stress reactivity and susceptibility to metabolic, immunological and neuropsychiatric diseases such as Alzheimer's, Parkinson's, schizophrenia, and autism (Nelson & Willoughby, 2000, 2002; Rantakallio et al., 1997; Shi et al., 2003). Nonetheless, the non-experimental nature of this body of findings does not allow us to disentangle whether the observed effects on the developing fetal brain are due to infection-induced maternal IRS activation or to infection itself. Evidence for a specific causal role of maternal cytokines in fetal programming is mainly based on animal studies (Mandal et al., 2013; Meyer et al., 2008; Straley et al., 2017). In a study using mouse models, maternal stress triggered placental inflammation, as indicated by upregulation of pro-inflammatory placental genes, and this, in turn, resulted in an offspring hyperactive phenotype. In addition, administration of anti-inflammatory drugs prevented gene upregulation and altered the phenotype in the offspring (Bronson & Bale, 2014). Furthermore, several studies on pregnant rats showed that peripheral injection of IL-6 produced a number of negative outcomes in the offspring, including structural changes in the hippocampus and reduced learning abilities, increased body weight and decreased insulin sensitivity and hypertension and hyperactivity of the HPA axis (e.g. Dahlgren et al., 2006; Samuelsson et al., 2004). Notably, maternal IRS activation induced by viral mimic during pregnancy resulted in a schizophrenia-like phenotype in the offspring, unless they were IL-6-knockout mice or an IL-6 antibody was added to the viral mimic (Smith et al., 2007), suggesting an important role of maternal IL- 6 in programming risk for later disease.

Only a few studies in humans have attempted to explore the association between antenatal maternal cytokine levels and offspring risk for schizophrenic spectrum disorders (Allswede et al., 2016; Brown et al., 2004; Buka et al., 2001),

generally, reporting no associations. In addition, when positive associations between specific markers and risk for psychosis in the offspring were found, these were not replicated in the other reports. In addition, to our knowledge, only one report related levels of maternal specific inflammatory markers during pregnancy to later risk of MDD in the offspring (Gillespie et al., 2016). The authors showed that maternal inflammation during pregnancy increased the risk for major depression in adulthood in offspring in a sex-dependent manner. Specifically, prenatal exposure to a higher concentration of maternal TNF-α and IL-10 were associated with a lower risk of depression among female offspring, and with a higher risk of depression among males.

As previously mentioned, maternal inflammation has been consistently linked to adverse pregnancy outcomes, such as preeclampsia, miscarriage and gestational diabetes (reviewed in Christian et al., 2012). In addition, a number of studies showed that increased levels of circulating inflammatory markers predict adverse birth outcomes, such as shorter gestational length, lower birth weight and shorter body length (e.g. Kuzawa et al., 2017; Pringle et al., 2015), while some others have suggested that an altered maternal Th1-Th2 balance during pregnancy favouring a Th1 response, rather than Th2, might be implicated in risk for preterm delivery (Ekelund et al., 2008; Sykes et al., 2012a).

Moreover, some preliminary studies have investigated the mediating role of maternal inflammation in the link between antenatal maternal stress and birth outcomes, and these have yielded inconsistent findings. Okun and colleagues (2013) reported an association between maternal increased IL-6 levels and smaller birth weights in depressed women, although the effect became non-significant in an adjusted model. Coussons-Read and colleagues (2012) reported an association between higher CRP, IL-6 and TNF-α levels and shorter gestation. In addition, they showed that maternal levels of IL-6 and TNF-α during pregnancy partially mediated the effects of antenatal maternal pregnancy-specific distress on gestational length, although the direction of findings was unexpected, with greater maternal distress being

associated with lower, rather than higher, inflammation and, in turn, less impact on gestational age. Lastly, Miller and colleagues (Miller et al., 2017) found that high maternal IL-6 levels during pregnancy mediated the association between maternal disadvantaged childhood and negative birth outcomes such as lower birth weight and preterm delivery.

A very limited number of studies have directly investigated the impact of prenatal levels of maternal inflammatory markers on child developmental outcomes, beyond birth outcomes. Graham and colleagues (2017) were the first to show that maternal averaged IL-6 levels across pregnancy was associated with newborn brain development. Specifically, higher IL-6 concentrations were associated with larger right amygdala volume and stronger bilateral amygdala connectivity to brain regions involved in sensory processing and integration, learning and memory. The alterations in newborns' amygdala, in turn, predicted lower impulse control at 24 months of age, after controlling for variations in mother-child relationship. Furthermore, very recent work from the same group of researchers additionally showed that maternal averaged IL-6 concentrations during pregnancy was associated with reduced functional anisotropy in the central portion of the uncinate fasciculus of newborns' brains, suggesting that maternal antenatal inflammation predicts reduced integrity of the main frontolimbic fibre tract (Rasmussen et al., 2018). Moreover, they showed a positive association between maternal IL-6 levels and rate of increase in functional anisotropy of the uncinate fasciculus across the first year of life, suggesting a compensatory postnatal growth, so that no association between maternal antenatal IL-6 and functional anisotropy was detected at 12 months of age. Maternal antenatal IL-6 levels also predict poorer cognitive development at 12 months of age and, importantly, this was mediated by the postnatal catch-up growth in functional anisotropy.

Another recent study by Osborne and colleagues (2018) showed that higher levels of maternal TNFα, vascular endothelial growth factor, and IL-10 in the third trimester of pregnancy were associated with infant outcomes, specifically, less optimal

neurobehavioral function, as assessed at the NBAS 6 days after childbirth, and higher cortisol levels in response to the immunization at 12 months of age, but not at 2 months of age. Lastly, Gustaffson and colleagues (2018) reported that maternal pro-inflammatory cytokines (indexed though a latent variable including IL-6, TNF-α, and monocyte chemoattractant protein-1) in late pregnancy mediate the link between prenatal depressive symptoms and 6-month-olds negative affect

Despite this evidence being only preliminary, findings are suggestive of a role of maternal inflammation during pregnancy in influencing fetal brain development, indicating that an inflammation-mediated pathway from antenatal depression to child altered outcomes deserves further investigation.

1.2 The current thesis

The studies described above provide an overview of accumulating evidence linking maternal antenatal stress to bio-behavioural outcomes in the child, suggesting that understanding pathways influencing fetal development should be a global health challenge. Nonetheless, the science of fetal origins of psychopathology poses several key questions.

First of all, there is a need for a clear characterization of maternal antenatal stress experience both from a psychological and biological perspectives. Studies relating specific maternal antenatal influences to specific neurodevelopmental outcomes hold greater promise in revealing underlying mechanisms.

Secondly, while biological and psychological stress measures are often assumed to be markers of the same underlying construct, evidence suggests that maternal psychological distress is not necessarily related to increased stress biomarkers and, similarly, higher levels of stress hormones do not seem to automatically reflect maternal experience (Harville et al., 2009; Sandman et al., 2012). Rather, as biological and psychological stress measures have both been found to be

related to child outcomes, it has been proposed that the effects of these factors on offspring development might occur through different pathways, although these still need to be elucidated (O'Donnell & Meaney, 2017).

Third, while it is known that under stress conditions both the stress and immune systems are activated and extensively interact with each other (e.g. Chrousos & Kino, 2005), the majority of studies in the field focus exclusively on neuroendocrine mediators of maternal stress experience. Studies evaluating multiple biological stress measures are scarce and, to our knowledge, no published study has measured concurrently the activity across the HPA axis, SNS and IRS.

Fourth, mounting evidence suggest that genetic and postnatal environmental factors play a role in programming later developmental trajectories. In particular, animal models suggest that early rearing conditions can moderate the impact of prenatal stress exposure on offspring development, however the generalizability of these findings to humans is still unknown. As a greater understanding of whether postnatal caregiving experiences might moderate the effects of antenatal stress exposure has considerable theoretical and practical significance, more research into the interplay between prenatal and postnatal environmental influences in humans is needed.

Building on existing knowledge, the current dissertation attempts to address these key issues by adopting a multi-systems approach in order to examine prospective associations among naturally occurring variations in maternal antenatal depressive symptoms, stress and inflammatory biomarkers concentrations and offspring's bio-behavioral outcomes from birth to three months of age, taking into account the role of postnatal maternal care.

A community sample of healthy pregnant women, together with their offspring, was studied from late pregnancy to three months after delivery as described in Chapter 2. In Chapter 3, cross-sectional associations between maternal depressive symptoms and levels of stress and inflammatory markers in late pregnancy and soon

after delivery were examined. Chapters 4 and 5 aimed at investigating the prospective associations between variations in maternal depressive symptoms, stress and inflammatory markers during pregnancy and infant behavioral and biological outcomes at birth (Chapter 4) and 3 months after delivery (Chapter 5). Lastly, Chapter 6 was designed to explore the role of postnatal maternal care in the association between prenatal maternal influences and 3-month-olds' bio-behavioral outcomes. To our knowledge, the present study is unique in the assessment of multiple biological markers of maternal stress and immune systems in association with infant outcomes. The neonatal assessment soon after birth provided a unique opportunity to examine the effects of antenatal maternal stress, largely independent from postnatal environmental factors. Furthermore, the evaluation of the quality of early maternal postnatal care, jointly with maternal postnatal symptoms, is rarely included in research within the DOHaD field and allowed to preliminarily examined the complex interplay between prenatal and postnatal environemental factors in influencing infants' development. Lastly, despite the most sensitive time in gestation for the adverse effects of antenatal maternal stress has not yet been established, the current dissertation focuses on examining effects of exposure in late pregnancy as this is a period of rapid infant growth and brain development (Grossman et al., 2003) in which exposure to maternal stress signals is thought to influence the developing fetal stress response system (Davis et al., 2011) and is associated with later risk for emotional problems (Rice et al., 2007).

Based on available literature, we tested the following hypothesis:

1) Women with higher depressive symptoms would showed heightened inflammation and an altered cortisol diurnal pattern during pregnancy and after delivery (Chapter 3).
2) Higher maternal antenatal depressive symptoms would be associated with more negative birth outcomes and greater stress reactivity in offspring at birth (Chapter

4) as well as with 3-month-olds' greater stress reactivity, negative emotionality and poorer cognitive/motor development (Chapter 5).

3) Variations in maternal diurnal cortisol in late pregnancy would predict newborns' birth outcomes and stress reactivity at birth (Chapter 4), as well as infants' stress regulation, temperament and cognitive/motor development at 3 months of age (Chapter 5).

4) We tested two main hypotheses concerning the role of maternal caregiving in the observed associations between prenatal maternal influences and infants' development: a) Higher maternal prenatal depressive symptoms would be associated with lower maternal sensitive caregiving, as measured through the Emotional Availability (EA) Scales (4th edition, Biringen et al., 2008), possibly mediating the influence of antenatal depression on infant's outcomes. b) Maternal sensitive caregiving would moderate the association between prenatal maternal influences and infant's outcomes at 3 months of age (Chapter 6). Due to limited available literature, we made a broad hypothesis that high maternal EA 3 months after childbirth would be able to buffer the negative effect of prenatal stress exposure (as indexed either by psychological or biological measures) on infants' development.

Furthermore, in Chapter 3, we conducted exploratory analyses to test whether maternal antenatal depressive symptoms would be associated with an altered diurnal sAA pattern as well as the association between stress and inflammatory markers between women with higher versus lower depressive symptoms. In particular, after Corwin and colleagues (2015), we hypothesized that women with higher depressive symptoms would show a positive association between stress and inflammatory markers during the perinatal period.

In Chapter 4 and 5, we investigated possible novel mediators of the effects of maternal antenatal stress on child outcomes involving the IRS and the SNS

functioning which have received very limited empirical support to date. In particular, exploratory analyses examined possible associations between either maternal antenatal inflammation or diurnal sAA levels and a wide range of outcomes in offspring including birth outcomes, stress reactivity, temperament, motor and cognitive development. Due to the lack of available literature, we made no a priori hypothesis concerning these associations.

Lastly, as perinatal depression and anxiety are strongly correlated (Falah-Hassani et al., 2017), in each chapter, we investigated whether the observed associations were specific to depression or can be replicated for anxiety. These exploratory analyses were expected to provide some preliminary insight into the prenatal risk phenotype that might be more implicated in child development.

Chapter 2: Materials and methods

2.1 The Effects of Depression on Infants (EDI) Study

The findings discussed in this dissertation come from a community sample of mother infant-dyads recruited as part of the Effects of Depression on Infants (EDI) Study, an ongoing longitudinal research, based at the Scientific Institute Eugenio Medea in Italy, investigating the effects of antenatal maternal depression on children bio-behavioral development. The EDI Study started in 2014 and it is still ongoing. It consisted of a screening phase at 30-33 weeks of gestation and of five assessment phases, respectively, at 34-36 weeks of gestation (phase 1), at delivery (phase 2), 12 weeks after delivery (phase 3), 13 months after delivery (phase 4) and 36 months after delivery (phase 5). As data coding and analyses for phases 4 and 5 is still ongoing and data collection for phase 5 is underway, only data from the first three phases (i.e. pregnancy, birth, 12 weeks) will be included in the current dissertation. Specifically, maternal data from phase 1 and 2 will be part of Chapter 3, maternal data from phase 1 and infants' data from phase 2 will be included in Chapter 4, while data collected in phase 1 and phase 3 will be object of Chapters 5 and 6. Only sample characteristics and methods pertinent to the aims of the current dissertation are described below.

2.2 Participants recruitment

Women in the early third trimester of gestation were consecutively recruited at the beginning of childbirth classes or through ads placed in the clinic waiting rooms of 3 hospitals located nearby the Medea Institute in Italy. A researcher from the team presented the EDI study at the first lesson of childbirth classes and women who were interested to participate were given an information pack and some screening questionnaires and forms to be filled out between 30 and 33 weeks of gestation.

Approximately 20% (N=237) of women informed about the study at 111 childbirth classes were willing to participate and return the questionnaires. In addition, 15 women contacted the team after seeing the ads in the hospitals to take part in the study. Thus, 252 women in the early third trimester of gestation (weeks of gestation: M=31.41, SD=1.61) took part in the screening phase of the study.

2.3 Inclusion/exclusion criteria

The following prenatal inclusion criteria were established: being between 18-45 years of age, less than 36-weeks pregnant, in good health, normotensive, with singleton uncomplicated pregnancy naturally conceived, anticipating a vaginal birth in one of the hospitals involved in the study, non-smoker, not afflicted by any disease that might affect the immune or endocrine systems and not taking any chronic medications, including anti-inflammatory, anti-depressant or steroid medications, not having done or anticipating intravenous anti-D immunoglobulin, and with no known substance/alcohol abuse problems or chronic psychiatric disorders (with the exception of depression and anxiety disorders). All inclusion criteria were adopted in order to limit possible conditions that might themselves be associated with an altered functioning of the biological systems examined. Furthermore, the upper limit of the age range was set to be 45 years old, as pregnancy in women aged over 45 years is uncommon and pregnancy complications (such as high blood pressure, diabetes during pregnancy, placental problems and birth complications) are more likely to occur. The lower limit was set to 18 years as the Italian legislation required to obtain consent from both parents before involving participants under 18 years within a research project, therefore mothers aged less than 18 years would have not been able to consent for their participation in the study as well as for their new-born's participation.

Postnatal criteria for inclusion were: infants born at term (>37 weeks of gestation) or late preterm (>35 weeks of gestation) and in good health. These criteria were adopted to ensure that effects of prematurity or health problems did not confound the results of the current studies. In addition, there were a number of study-specific postnatal inclusion criteria for studies included in Chapter 3, 4 and 6. Postnatal criteria for continued inclusion in the study presented in Chapter 3 were: having a vaginal delivery of a live infant, without experiencing hemorrhage or transfusion. Postnatal criteria for continued inclusion in the study presented in Chapter 4 were: delivering in one of the three hospitals involved and available videotaped data of the heel-stick procedure. Postnatal criteria for continued inclusion in the study presented in Chapter 6 were: available data on maternal sensitivity.

2.4 Sample

Sample for Chapter 3: From the screened sample, 2 women were excluded for smoking during pregnancy, 3 for taking chronic medications, 2 for having a chronic disease, 10 for gestational diabetes, 13 for hypothyroidism, 14 for antenatal intravenous anti-D immunoglobulin, 7 for antenatal progesterone treatment, 1 for antenatal betamethasone administration, 1 for twin pregnancy, 4 for artificial conception, 5 for delivering before phase 1 was done, 2 for being more than 36-weeks pregnant and 11 for, anticipating delivery in a hospitals not involved in the study. In addition, 65 women did not agree to participate to the subsequent study phases. Thus, 110 women (mean age= 33.00; SD= 3.85) were included in the study. The final sample consisted of middle-high class (90%), mostly Italian (96.4%), married (62.7%) or cohabiting (35.5%), with at least high school diploma (89.4%) and primiparous (90%) women. The sociodemographic characteristics of the sample reflect the low-risk nature of the sample. Sociodemographic characteristics as well as depression and anxiety scores for the three groups (i.e. participants, excluded women and women who withdrew their participation) are reported in Table 2.1.

Table 2.1 - Sociodemographic and clinical characteristics of women included in the study, excluded or who withdrew their participation after the screening phase.

Variables	Participants (N=110)	Excluded (N=77)	Withdrawn (N=65)	p-value
Age	M=33.01, SD=3.85	M=32.19 SD=4.63	M=31.92 SD=5.02	0.24
Education *				0.34
< 10 years	11 (10.0%)	8 (10.4%)	11 (16.9%)	
> 10 years	99 (90.0%)	67 (87.0%)	53 (81.5%)	
Family Socio-Economic Status (SES) *				0.02
Low	5 (4.5%)	2 (2.6%)	8 (12.3%)	
Middle	46 (41.8%)	32 (41.6%)	33 (50.8%)	
High	53 (48.2%)	33 (42.9%)	17 (26.2%)	
Marital Status *				0.92
Married	68 (62.7%)	45 (58.4%)	37 (56.9%)	
Cohabiting	39 (35.5%)	30 (39.0%)	26 (40.6%)	
Divorced	1 (0.9%)	0 (0.0%)	0 (0.0%)	
Single	1 (0.9%)	1 (1.3%)	1 (1.5%)	
Gestational age at screening	M=31.41, SD=1.40	M=31.62, SD=1.75	M=31.16, SD=1.78	0.24
Parity				0.87
Primiparous	99 (90.0%)	71 (92.2%)	59 (90.8%)	
Multiparous	11 (10.0%)	6 (7.8%)	6 (9.2%)	
Baby's gender				0.89
Male	59 (53.6%)	36 (46.8%)	31 (47.7%)	
Female	48 (43.6%)	38 (49.4%)	32 (49.2%)	
Unknown	3 (2.7%)	3 (3.9%)	2 (3.1%)	
Prenatal EPDS	M=5.37, SD=4.41	M=4.26, SD=3.09	M=5.56, SD=4.34	0.10
Prenatal STAI-T	M=36.38, SD=9.62	M=35.72, SD=8.67	M=37.38, SD=8.94	0.56

* Percentages for Education, Family SES and Marital Status do not add to100% due to missing values. EPDS, score at the Edinburgh Postnatal Depression Scale (Cox et al., 2010); STAI-T, score at the State-Trait Anxiety Inventory trait scale (Spielberger et al., 1970)

Excluded women as well as women who withdrew their participation did not differ significantly from participants on depression and anxiety scores and on any demographic variables with the exception of socioeconomic status, as assessed with the 9-point Hollingshead (1957) scale for parental occupation. Specifically, women who did not consent to participate to the follow-up assessment phases were more likely to be from a low socio-economic class than participants or women who were excluded ($\chi^2(4, N=229)=11.93$, $p=.02$). Nineteen caesarean sections and 1 intrauterine

death at 38 weeks due to umbilical cord accident were excluded from the postnatal phase of Chapter 3, while 1 woman withdrew her participation due to her newborn's health problems, leaving 89 women with available postnatal data for Chapter 3.

Sample for Chapter 4: From the initial sample of 110 women included in the prenatal phase, 1 intrauterine death at 38 weeks, 1 delivery in a different hospital and 3 women with no behavioral data (due to delay in giving notice of the delivery) were excluded from the study presented in Chapter 4. Additionally, 1 woman withdrew her participation after delivery due to her newborn's health problems. Thus, the sample for Chapter 4 consisted of 104 infants (mean postnatal age=1.96 days, SD=0.68; 51.9% males) and their mothers (mean age=33.04, SD=3.83). Eighty-six babies (82.7%) were born by vaginal delivery and eighteen (17.3%) by caesarean section. Two newborns were born respectively at 35 and at 36 gestational weeks but as they were in good health and did not require any intervention, they were included in the sample.

Sample for Chapter 5: From the initial sample of 110 women, one intrauterine death and 1 infant with serious health problems at the 12-weeks assessment were excluded from the initial sample. Additionally, 1 woman withdrew her participation after delivery due to her newborn's health problems. Thus, 107 infants (mean postnatal age=12 weeks, SD=1.84; 52.3% males) and their mothers were included in the study presented in Chapter 5.

Sample for Chapter 6: From the sample of 107 infants participating in Study 3, 3 women did not have data on maternal sensitivity because they did not attend the postnatal assessment session at the Medea Institute due to logistic reasons (i.e. living too far from the Medea Institute). Thus, the final sample for Study 4 consisted of 104 infants (mean postnatal age=12 weeks, SD=1.84; 51% males) and their mothers.

2.5 Assessment protocol

The assessment protocol of the phases of the EDI study included in the current dissertation is presented in Figure 2.1 and is summarized in what follows.

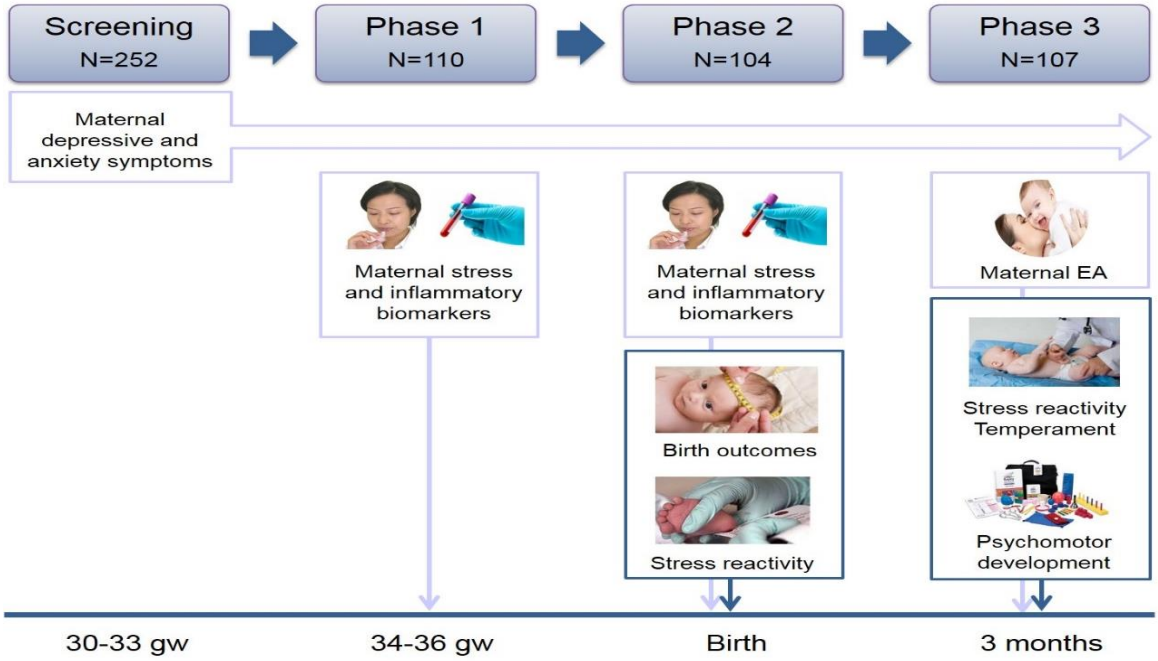

Figure 2.1 - Schematic representation of the longitudinal assessment protocol of the current dissertation. In light blue are reported maternal assessment, while in dark blue infant assessment

Screening phase: between 30-33 weeks of gestation, pregnant women filled out questionnaires on depression and anxiety and a demographic and pregnancy-related information form. Women who gave written informed consent to participation to the follow-up phases and fulfil the inclusion criteria were included in the study.

Phase 1: between 34-36 weeks of gestation women were invited to attend a morning session at the Medea Institute or at the hospitals involved. During this session, the Structured Clinical Interview for DSM-IV-TR Axis I Disorders (SCID-I; First et al., 2002) was administered, together with the Raven Standard Progressive Matrices (RSPM, Raven & Court, 1998). In addition, women were asked to donate a blood sample and to return their saliva samples collected during the previous days. In that occasion, women were reminded to inform the research team once their babies were born through a text message and to perform once again the saliva collection from the morning after delivery. Additionally, one week before the due date and, eventually, on due date, women were contacted once again to remind to give notice of delivery.

Phase 2: between 48-72 hours after delivery (mean hours after delivery=52.36;

SD=19.70), women were asked to fill in once again the questionnaires on depression and anxiety, to provide once again a blood sample and to collect their saliva samples beginning from the morning after delivery (mean hours after delivery=17.64; SD=10.16) according to the same procedures adopted during pregnancy. Women were also asked to fill out a form on health and delivery, while data on birth outcomes were extracted from medical records. Lastly, infants' behavioral and cortisol response to the heel-stick was assessed.

Phase 3: at 12 weeks of age, infants' behavioral and cortisol response to the inoculation was evaluated during the routine first inoculation visit at the infant's pediatric health center. Infant cognitive and motor development, as well as maternal sensitivity were assessed in a subsequent session at the Medea Institute. Additionally, women were asked to complete a form on infants' health and two questionnaires on infants' temperament, daily sleep and crying behaviour, as well as to fill out once again questionnaires on depression and anxiety.

Women's written informed consent was required at each phase of the study. In addition, written informed consent was required from both parents for the participation of their baby in each postnatal phase. The Ethics Committee of University College London, of Scientific Institute Eugenio Medea and of the hospitals and pediatric health centers involved approved the study protocol.

2.6 Maternal measures

Maternal depression was assessed through the *Edinburgh Postnatal Depression Scale* (EPDS; Cox, Holden, & Sagovsky, 2010) during pregnancy, after delivery and 12 weeks after delivery. The EPDS is the most widely used self-report questionnaire to screen for perinatal depression (Underwood et al., 2016). Although originally developed to screen for postnatal depression, it has been validated also for pregnancy, demonstrating excellent reliability and validity (Murray & Cox, 1990). As compared to other similar screening instruments, the EPDS is more specific to the

perinatal period and less reliant on somatic symptoms (such as sleep and appetite dysregulation) which are normative in pregnancy. It consists of 10 items each scored on a 4-points Likert scale ranging from 0 to 3, giving scores ranging from 0 (not depressed) to 30 (very depressed). Currently, there is no clear consensus about the most optimal cut-off to significantly screen for a risk of antenatal depression and a range of cut-offs have been employed (from 9/10 to 12/13). A continuous total sum score was employed in the main analyses because the study sample was a community sample drawn from the general population and the frequency of women scoring above the cut-off was expectedly low.

Maternal anxiety was evaluated in parallel with assessments of maternal depression through the *State-Trait Anxiety Inventory (STAI;* Spielberger et al., 1970) a well-validated self-report measure of anxiety. The STAI consists of a state (STAI-S) and a trait (STAI-T) subscale, each constituting of 20 items to be rated on a 4-points scale ranging from 1 'not at all' to 4 'very much'. The maximum score for each questionnaire is 80, with higher scores indicating higher anxiety. Trait anxiety or anxiety as a general trait reflects a dispositional anxiety proneness, while state anxiety measures anxiety at the moment of the rating and is supposed to reflect a transient anxiety condition. The STAI has been widely employed during pregnancy (e.g. Pluess et al., 2010; Davis et al., 2011), and has been translated and validated also in Italian (Pedrabissi & Santinello, 1989), showing good internal consistency with coefficients ranging from 0.89 to 0.91 and both construct and concurrent validity (Barnes, Harp, & Jung, 2002). In the current sample, trait anxiety as measured through the STAI, was strongly correlated with state anxiety on the STAI at all time points (pregnancy r=.78, delivery r=.64 and 12-weeks after delivery r=.73 p<.001), in line with previous reports (e.g. Huizink et al., 2017). The trait anxiety total sum score was employed in all main analyses because there is some evidence suggesting that it might account also for a proportion of pregnancy-related anxiety which is often found to be highly predictive of offspring outcomes in low risk samples (e.g. Tollenaar et al., 2011). Supplementary

analyses including measures of state anxiety rather than trait anxiety lead to substantially similar results as those using trait anxiety as a predictor.

Maternal diagnostic status was assessed during pregnancy through the *Structured Clinical Interview for DSM-IV-TR Axis I Disorders, Research Version, Non-Patient Edition (SCID-I RV-NP;* First et al., 2002). The SCID–I is a semi-structured diagnostic interview, widely used in psychiatric research studies. It consists of standardised diagnostic questions arranged in modules corresponding to each DSM–IV-TR Axis-I disorder (American Psychiatric Association, 2000). The test–retest reliability of SCID–I falls between reported values for similar instruments (First et al., 1995) . All women were interviewed by two trained clinical psychologists in order to ascertain the presence of a current depressive/anxiety disorder and exclude other diagnoses. Five women (4.5%) fulfilled the criteria for a diagnosis of Major Depressive Episode, while 4 women (3.6%) fulfilled the criteria for a diagnosis of Anxiety Disorder during pregnancy. Considering the low rate of clinically depressed or anxious women in the current study sample, which is in line with reported antenatal prevalence for these disorders in low-risk samples (Bennett et al., 2004), the diagnostic status was not employed in the analyses presented in the current dissertation and the SCID-I was employed exclusively to ascertain that women did not have any substance/alcohol abuse problems or chronic psychiatric disorders with the exception of depression and anxiety disorders.

Maternal cognitive function was assessed during pregnancy through the *Raven Standard Progressive Matrices (RSPM*, Raven & Court, 1998), a widely employed test that evaluate non-verbal general cognitive ability. It consists of 60 multiple choice items divided in five set of 12 items each administered in ascending order of difficulty. Each item requires participants to identify the missing element that completes a pattern from a number of options. The RSPM was administered individually by a clinical psychologist and administration time was limited to 30 minutes. The RSPM has shown good validity and acceptable test-retest reliability coefficients ranging from 0.76

to 0.91 in various cross-cultural studies of intelligence (Raven et al., 1998). In addition, although the RSPM offers substantially shorter administration, good correlations with other measures of intelligence and with educational level have been reported (e.g. Nikolaos et al., 2016; O'Leary, Rusch, & Guastello, 1991). The RSPM raw scores, as a measure of individual general cognitive ability, were employed in the analyses.

Maternal sociodemographic characteristics were evaluated at the screening phase through an ad-hoc form. Women were asked to report for themselves and for the father of their baby the following sociodemographic information: birthdate, nationality, education, actual employment, income, marital status, parity and, eventually, birthdate and sex of other children. Actual employment were used to calculate maternal, paternal and family socio-economic status (SES) according to the 9-point Hollingshead (1957) scale for parental occupation.

Health and pregnancy-related information were collected at the screening phase through an ad-hoc form. Specifically, participants were asked to provide data concerning: due date, date of last menstruation, fetal sex, natural conception, fertility treatment, any complication or risk or medical disorder related to pregnancy, any disease or dysfunction not related to pregnancy, parity, actual and pre-pregnancy weight and height, any medication during pregnancy (including over-the-counter drugs and vitamins, immunization shots -e.g. measles shot, a flu shot, a shot for Rh incompatibility-, antibiotics, antivirals or antifungals), use of cigarettes, alcohol or other substances during pregnancy, ever seek professional help for psychological problems. Self-reported height and weight were used to estimate pre-pregnancy BMI.

Health and delivery-related information were collected after delivery through an ad-hoc form. Participants were asked to report on: date and time of delivery, length of labor, induction of labor, type of delivery, assisted vaginal delivery, episiotomy, perineal tear, any stitches, any complications at site of episiotomy/stitches, anaethesia, rupture of membranes before delivery and eventual time, fever during

labor, any complication/abnormal sign/blood transfusion, any medication, expected time of discharge.

Maternal Emotional Availability was evaluated at the 12-weeks assessment during a 15-minutes videotaped free-play session. Mothers were left with their 3-month-olds in a room equipped with a standard set of age-appropriate toys and were told to interact with their child as they normally would do at home. The quality of maternal caregiving was evaluated through the *Emotional Availability (EA) Scales, Infancy/Early Childhood Version* (4th edition, Biringen, 2008), as described in section 3.5.3. The EA scales integrate attachment (Ainsworth et al., 1978) and emotional perspectives (Emde & Easterbrooks, 1985), emphasizing the emotional quality of the interaction. An increasing number of studies have employed this instrument to investigate caregiving behaviors and mother-child relationship both in typical and atypical populations (e.g. Lock & MacMahon, 2006, Trapolini et al., 2008, Austin et al., 2017). The EA scales have been validated also for very young children (Carter et al., 1998) and have been shown to consistently predict infants' attachment classifications (e.g. Easterbrooks, Biesecker, & Lyons-Ruth, 2000). The validity and stability of the EA construct over time have been well demonstrated (Biringen et al., 2014). The EA scales included four global ratings of caregiver behaviors, specifically: 1) Sensitivity, which refers to the caregiver's ability to be warm and emotionally connected with the child, show appropriate and authentic affect and be responsive to the child's signals; 2) Structuring, which refers to the caregiver's ability to appropriate scaffold and structure the child's play in a way that is well received by the child; 3) Non-intrusiveness, which refers to the caregiver's ability to be available to the child without interfering with the child's age-appropriate autonomy; 4) Non-hostility, which refers to a style of interaction with the child that is not negative, abrasive, impatient or antagonistic. Each scale is rated with a direct score ranging from 1 (non-optimal) to 7 (optimal), as well as along 7 sub-scales generating a total score ranging from 7 to 29. In order to maximize variability in the EA scores, total scores, rather than the direct

scores, were employed in data analyses as previously done (Austin et al., 2017). Additionally, as the four maternal EA scales were moderately inter-correlated (rs= 0.73–0.50) in order to reduce the number of variables for analyses, they were standardized and summed to create an overall index of maternal EA (Cronbach's α = .85), as done in prior work (Taylor-Colls & Fearon, 2015). Two clinical psychologists, both trained by Biringen and reliable in the use of the EA scale, 4th Edition, both with the author, Zeynep Biringen, and with each other, coded independently the videotaped interactions after the end of data collection. Both raters were blind to all prenatal and postnatal data. A subsample of 20 randomly chosen dyads (around 19.5% of the sample) were coded by both raters. Intra-class correlation (ICC) coefficients ranged from .75 to .91 with a mean ICC of .84.

2.7 Child measures

Birth outcomes were extracted from medical records after delivery. The following information concerning newborns' health were collected: sex, gestational age, weight, body length and head circumference at birth, Apgar scores, any evidence of fetal distress during labor, any fever, any evidence of infection or congenital anomaly, any medication, any treatment/intervention, breast or formula feeding, expected time of discharge were collected.

Health-related information were collected at 12 weeks through an ad hoc form that a researcher filled out with the mother. More specifically, the following information concerning physical health, feeding and sleep were collected: actual weight, body length, day spent in hospital after birth, any disease/disorder after birth, any medication or treatment from birth to date, any hospitalization/outpatient procedures/surgery from birth to date, actual health and health in the past 2 weeks, breastfeeding or formula feeding or mixed, daily feeding schedule, anyone smoking in family, any day-care.

Temperament was assessed at 12 weeks of age through the *Very Short Form*

of the Infant Behavior Questionnaire (IBQ-R-VSF; Putnam et al., 2014). The IBQ is a well-established standardized measure designed to evaluate individual differences in temperament in infants aged from 3 to 12 months by caregiver report. The IBQ-R-VSF consists of 37 items describing specific infants' behaviors in common situations. Parents are asked to report on a 7-point scale, which ranges from "never" to "always", the frequency of each behavior during the past 2 weeks. A "not applicable" response is also possible when the infant has never experienced the situation described. The items measured three broad, empirically derived, temperamental dimensions, namely negative affectivity, positive affectivity/surgency, and orienting/regulatory capacity, that are proposed to be comparable to three of the Big Five personality traits found in adults, Neuroticism, Extraversion, and Conscientiousness, respectively. The negative affectivity dimension measures sadness, distress to limitation and fear. The positive affectivity/surgency dimension included measures of approach, vocal reactivity, high-intensity pleasure, smiling and laughing, activity level and perceptual sensitivity. The orienting/regulatory capacity dimension evaluates duration of orienting, low-intensity pleasure, cuddliness and soothability. Levels of reliability and stability of the IBQ-R-VSF are similar to those obtained with the longer versions of the scale and other temperament measures (Putnam et al., 2014).

Crying was assessed at 12 weeks of age through the *Baby's Day Diary* (R. Barr, 1985), a 24-hour record of infant behaviors to be completed at home by the caregiver. The Baby's Day Diary has been previously employed with infants as young as 4 days (Fujiwara et al., 2011), as well as in the first months after delivery (Radesky et al., 2013). Mothers were instructed to record over a 24-hour period the durations of 6 infant behavioral states (i.e. awake and alert, awake and fussing, awake and crying, awake and inconsolable crying, feeding, sleeping). Inconsolable crying was defined as "infant crying that cannot be soothed." Total minutes of infant distress (fussing, crying, and inconsolable crying) per day and minutes of inconsolable crying per day were abstracted from the diary. The assessment of frequency and duration of crying, as

evaluated through the Baby's Day Diary, has been shown to strongly correlate with audiotape recordings (Barr et al., 1988; St James-Roberts, Hurry, & Bowyer, 1993). Additionally, the evaluation of infant's behavior through the Baby's Day Diary has been demonstrated to be independent from postnatal maternal depressive symptoms (A. R. Miller, Barr, & Eaton, 1993).

Mental and motor development was evaluated at 12 weeks of age through the *Bayley Scales of Infant and Toddler Development – Third Edition (Bayley III,* Bayley, 2006). The Bayley III are a well-standardized and widely employed developmental assessment. The examination was performed by two trained clinical psychologists blind to prenatal data, according to the standardized protocol. The Bayley III provides four types of norm-referenced scores: scaled scores for the 5 subtests (Cognitive, Expressive Communication, Receptive Communication, Fine Motor, Gross Motor), composite scores for cognitive, language, and motor domains, percentile ranks and growth scores. In the current study we focused on age-standardized composite scores for the Cognitive and Motor domain calculated by using test norms (mean= 100; SD= 15). The Bayley III have been widely employed in research and are generally recognized as the 'gold standard' tool to evaluate development of very young children (Bedford, Walton, & Ahn, 2013).

Stress regulation was assessed both at birth and at 12 weeks of age. On both occasions, infants' cortisol and behavioral responses to a painful stressor (i.e. heel-stick at birth and inoculation at 12 weeks of age) were evaluated, although the procedures were slightly different. Both the heel-stick and the inoculation have been previously extensively employed to evaluate infants stress reactivity (heel-stick e.g. Davis et al., 2011; Giesbrecht et al., 2017; inoculation e.g. Braithwaithe et al., 2016) and demonstrated to activate significantly the HPA axis soon after birth up through 6 months of age (for a review see Gunnar, Talge, & Herrera, 2009; Jansen et al., 2010). On both occasions, the entire procedure was videotaped to be later coded and efforts were made to keep infant's body, and specifically face, in full view. At birth, the

procedure included 10-minute baseline period, followed by the heel-stick and by 5-minutes recovery. The heel-stick was performed by a neonatal nurse: the baby's heel was disinfected with an alcohol swab, lanced with an automatic device and repeatedly squeezed to collect the blood sample. Average length of the heel stick was 3.99 minutes (SD=2.32). At 12 weeks of age, the procedure began with undressing of the baby until 2 minutes after the last needle was retracted. Specifically, the infant was undressed and laid down and either a doctor or a nurse administered two injections, respectively, for the hexavalent vaccine (tetani, diphtheria, pertussis, hepatitis B virus, polio virus, haemophilus influenza type B) and pneumococcal vaccine in the infants' thigh. Then the infant was given to the mother, who was free to soothe the baby. Mean duration of the shot administration was 1.06 minutes (SD=00.30). Videotaped infants behaviors were coded as described below.

2.8 Behavioral reactivity coding

Behavioral stress reactivity at birth. Newborns' behaviour in response to the heel-stick procedure was evaluated using a modified version of a coding system employed by Davis et al. (2011). Specifically, infants' videotaped behaviour during the heel-stick procedure was coded every 20-seconds on a 5-point scale (i.e. sleep, drowsy, awake and alert, awake and fussy, and crying). Despite the original system employed by Davis (2011) included two different levels of sleep, specifically, quiet and active sleep, after consulting with the author, the two categories were combined in a single category named sleep, as it was often hard to distinguish between the two types of sleep from video-recorded observation. The highest state observed during each epoch was coded. As the length of the heel-stick was variable, the first 2 minutes from the beginning of the blood draw were coded for all infants to ensure complete data for the whole sample. The average state score for each of the three phases (10-minutes baseline, 2-minutes response, 5-minutes recovery) was calculated. For approximately 10% of cases (N=10), two observers, blinded to all prenatal and postnatal information,

independently coded behaviour. Intra-class correlations were, respectively, equal to 1.0 for baseline, 0.99 for response and 0.99 for recovery, ps<.001.

Behavioral stress reactivity at 12 weeks of age. Infants' behaviour in response to the inoculation was evaluated continuously once the last needle was retracted for the subsequent 2 minutes at 5-seconds intervals according to Jahromi and colleagues coding system (2004). Specifically, infants' distress was coded on a 4-point scale indicating an increasing in the intensity of the negative affect: 0 (no vocalization), 1 (fussing, whining or whimpering), 2 (low-intensity crying), 3 (very intense loud crying with a out-of-control quality and typically accompanied by red face, squinted eyes and open mouth). The predominant (>2.5 seconds) level of intensity during each 5-seconds interval was scored. A measure of overall crying intensity was obtained by averaging scores across the intervals, while a measure of overall cry duration was calculated by adding the number of intervals during which the infant was distressed (i.e. received a rating >0) and multiplying by 5. The videotapes were coded by a trained graduate student with a MSc in Developmental Psychology, blinded to all prenatal and postnatal data. Twenty-one percent of the observations were coded by an independent trained coder. Intra-class correlations were 0.998, p<.001.

2.9 Biological measures

2.9.1 Samples collection

Infants' saliva collection. On both occasions, infants' saliva was collected by introducing a specifically designed swab (Salimetrics Infant Swab) in the infant's mouth until it was saturated. The saturated portion of the swab was then placed in a 5mL syringe to extract saliva by compression and immediately checked for saliva volume. This method of collection is safe and non-invasive, and has been shown not to be disturbing for the infants (e.g., Davis et al., 2004). Baseline salivary cortisol samples were collected before the beginning of the heel-stick after the baseline period at birth and right before entering in the doctor room for the inoculation procedure at 12

weeks of age. Additionally, saliva samples were collected after 20 and 40 minutes from the end of the procedures in order to capture the peak cortisol response to the painful stressor (Gunnar et al., 2009). Infants were not fed in the 30 minutes before the heel-stick (mean time from last feeding=68.50 minutes, SD=50.05) and before the inoculation (mean time from last feeding=102.99 minutes, SD=54.60), with the exception of 1 infant who was fed 11 minutes before the baseline saliva collection at the 12-weeks assessment. On both occasions, all infants were not handled during the study protocol besides that which was strictly required for the examination. Previous studies suggested that there is no diurnal rhythm of salivary cortisol shortly after birth, while an adult-type circadian rhythm appear to emerge around 2–3 months after birth (Spangler, 1991). Thus, concerted efforts with the paediatric health centres were made to arrange all inoculations in the morning between 09.00 and 12.00, with the exception of 4 infants who were examined at 2 pm. Time of the day was examined as a covariate in all cortisol models both at birth and at 12 weeks of age. In addition, information on additional variables potentially affecting cortisol concentrations, such as time of last feeding, time of last sleeping etc., were recorded on both occasions.

Maternal saliva collection. Women were asked to collect saliva samples on two consecutive days immediately upon awakening, 30 minutes after awakening and before going to bed between the 34th and 36th gestational weeks and the morning after delivery. The times of saliva collection throughout the day were chosen as they are thought to provide an index of cortisol and sAA diurnal pattern (O'Donnell et al., 2013), while minimising inconvenience for participants. During pregnancy samples were collected at home, while after delivery they were collected in the hospital, according to the same protocol. Participants were carefully instructed to collect saliva and were given a saliva sampling pack which included six 2.0 mL colour-coded polypropylene cryovials, six shorten plastic straws, instructions and a diary. Whole unstimulated saliva samples were collected by passive drool as generally recommended (Granger

et al., 2007). Specifically, women were instructed to: rinse out their mouth with water before saliva collection, allow saliva to pool in the mouth and gently drool through the straw into the tube, place it in their home refrigerator (or in the fridge of the maternity ward when the sampling occurs in hospitals) and fill in the diary. Additionally, participants were asked to avoid: eating, tooth-brushing and exercising in the 30 minutes before collection and eating a major meal in the hour before the evening sample. On the diary, participants were asked to report: date, time of collection, hours of sleep, nocturnal awakening, occurrence of eating/toot-brushing/exercising, medication, presence/absence of blood in the oral cavity or unhealed dental caries. Women were reminded of the collection through pre-scheduled phone callings and were instructed to employ a cooler for returning samples at the pregnancy assessment. All samples were kept on ice until reaching the laboratory. Times recorded on the diary were used as an index of compliance with sampling procedure and were check for correspondence to planned sampling time. During pregnancy the mean time from the awakening collection and the 30-minutes post-waking collection was 30.82 minutes on day 1 (SD=3.64, range: 20.00-60.00) and 31.44 minutes on day 2 (SD=6.81, range: 20.00-90.00). After delivery, the mean time from the awakening collection and the 30-minutes post-waking collection was 32.05 minutes on day 1 (SD=6.53, range: 20.00-65.00) and 35.89 minutes on day 2 (SD=18.53, range: 29.00-140.00). As 1 pregnant woman and 4 women after delivery, according to diaries, collected the second sample more than one hour from awakening, and the decline in cortisol usually occurs one hour after wake up, these samples were excluded from analyses (O'Donnell et al., 2013; O'Connor et al., 2014). In addition, in order to guarantee the data quality of salivary samples, all individual samples were visually inspected for contamination and checked for reporting of potentially interfering behaviors on the diary. All saliva samples were stored frozen at -80° until assayed. On day of assay, the saliva samples were thawed, vortexed and centrifuged at 1500 x g for 15 minutes in order to remove mucins.

Maternal blood collection. Blood samples were drawn by venipuncture by qualified nurse at the hospital or at the Medea Institute using specific polypropylene test tubes. Sample were kept refrigerated at +4°, following well-established practices (e.g. Skogstrand et al., 2008), until they reach the Biological Lab of Medea Institute were serum was centrifuged, aliquoted, and stored at −80 °C until analyzed. Diurnal variations in inflammatory markers have been reported, although phase estimates are conflicting (reviewed in Nilsonne et al., 2016). In order to attempts to account for diurnal variations, all blood sampling was conducted in the morning, with the exception of 8 postpartum samples collected between 14:00 and 16:00 due to organizational reasons. Time of the day was examined as a potential factor affecting inflammatory markers concentrations both during pregnancy and after delivery.

2.9.2 Biochemical assay

Salivary Cortisol Assay. Salivary cortisol assay was performed at the Biological Lab of Medea Institute using a high sensitivity enzyme immunoassay kit (Expanded Range High Sensitivity Cortisol EIA Kit, Salimetrics), specifically designed and validated for the quantitative measurement of salivary cortisol, following the protocol suggested by the manufecturer. This specific kit is a competitive immunoassay where cortisol in saliva samples and standards, competes with horseradish peroxidise-linked cortisol for antibody binding sites on a microtitre plate. After one-hour incubation, unbound components are washed away. Bound cortisol is measured by the reaction of the horseradish peroxidase enzyme to a second incubation (25 min) in the dark with tetramethylbenzidene that results in a blue color. This reaction is stopped by adding an acidic solution that produce a change in color from blue to yellow. The optical density is then spectrophotometrically measured in a standard laboratory plate reader at 450 nm. The amount of bound cortisol detected is inversely proportional to the amount of cortisol in the sample. The average optical density for all duplicate readings is then computed and the percent bound for each standard, control, and sample is computed

by dividing the optical density of each well by the average optical density for the zero. Then a standard curve is generated using a data reduction software and the concentrations of the controls and saliva samples is determined with reference to the optical density of the standards of known concentration. Results are computed in µg/dl. This assay has shown good sensitivity, with lowest limit of detection equal to 0.007 µg/dL. In addition, salivary cortisol assayed through this method has been shown to highly correlate with serum cortisol ($r(47)= 0.91$, $p<0.0001$). All samples from any individual participant were run on the same assay to minimize method variability. The assay requires 25µl of saliva; salivary cortisol assays were run in duplicate, except for 2 maternal samples, 10 infants' samples collected at birth and 8 infants samples collected at 12 weeks of age with minimal volume. Average intra- and inter-assay coefficients for maternal samples were <6% and <8%, respectively, and <7% and <10% for infants' samples.

Salivary AA Assay. Assay of sAA was performed at the Salimetrics Centre of Excellence testing lab at Anglia Ruskin University where maternal salivary samples were shipped in dry ice at the end of the data collection. Due to budget restrictions and limited saliva volume at delivery sAA was examined only in pregnancy. Samples were assayed for sAA using a kinetic enzyme assay kit (Salimetrics α-Amylase Kinetic Enzyme Assay Kit) specifically designed and validated for the kinetic measurement of sAA activity. This method requires 10 µL of saliva and employs a chromagenic substrate, specifically 2-chloro-p-nitrophenol linked with maltotriose. Before the beginning of the assay, saliva samples are diluted (with a final dilution of 1:200) using an automated liquid handler (Tecan Evo). Then the diluted saliva samples and controls are plated out by the liquid handler and the heated substrate is added to all wells using a Biohit 1200µl multichannel pipette. The enzymatic action of sAA on this substrate yields 2-chloro-p-nitrophenol, which is spectrophotometrically measured at 405 nm one minute and three minutes after the substrate is added in a 96-well microtiter plate with controls provided. The amount of sAA in the sample is directly proportional to the

increase in absorbance at 405 nm from one-minute reading to three minutes reading. Results are computed in U/mL. A random 10% of the sAA assays were run in duplicate to confirm reliability. The intra-assay coefficient of variation was < 3%.

Serum CRP and IL-6 assay. Serum CRP and IL-6 concentrations were assayed at LaboSpace in Milan where maternal samples were shipped in dry ice. Samples were assayed using Quantikine High Sensitivity ELISA kits (R&D Systems Europe, LTD), specifically designed for the quantitative measurement of IL-6 and CRP and widely employed in research (e.g. Blackmore et al., 2011, Coussons-Read et al., 2012). This method employs the quantitative sandwich enzyme immunoassay technique and requires 100 µL of serum for IL-6 assay and 50 µL of diluted (100-fold dilution) serum for CRP assay. On the day of assay, serum samples were thawed and bring to room temperature. Standards and serum samples are then pipetted into the wells of a microplate pre-coated with a monoclonal antibody specific for IL-6 or CRP and incubated for 2 hours at room temperature. Any IL-6 or CRP present in the samples is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-6 or CRP is added to the wells and incubated for other 2 hours. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. After one-hour incubation period, an amplifier solution is added and, during 30 minutes incubation, color develops in proportion to the amount of IL-6 or CRP bound in the initial step, until color development is stopped by a specific solution. The optical density is then measured using a microplate reader set to 490 nm for IL-6 and 450 nm for CRP. The duplicate readings for each standard, control and samples are then averaged and the average zero standard optical density is subtracted. Then a standard curve is generated through computer software to determine the CRP and IL-6 concentrations of the samples. The CRP concentrations read from the standard curve are then multiplied by the dilution factor. Results were computed in pg/mL for IL-6 and ng/mL for CRP. Both CRP and IL-6 assays have shown good sensitivity, with lowest limit of

detection equal to 0.039 pg/mL for IL-6 and 0.010 ng/mL for CRP. All samples from one participant were run in duplicate and on the same assay to minimize method variability. Intra-assay coefficient of variation (CV) was <6% for IL-6 and <3% for CRP, inter-assay CV was <10% for both markers.

2.10 Missing data

Attrition. The drop-out rate from the prenatal phase to the postnatal ones was very low. Specifically, less than 1% from phase 1 to phase 2, with only one woman withdrawing her participation at the postnatal phases. Additionally, there were no withdrawn from phase 2 to phase 3, although less than 3% of women from the initial sample attended only one of the two sessions included in phase 3. Women who were excluded or withdrew from each postnatal phase did not differ from participants on any demographic variables, depression or anxiety scores, thus suggesting no selective attrition within this sample.

Behavioral missing data. Data concerning infants' motor and cognitive development and maternal EA were available for 104 dyads out of 107, as the remaining dyads did not attend the second postnatal session at the Medea, scheduled 12 weeks after delivery, due to logistic reasons (i.e. living too far from the institute). In addition, both behavioral and biological data concerning the inoculation at 12 weeks of age were available for 94 infants out of 107, as for 2 infants' parents did not consent to the inoculation, while 11 infants were vaccinated in a district outside the province of Lecco and Como that were involved in the project and for which we gained the approval of their ethics committees'.

Biological missing data. All women provided completed salivary samples during pregnancy, while after delivery 68 women out of 89 women, included in the postnatal phase of Study 1 provided completed salivary data. Twelve women have uncompleted postnatal salivary data (specifically, 5 women had no data at bedtime on day 2 because of being discharged, 4 women were missing the 30 minutes post awakening

collection either on day 1 or 2, 2 women were missing data from 2 sampling occasions on day 1 and 1 woman provided no salivary data on day 2), while 9 women were not able to perform the saliva collection after delivery due to postpartum pain. In addition, 97 women out of 110 agreed to blood sampling in pregnancy and 66 out of 89 after delivery.

Concerning infants' cortisol data, at birth complete salivary data were available for 49 infants, while one or two sample were missing for 30 infants and 25 infants had no data due to insufficient saliva volumes. At the 12 weeks-assessment, complete cortisol data were available for 78 infants out of 94 undergoing the inoculation, while one or two sample were missing for 16 infants. Despite the amount of missing data for infants' salivary cortisol, particularly at birth, is considerable, it is not unusual in early infancy (e.g. Azak et al., 2013; Thayer & Kuzawa, 2014; Thayer & Kuzawa 2014) and is typically found in infants who are not familiar with pacifiers such as 48-72 hours-old newborns (Harmon et al., 2007; Goldberg et al., 2003; Granger et al., 2007). Although specific swabs designed for the collection of saliva in newborns (Salimetrics Infants Swabs) were employed in the current study, the nature of this problem involves that saliva is very scarce in newborns soon after birth (Gunnar, 1992) and volumes become even less after stress. While saliva stimulants might be an option, they have been shown to compromise sample integrity and interfere with analytical methods (Schwartz et al., 1998). It is generally acknowledged that saliva collection through a stress-free non-invasive method in very young research participants is challenging (e.g. Granger et al., 2007) and, indeed, there is a paucity of work assessing salivary cortisol in newborns soon after birth although it represents a unique opportunity to investigate fetal programming processes independently of postnatal influences (Egliston, McMahon, & Austin, 2007). As will be discussed later, multilevel models were chosen as they also allow to better handle missing data by maximizing all valid data points.

Non-compliance analyses. In order to control for the missing data issue post-hoc, non-compliance analyses were run to check for differences between children with complete, partial or missing behavioral/biological data on any sociodemographic, maternal, infants' or situational (e.g time of the day, length of the inoculation etc.) variables. No significant differences were found, suggesting that there was no systematic pattern of missing data. Women who did not agree to blood sampling did not differ from women who provided blood samples on demographic and pregnancy or delivery-related variables, depression or anxiety scores, with the exception of maternal age, with younger women being more likely to refuse antenatal blood sampling as compared to older women ($F=4.08(1, 108)$, $p=.05$). No differences between women with complete, partial or missing salivary data after delivery on any sociodemographic or delivery-related variables was found, suggesting that there was no systematic pattern of missing data.

2.11 Statistical analyses

2.11.1 Data transformation.

In all studies, numeric variables were first examined for outliers and skewness. Specifically, samples greater than 3 SD from the mean were removed and variables were tested for normality through the Kolmogorov-Smirnov Test. Skewed data were natural log transformed prior to analysis to approximate normal distributions. Model parameters for ln-transformed values are presented in tables, while non-transformed values are employed in the descriptive statistics table and figures to facilitate interpretation.

Given the low rate of women scoring above the EPDS cut-off and the focus of the current dissertations on naturally occurring variations in maternal symptoms across the whole normative range, the total score of the EPDS or STAI was employed as a continuous variable in the analyses.

Four summary parameters were calculated to index different aspects of maternal cortisol and sAA diurnal activity, namely, waking levels, response to awakening, diurnal slope and total diurnal output. Specifically, as cortisol and sAA values at each time points across the two days were highly correlated (rs between .51 and .59 for cortisol and .55 to .78 for sAA), they were averaged over days for each time points (i.e. wake, 30 min post wake and bedtime) and mean values were used as done in prior works (e.g. Pluess et al., 2010; De Weerth and Buitelaar, 2005, Braithwaithe et al., 2015). Response to awakening was calculated by subtracting waking values from the 30min post-waking levels, while diurnal slope was calculated by subtracting the bedtime values from the waking values, as done in several prior studies (e.g. de Weerth et al., 2013). Additionally, daily average cortisol and sAA were calculated as the area under the curve (AUCg) using the trapezoid method with respect to the ground (Pruessner et al., 2003) for each day separately and, as the two values were highly correlated ($r=.58$, $p<.001$, for cortisol, $r=.74$, $p<.001$ for sAA), the mean of the two days was used (e.g. Shea et al., 2007). These composite measures are widely employed (e.g. De Weerth et al., 2013; Giesbrecht et al., 2017) and are thought to reflect different aspects of cortisol and sAA physiology. In particular, the response to awakening and diurnal slope reflect diurnal changes related to the HPA axis or SNS daily functioning, while the AUCg provide unique information about the overall secretion over the day (Adam & Kumari, 2009). As reported in previous studies (e.g. De Weerth et al., 2013), the awakening response and diurnal decline were moderately interrelated suggesting that a greater response to awakening was related to a flatter diurnal slope both for cortisol and sAA (respectively, $r=-.47$ and $r=-.55$, $p<.001$).

2.11.2 Preliminary analyses.

Unadjusted associations between maternal antenatal variables and infant outcomes, as well as among maternal biological markers and self-reported depressive

symptoms were explored using Pearson product-moment correlation coefficient. Additionally, in all studies, preliminary analyses were performed to identify potential confounders of the associations under investigation. Specifically, in Chapter 3, the potential effects of variables known to potentially affect maternal stress and inflammatory markers concentrations were examined. These included demographic and pregnancy-related variables such as age, marital status, employment, income, education, SES, weeks of gestation, foetal sex, parity, body mass index (BMI) actual and prior to pregnancy, medications and use of prenatal vitamins, sampling time. Pre-pregnancy BMI was highly correlated with current BMI ($r=.95$ $p<.001$). Because current BMI was influenced by pregnancy-related weight gain, pre-pregnancy BMI was employed in all subsequent statistical analyses. Additionally, recent food or drink intake, brushing teeth, physical activity, dental work, presence of blood in the oral cavity, smoking, disrupted sleep, hours of sleep, spontaneous awakening, working versus weekday of collection, wake time, sampling time and time between collections were all examined as potential confounds for the salivary cortisol and sAA assay. The occurrence of eating and brushing teeth shortly before sampling were rare both during pregnancy and after delivery (respectively, 0.9-3.6% during pregnancy and 2.6-7.8% after delivery for eating, while 0.9-9.1% and 6.6-7.9% for brushing teeth). Drinking water within 30 minutes of sample collection was more common, 5.5-15.5% during pregnancy and 7.6-23.7% after delivery. Lastly, a series of variables related to labor and delivery were examined as potential confounds on biological assay performed postpartum. Specifically, length of pregnancy, length of labor, induction of labor, amniotomy, episiotomy, perineal injury, vacuum-assisted delivery, epidural anhestesia, fever during labor, any complication, blood transfusion, medication, time from delivery and breastfeeding were included

In Chapter 4, 5 and 6 the effects of sociodemographic factors (e.g. maternal age, marital status, education and SES), pregnancy- and delivery-related factors (e.g. parity, mode of delivery, assisted delivery, length of labor), infant factors (e.g.

gestational age, birth weight, gender, Apgar scores, postnatal age), situational factors (e.g. length of the heel-stick procedure, time of the day and time from last feeding) on child outcomes were examined. In additions, in Chapter 5 and 6 the potential effect of maternal postnatal symptomatology was also investigated. The associations between continuous confounders (e.g. maternal age) and outcomes were measured using Pearson product-moment correlation coefficient, while the effects of categorical confounders (e.g. fetal sex) on the outcomes investigated were examined through univariate analysis of variance (ANOVA). All variables found to be significantly associated with levels of biological markers were included in the following models as covariates.

2.11.3 Hierarchical Regression Analyses

In Chapter 4, 5 and 6 separate hierarchical regression analyses were performed in order to evaluate the effects of maternal variables (i.e. antenatal depressive symptoms, antenatal stress and inflammatory markers, postnatal EA) on all infants' outcomes investigated, with the exception of stress reactivity, while adjusting for covariates. In chapter 4 and 5, covariates were entered in the first step. The EPDS scores was entered in a second step to assess the independent effect of prenatal maternal depressive symptoms on birth outcomes, while the biological markers concentrations were included in a third step to evaluate the unique contribution of maternal antenatal physiology to child outcomes. In Chapter 6, prenatal maternal variables were entered in a second step, following covariates, jointly with maternal EA scores, while the interacting effect of maternal prenatal and postnatal variables in predicting child outcomes was evaluated in the last step.

Preliminary analyses and Hierarchical Regression Analyses were performed using SPSS 24. All statistical tests were two-sided and a $p<.05$ was considered statistically significant.

2.11.4 Hierarchical Linear Models (HLMs)

Hierarchical Linear Models (HLMs) were employed in all studies in order to model diurnal or stress reactivity data. While study-specific aims and model fitting are described in each study, the general principles and advantages of HLMs are briefly discussed in what follows.

HLMs (Raudenbush & Bryk, 2002), also often referred as Multilevel Models (H. Goldstein, 2011) or Mixed Models (Little et al., 1996), are increasingly employed in data analysis because they provide a powerful tool to model data simultaneously at different levels (i.e. between occasions and between individuals), to estimate how much variance can be attributed to each of these levels, and to investigate whether and how predictors of interest can predict variations at these different levels (Goldstein, 2011; Singer et al., 2003; Steenbergen & Jones, 2002). As compared to conventional techniques, such as repeated measures ANOVA, they present several advantages, including that they do not require each individual to have the same number or complete observations or observations regularly spaced in time (Goldstein, 2011). Furthermore, HLMs allow to accurately model nested data (Goldstein et al., 2011). Typically, neuroendocrine data, such as cortisol or sAA diurnal data, are characterized by a hierarchical structure. For example, samples from one individual are collected on a number of occasions during the day (e.g. wake up, 30 minutes after waking, bedtime) over repeated days (e.g. day 1 and 2). Thus, as shown in Figure 2.2, sampling occasions (level 1, within-day variance) are nested within two time-points (level 2, between days variance) and time-points are nested within individuals (level 3, between-individuals variance). HLMs assume that the total variance in data can be partitioned at different levels and allow to compute the variance at each level relative to the total variance (Hruschka, Kohrt, & Worthman, 2005). A clear partition of the sources of variance is fundamental to avoid incorrectly interpreting within-individual variations as an indication of between individual differences (Hruschka et al., 2005). HLMs allow to attribute the total variance in cortisol or sAA diurnal data to one of three

levels (i.e. within-day, between days and between individuals) and estimated the relative contribution of each level to the total variance in cortisol or sAA data can be obtained. Similarly, data on acute stress reactivity, whether behavioral or physiological, are characterized by a nested structure in their design. There are typically repeated sampling occasions (e.g. baseline, response and a recovery, level 1) nested within individuals (level 2). HLMs can be a useful tool to model the individual pattern of response to stress, while accounting for the nested structure of the design.

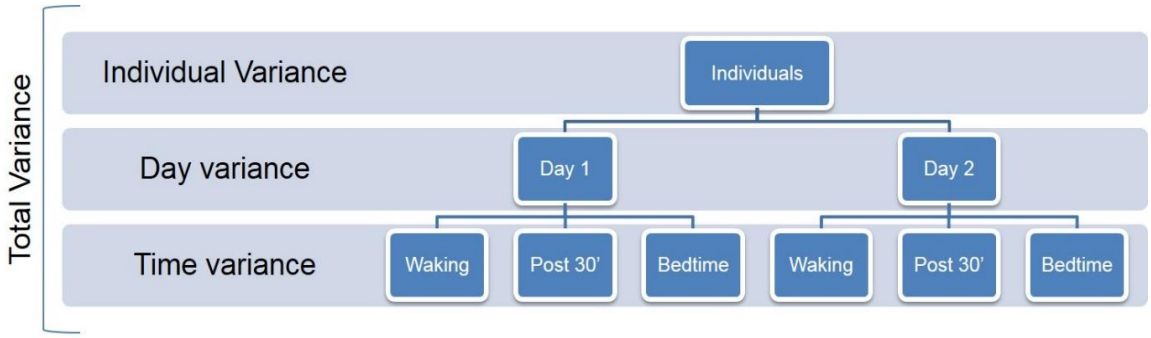

Figure 2.2- Illustrative hierarchical model for salivary cortisol or AA diurnal data

Moreover, HLMs allow to compare the individual diurnal profiles or trajectories of response, rather than just probing association with mean levels at a given time point, while controlling for the effects of initial values. In particular, with HLMs the influences of predictor variables on parameters that are relevant to cortisol or sAA diurnal or reactivity profile can be examined. In Study 1, three cortisol or sAA parameters were tested for association with the predictors investigated: 1) intercept, representing waking level; 2) morning slope representing the change from waking to 30 minutes after; 3) afternoon slope representing the slope to bedtime. In Study 2, 3 and 4, the following parameters of infants' cortisol/behavioral reactivity were examined: 1) intercept, representing baseline level; 2) linear slope, representing the linear increase/decrease in response to stress; 3) quadratic slope, representing the curvature of the trajectory of stress response.

An additional strength of HLMs is the ability to explore both fixed and random effects. Similarly to conventional analyses, such as linear regression, HLMs allow to determine the relationship between a predictor variable and an outcome of interest (fixed effect). Additionally, it allows to determine if this effect varies from participant to participant (random effect). For example, when examining cortisol diurnal pattern, as shown in Figure 2.3, the inclusion of a random intercept allows for between-person variability in cortisol waking values, while the inclusion of a random linear slope allows for between-person variability in the diurnal cortisol slope.

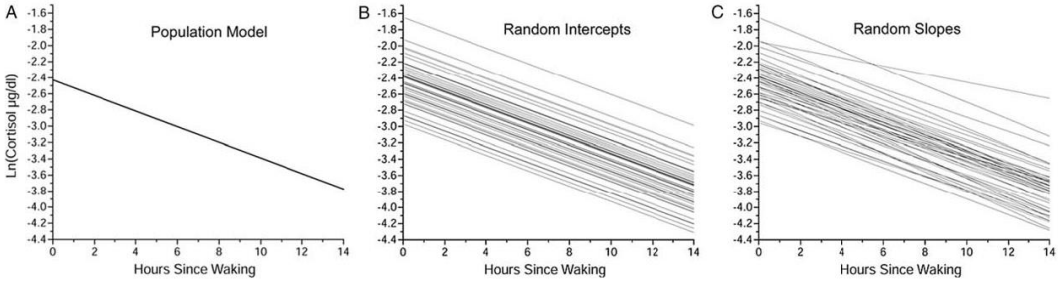

Figure 2.3 - Increasing model complexity for diurnal cortisol data. Model A: population average diurnal slope (typically negative). Model B: individual means over the day (random intercept). Model C: individual means and diurnal slopes (random intercept and random linear slope). (From Hruschka et al., 2005)

An additional advantage of HLMs is its robustness for handling missing data (Tabachnick & Fidell, 2007) by maximizing all valid data points. Indeed, HLMs do not rely on means values and do not exclude a case if there are any missing data for that case, rather all model parameters are modelled using all available data.

Study-specific model fitting is described in each study methods section, however, in all studies model fitting proceeded in three stages. First, unconditional means models were computed to evaluate the relative contributions of each level (e.g. within-day, between days and between individuals) to the total variance. Secondly, baseline models including all the parameters of interest that describe individual trajectories were fitted. Third, explanatory models including level-2 predictors

(beginning with covariates and then adding predictors of interest) were fitted. Explanatory variables were centered around the grand mean and entered in the model one-by-one. Lastly, cross-level interactions between level-2 predictors of interest and level-1 parameters are included to evaluate the extent to which intercepts and slopes vary accordingly to level-2 predictor. Model fit was tested with likelihood deviance difference test for nested models. Specifically, variables were kept in the model when their presence resulted in a significant ($p<.05$) reduction of the likelihood ratio statistic. To test the statistical significance of the regression coefficients, the Wald's chi-square test was performed. All HLM analyses were carried out using MLWiN 2.29. All statistical tests were two-sided and a $p<.05$ was considered statistically significant.

2.11.5 Supplementary analyses

Given the high correlation between antenatal depression and anxiety scores, comparable analyses with anxiety scores as individual-level predictor, rather than depression were performed in all studies. In order to avoid multicollinearity issues and as maternal anxiety and depression are expected to differ in their underlying biology (O'Donnell & Meaney, 2017), they were entered separately in all models. Analyses including maternal anxiety rather than depression are reported in Supplementary Sections.

In addition, in Chapter 3, graphical analyses were used to explore the relationship between stress and inflammatory markers among women with higher versus lower depressive symptoms according to the EPDS median.

Chapter 3: Exploring the biological underpinnings of perinatal depression through a multisystem approach.

3.1 Introduction

Pregnancy and the postpartum period are considered times of heightened risk for women to develop depression (Kessler, 2003), with 8-17% of women in high-income countries experiencing depressive symptoms antenatally, and around 9-13% in the twelve months following delivery (for a review Woody et al., 2017). There is now considerable evidence of an association between depressive symptoms during the perinatal period and a wide range of adverse physical and psychological outcomes for both women and their offspring (reviewed in Gentile, 2017). Thus, in the light of the substantial personal, social and economic burden of perinatal depression, understanding possible risk factors, mechanisms and treatment is a key priority for maternal-child health worldwide.

The major physiological changes and hormonal fluctuations occurring during pregnancy and after delivery have been argued to play a role in increasing women's vulnerability to developing depression during the perinatal period (Kammerer, Taylor, & Glover, 2006; Corwin & Pajer, 2008). For this reason, in the last decades, the attention of scholars has been focused on identifying the biological factors that might be involved in risk for perinatal mood disorder and potentially relate to poor obstetric outcomes and alterations in offspring development. To date, the most studied candidate has been the Hypothalamic-Pituitary-Adrenal (HPA) axis, followed by the Inflammatory Response System (IRS). In contrast, the Sympathetic Nervous System (SNS), which is thought to play a crucial role in the stress response jointly with the HPA axis, has been much less investigated. As described in Chapter 1, the regulation of all these systems changes dramatically during pregnancy and after delivery in order

to support fetal development and prepare for childbirth and nursing. In light of the major neuroendocrine and immune alterations occurring during the perinatal period, it has been questioned whether well-established research findings of an association between depressive symptoms and an anomalous functioning of the stress and inflammatory response systems in non-pregnant adults (e.g. Dowlati et al., 2010; Knorr et al., 2010), might extend to pregnant women. That is, the association between depressive symptoms and levels of stress or inflammatory markers may be different in pregnancy or may be confounded by the substantial neuroendocrine resetting associated with pregnancy, thus making research into psychobiological factors associated with perinatal depression a complex as much as intriguing challenge. In the next sections, we will examine available evidence on the relationship between perinatal depression and the functioning of the stress response system (SRS) and IRS.

3.1.1 HPA axis and perinatal depression

Dysregulation of the HPA axis, as indexed by altered cortisol levels in most studies, in depressed non-pregnant adults is among the most consistent and robust findings in psychiatry and it has been proposed as a possible biomarker for depression (Holsboer, 2000; Ising et al., 2007). A meta-analysis by Knorr and colleagues (2010) including 20 case-control studies, found a small but significant increase in both morning and evening salivary cortisol levels in depressed patients as compared to controls. However, the picture is far less coherent in pregnancy where either no significant associations between basal levels of maternal cortisol and depressive symptoms (e.g. Hellgren et al., 2013; Katz et al., 2012; Bleker et al., 2017; Iliadis et al., 2015) or slight positive associations during specific gestational time windows have been reported (e.g. Voegtline et al., 2013; Field et al., 2006; Lommatzsch et al., 2006). Furthermore, a small number of studies on low risk samples found lower levels of cortisol in pregnant women with depressive symptoms (e.g. Harris et al., 1996;

Tsubouchi et al., 2011). In a recent systematic literature review, Seth and colleagues (2016) selected “higher quality” studies, based on the quality of samples and assessments as well as greater statistical power, and concluded that there is no evidence of an association between antenatal depression and cortisol levels during pregnancy. The association between cortisol and postnatal depressive symptoms is even more uncertain. The majority of studies have focused on non-clinical samples; among these, a number of studies reported higher cortisol levels in women with depressive symptoms from 1 to 8 weeks postpartum (e.g. Taylor et al., 2009; Iliadis et al., 2015; Nierop et al., 2006), while others found either lower cortisol levels (e.g. Groer & Morgan, 2007) or no association at all (e.g. Shimizu et al., 2015; Davis et al., 2007; O’Keane et al., 2011).

Despite extensive research investigating the associations between perinatal depression and cortisol (reviewed in Orta et al., 2018; Seth et al., 2016), mood-related alterations in the diurnal cortisol pattern have been poorly investigated. A lower waking cortisol level (O’Connor et al., 2014), a blunted cortisol awakening response (CAR; Osborne et al., 2018), a flatter diurnal decline (O’Connor et al., 2014), increased evening (O’Keane et al., 2011; Osborne et al., 2018) and averaged daily cortisol (O’Connor et al., 2014; Osborne et al., 2018) in clinically depressed pregnant women have been found in some studies, though not all (Shea et al., 2007; Hellgren et al., 2013). Likewise, a blunted CAR have been reported in depressed women at 6-8 weeks (Taylor et al., 2009) and 6 months (De Rezende et al., 2016) postpartum, whereas Corwin and colleagues (2015) reported higher daily cortisol in depressed women 14 days, but not 7 days or 6 months after delivery. Conversely, studies on non-clinical samples have failed to detect any associations between depressive symptoms and diurnal cortisol measures both antenatally (Rash et al., 2015; Heuvel et al., 2018) and postnatally (Cheng & Pickler, 2010, Scheyer & Urizar, 2016). Lastly, few studies have investigated the associations between cortisol levels and depressive symptoms at both points - antenatally and postnatally (O’Keane et al., 2011; Iliadis et al., 2015;

Scheyer & Urizar 2016), and they have all adopted a cross-sectional design, thus it is still unknown whether antenatal depressive symptoms are associated with changes in diurnal cortisol from pre- to post-pregnancy.

3.1.2 SNS and perinatal depression

Despite existing evidence for a dysregulation of the SNS, as indexed by higher levels of catecholamines (e.g. Veith et al., 1994), in depressed adults, few studies have evaluated the association between SNS activity and perinatal depression, and these have yielded mixed results. Higher norepinephrine and lower dopamine levels have been found in depressed pregnant women in some studies (Lundy et al., 1999; Field et al., 2004; Diego et al., 2006), while others failed to find any association (Field et al., 2006; Shimizu et al., 2015). Recently, salivary α-amylase (sAA), an enzyme produced by the salivary glands, has been proposed as a non-invasive and reliable marker of SNS activity (Nater & Rohleder, 2009). A small number of studies have evaluated sAA levels in depression and reported generally higher sAA levels in patients with major depression compared to controls (Tanaka et al., 2012; Veen et al., 2013; Ishitobi et al., 2010; Booij et al., 2015) and remitted patients (Bagley et al., 2011; Veen et al., 2013; Ishitobi et al., 2010). Although sAA has been shown to provide a convenient index of SNS even in pregnancy (Nierop et al., 2006), to our knowledge, only two studies have evaluated the association between prenatal sAA diurnal levels and depression. Specifically, Braithwaite and colleagues (2015) found higher sAA levels at awakening in a very small sample of women reporting higher depressive symptoms (N=9), as compared to non-depressed controls (n=26), in the third trimester of pregnancy but not in the second. However, the small sample size, particularly of the at risk for depression group, the very large variability in sAA concentrations reported in the study and the use of composite measures to index sAA diurnal pattern constitute limitations of the study and limit the impact and generalizability of the findings.Giesbrecht and colleagues (2013) examined the association between

maternal mood and diurnal sAA in a community sample of 83 pregnant women using multilevel models. The authors found no association between the average sAA trajectory throughout the day and depression, although momentary depression, positive mood and trait anxiety were all positively associated with increases in sAA over the day during pregnancy.

3.1.3 IRS and perinatal depression

Immune dysregulation is increasingly regarded as a central mechanism in the pathophysiology of major depression (Miller et al., 2009). Recent meta-analytical findings have pointed out significant associations between depression and increased levels of a number of inflammatory markers, including pro-inflammatory cytokines, such as interleukine-6 (IL-6), or acute phase proteins, such as C-reactive protein (CRP), in depressed non pregnant adults (e.g. Valkanova et al., 2013; Dowlati et al., 2010; Liu et al., 2012). On the basis of this evidence, several studies over the last two decades have sought to elucidate the link between inflammation and perinatal depression, although findings do not yet allow firm conclusions to be drawn (see Osborne & Monk, 2013 for a review). IL-6 and CRP, which have been found consistently increased in patients with depression (Valkanova et al., 2013), are among the most studied markers. Either positive (e.g. Scrandis et al., 2008; Christian et al., 2009; Liu et al., 2016) or null associations (e.g. Blackmore et al., 2011; Buglione-Corbett et al., 2018; Skalkidou et al., 2009) between depressive symptoms and IL-6 and/or CRP levels have been reported during pregnancy, as well as following birth-to-6 months postpartum. Few studies have investigated the cross-sectional relationship between depression and inflammation both antenatally and postnatally (Accortt et al., 2016; Bränn et al., 2017; Corwin et al., 2015; Maes et al., 2000; Scrandis et al., 2008) and, to our knowledge, only Osborne and colleagues (2019) have examined how changes in cytokines levels across the perinatal period relates to depressive symptoms. Those authors reported a greater increase in pro-inflammatory cytokines

levels, including IL-6, from the second to the third trimester of gestation, followed by a greater decrease at 6 weeks postpartum in a small group of women (N=12) with higher antenatal depressive symptoms, as compared to non-depressed women (N=37).

3.1.4 Coordination between SRS and IRS

Besides their individual effects, the stress and inflammatory response systems exhibit significant bidirectional interactions (Kuhlman et al., 2017). The principal stress hormones, glucocorticoids and catecholamines, exert regulatory influences on the functioning of the immune system (Elenkov & Chrousos, 2006). In particular, glucocorticoids have been shown to down-regulate inflammation within a well-documented cytokine-glucocorticoid feedback circuit (Elenkov, 2008). Failure of glucocorticoids to limit inflammation (e.g. Miller, Cohen, & Ritchey, 2002), leads to dysregulation of both cytokines production and cortisol secretion (Corwin et al., 2013) and is hypothesized to play a role in the development of several disorders (Raison & Miller, 2003), including depression (Pace, Hu, & Miller, 2007; Pariante, 2017). More than two decades ago, Chrousos and Tsigos proposed that a disruption of the bidirectional relationship between inflammation and the HPA axis might increase vulnerability to developing postpartum depression (Chrousos, 1995; Tsigos & Chrousos, 2002). However, to date, a limited number of studies investigated concurrently the functioning of the SRS and IRS during the perinatal period as well as possible associations with symptoms of depression. Walsh and colleagues (2016) found a positive association between cortisol and IL-6 levels in a high-risk sample of pregnant teenagers, while Shelton and colleagues (2015) reported an inverse association among cortisol and pro-inflammatory cytokines concentrations in a low risk sample of pregnant women. However, none of these studies investigated possible link with depressive symptoms. To our knowledge, only Corwin and colleagues (2015) has investigated the interplay between cytokines and cortisol in influencing mood. They found a positive association between pro-inflammatory cytokines and cortisol two

weeks after delivery in women who were experiencing significant postnatal depressive symptoms, while no association in euthymic women, suggesting that variability in the bi-directional interactions between the HPA-axis and IRS soon after delivery might influence the risk for postpartum depression.

3.2 Summary and study hypotheses

Headway has been made towards a better understanding of the link between depressive symptoms and an altered functioning of the neuroendocrine and immune systems during the perinatal period, however many questions remain unanswered. Despite mounting evidence for a role of a dysregulation of the stress and inflammatory response system in the pathophysiology of depression outside the perinatal period (e.g. Juruena et al., 2018; Khulman et al., 2017; Pariante et al., 2017), evidence of an association between perinatal depressive symptoms and an alteration of these systems during pregnancy and after delivery is mixed. Heterogeneity in the assessment of mood, as well as in timing of assessment (from early pregnancy up to months after delivery) makes comparisons across studies within the existing literature difficult. Similarly, large variability in cortisol and sAA diurnal measures may play a role in findings inconsistencies with studies differing in the selection of composite scores (e.g. CAR, decline, etc.) and in the way they are calculated. More advanced statistical techniques, such as multilevel models, have been recommended for analysing repeated measures of stress markers (Hruschka et al., 2005; Zijlmans et al., 2015) and might help to detect alterations in cortisol or sAA diurnal pattern related to perinatal depressive symptoms, yet they are scarcely applied. Additionally, while it is now increasingly acknowledged that the stress and immune systems extensively interact with each other to prepare the organism to adapt in the face of challenge (e.g. Chrousos & Kino, 2005), the majority of studies focused on a single biological system, under the assumption that a dysfunction of this system might be a central mechanism involved in perinatal depression. However, inconsistent findings in the literature might

suggest that a better understanding of the link between perinatal depressive symptoms and altered psychophysiology may be obtained by measuring concurrent activity across multiple systems. To date, several studies have investigated associations between perinatal depressive symptoms and either HPA axis or IRS functioning; however, few studies have investigated the concurrent activity of these two systems (Corwin et al., 2013; 2015; Shelton et al., 2015; Walsh et al., 2016) and, to our knowledge, none have included SNS markers. Lastly, studies have largely been cross-sectional, thus the relationship between mood and change in biomarker levels over the perinatal period is mostly unknown.

The current study begins to fill these gaps by investigating the cross-sectional and prospective associations among depressive symptoms and variations in multiple stress (i.e. diurnal cortisol and sAA) and inflammatory (i.e. IL-6 and CRP) markers in late pregnancy and after delivery. Based on existing evidence, we hypothesized that higher depressive symptoms would be associated with heightened inflammation and an altered cortisol diurnal pattern, as detected through multilevel models. Conversely, no formal hypothesis was made regarding the association between depressive symptoms and diurnal sAA due to limited available literature. Additionally, supplementary analyses investigated an exploratory hypothesis and examined the robustness of the associations found for depression. Specifically, we sought to extend findings from Corwin and colleagues (2015) by exploring the association among stress and inflammatory markers in women with higher versus lower depressive symptoms pre- and post-partum. Based on findings from Corwin and colleagues (2015), a positive association between inflammatory markers and stress hormones in women with higher depressive symptoms would be expected. Furthermore, as perinatal depression and anxiety are strongly correlated (Falah-Hassani et al., 2017), we explored whether the observed biological alterations were specific to depression or can be replicated for anxiety.

3.3 Material and methods

3.3.1 Participants

Women in the early third trimester of gestation were consecutively recruited at the beginning of child birth classes or through ads placed in clinic waiting rooms of 3 hospitals located nearby the Medea Institute in Italy, as part the Effects of Depression on Infants (EDI) Study, a wider longitudinal project investigating the effects of maternal antenatal depressive symptoms on newborn's bio-behavioral development.

Two hundred and fifty-two women participated in the screening phase (weeks of gestation: M=31.41, SD=1.61). Prenatal inclusion criteria were: aged 18-45 years, normotensive, with singleton uncomplicated pregnancy, non-smoker, not afflicted by any disease, not taking any chronic medications, and with no known substance/alcohol abuse problems or chronic psychiatric disorders (except for depression and anxiety). From the initial sample of 252 women, 77 women were excluded because they did not meet the inclusion criteria and 65 women did not agree to participate in the subsequent study phases. Thus, the final sample consisted of 110 women (mean age= 33.00; SD= 3.85). Excluded women as well as women who withdrew their participation did not differ significantly from the remaining participants on depression and anxiety scores or on any demographic variables with the exception of socioeconomic status, as assessed with the 9-point Hollingshead (1957) scale for parental occupation. Specifically, women who did not consent to participate in the follow-up assessment phases were more likely to be from a low socio-economic class than participants or women who were excluded ($\chi^2(4, N=229)=11.93$, $p=.02$). The majority of participants were Italian (96.4%), middle-upper class (90%), as assessed by the 9-point Hollingshead (1957) scale, and at their first pregnancy (90%). The average score on the EPDS was 5.37 (SD= 4.41) during pregnancy and 5.53 (SD= 4.77) after delivery; 19 women (17.3%) scored at or above a clinical cut-off of 10 during pregnancy and 18 (16.8%) after delivery.

Nineteen caesarean sections and 1 intrauterine death at 38 weeks due to umbilical cord accident were excluded from the postnatal phase while 1 woman withdrew her participation due to her newborn's health problems. Thus, data from 89 women were available at the postnatal phase. Women who were excluded from the postnatal phase did not differ from participants on any demographic variables, depression or anxiety scores.

3.3.2 Procedure

Pregnant women, between 30-33 weeks of gestation, filled out the Edinburgh Postnatal Depression Scale (EPDS), the State-Trait Anxiety Inventory (STAI) and a demographic and pregnancy information form. Women who gave written informed consent to participation in the follow-up phases and fulfilled the inclusion criteria were invited to attend a morning session between 34-36 weeks of gestation. During this session, the Structured Clinical Interview for DSM-IV-TR Axis I Disorders (SCID-I) was administered and women were asked to donate a blood sample and to return their saliva samples collected during the previous days. Beginning from the morning after delivery (mean hours after delivery=17.64; SD=10.16) women were asked to once again collect their saliva samples. In addition, between 48-72 hours after delivery (mean hours after delivery=52.36; SD=19.70), they were asked to provide another blood sample and to once again fill in the EPDS and STAI as well as a form on health and delivery.

The Ethics Committee of University College London, of Scientific Institute Eugenio Medea and of the hospitals involved approved the study protocol.

3.3.3 Instruments

Edinburgh Postnatal Depression Scale (EPDS; Cox, Holden & Sagovsky, 1987). The EPDS is a 10-items self-report questionnaire widely used to screen for perinatal depressive symptoms on a 4-point Likert scale. A cut-off of 10 has been

shown to indicate a significant risk for depression (e.g. Bergink et al. 2011) and is the recommended cut-off for the validated Italian version (Benvenuti et al., 1999).

State-Trait Anxiety Inventory (STAI) (Spielberger, Gorsuch & Lushene, 1970). The STAI-T is a well-validated 20 item self-report measure of anxiety that measures the "trait anxiety" or anxiety as a general trait. It is widely employed during pregnancy (e.g. Pluess et al., 2010), and has been translated and validated also in Italian (Pedrabissi & Santinello, 1989).

Structured Clinical Interview for DSM-IV Axis I Disorders, Research Version, Non-Patient Edition (SCID-I RV-NP; First et al., 2002*).* The SCID–I is a semi-structured diagnostic interview, widely used in psychiatric research to assess DSM–IV-TR Axis-I disorder (American Psychiatric Association, 2000). Due to the low number of women fulfilling the criteria for a diagnosis of Major Depressive Episode and Anxiety Disorder (respectively 4,5% and 3,6%) in the current sample, the diagnostic status was not employed in the analyses and the SCID-I was employed exclusively to exclude any substance/alcohol abuse problems or other chronic psychiatric disorders.

Pregnancy- and delivery-related forms. An ad-hoc prenatal form was used to collect pregnancy and health-related data (e.g. due date, any complication/medical disorder, pre-pregnancy weight and height, medication). Self-reported height and weight were used to estimate pre-pregnancy BMI. After delivery, an ad-hoc postnatal form was employed to collect health and delivery-related data (e.g. length of labor, type of delivery, any episiotomy, epidural anaesthesia, medication).

3.3.4 Biological collection and assay

Venous blood samples and analyses of cytokine concentrations. Blood samples were drawn by venipuncture by a qualified nurse and kept refrigerated at +4° until they reached the Biological Lab of Medea Institute where serum was centrifuged, aliquoted, and stored at −80 °C until analyzed. All blood samples were collected in the morning, with the exception of 8 postnatal sampling that were performed between

14:00 and 16:00 due to organizational reasons. 97 women out of 110 agreed to blood sampling in pregnancy and 66 out of 89 after delivery. Serum CRP and IL-6 concentrations were assayed in duplicate by using Quantikine High Sensitivity ELISA kits (R&D Systems Europe, LTD) at LaboSpace in Milan according to the instructions of the manufacturer. Intra-assay coefficient of variation (CV) was <6% for IL-6 and <3% for CRP, inter-assay CV was <10% for both markers.

Saliva collection and analyses of salivary cortisol and alpha amylase concentrations. Participants collected whole unstimulated saliva samples on two consecutive days immediately upon awakening, 30 minutes post-wakening and before going to bed, in order to capture cortisol and sAA diurnal pattern. Participants were asked to refrain from eating, tooth-brushing and exercising 30 minutes prior to collection and eating a major meal 60 minutes prior to the evening sample. In addition, women were asked to report time of collection on a diary and were reminded of the collection through pre-scheduled phone callings. All samples were kept on ice until reaching the laboratory where they were stored frozen at -80° until assayed. One hundred and ten women provided completed saliva samples during pregnancy. After delivery, 68 women provided completed salivary data, 12 women had incomplete salivary data while 9 women were not able to perform the saliva collection after delivery due to postpartum pain. During pregnancy, the mean time from the awakening collection and the 30-minutes post-waking collection was 30.82 minutes on day 1 (SD=3.64, range: 20.00-60.00) and 31.44 minutes on day 2 (SD=6.81, range: 20.00-90.00). After delivery, the mean time from the awakening collection and the 30-minutes post-waking collection was 32.05 minutes on day 1 (SD=6.53, range: 20.00-65.00) and 35.89 minutes on day 2 (SD=18.53, range: 29.00-140.00). As 1 pregnant woman and 4 women after delivery, according to diaries, collected the second sample more than one hour from awakening, and the decline in cortisol usually occurs one hour after waking up, these samples were excluded from analyses (O'Donnell, 2013; O'Connor, 2014).

Salivary cortisol assay was performed at the Biological Lab of Medea Institute whereas sAA samples were shipped in dry ice to the Salimetrics Centre of Excellence testing lab at Anglia Ruskin University. All samples from one woman were run in the same assay to minimize method variability. Due to budget restrictions and limited saliva volume at delivery sAA was examined only in pregnancy. A competitive high sensitivity enzyme immunoassay kit (Expanded Range High Sensitivity Cortisol EIA Kit, Salimetrics), specifically designed and validated for the quantitative measurement of salivary cortisol, was used for the assessment of cortisol following the manufacturer's procedures. Salivary cortisol assays were run in duplicate, except for 2 samples with minimal volume. Average intra- and inter-assay coefficients were <6% and <8%, respectively. Results were computed in μg/dl. Samples were assayed for sAA using a kinetic enzyme assay kit (Salimetrics α-Amylase Kinetic Enzyme Assay Kit) specifically designed and validated for the kinetic measurement of sAA activity. A random 10% of the sAA assays were run in duplicate to confirm reliability. The intra-assay coefficient of variation was < 3%. Results were computed in U/mL.

3.3.5 Statistical analyses

Given the low rate of depression in the sample according to the EPDS cut-off of 10 (17.3%), the total score of the EPDS was employed as a continuous variable in the main analyses. As typically done in literature (e.g. Christian et al., 2009), each biomarker was entered in the statistical models as the outcome variable in order to model the trajectory over time and control for possible confounders when investigating the association between depressive symptoms and physiological levels.

Variables were first examined for outliers and skewness. Distributions of biological markers were positively skewed even after removing samples greater than 3 SD from the mean (n=7 for prenatal cortisol, n=8 for postnatal cortisol, n=4 for sAA, n=3 for prenatal IL-6), thus measures were natural log transformed prior to analysis to approximate normal distributions. Daily average salivary cortisol and sAA were

computed for each day as the area under the curve according to the trapezoid method with respect to the ground (AUCg, Pruessner et al., 2003). As the correlations between AUCg values across the 2 days ($r(104)=.57$, $p<.001$ for cortisol and $r(98)=.22$, $p<.05$ for sAA) were comparable to those found in previous studies (Wust et al., 2000; Shea et al., 2007), the mean of the 2 days was used. Preliminary Pearson bivariate correlations and univariate analysis of variance (ANOVA) were undertaken to evaluate the potential effect of variables known to affect stress and immune physiology.

Hierarchical Linear Models (HLMs) were estimated to investigate the influence of maternal depressive symptoms on diurnal levels of biological markers during pregnancy and after delivery as well as on the markers' longitudinal trajectory from pregnancy to delivery, while accounting for the hierarchical structure of the data (individual samples nested within two time-points and time-points nested within individuals). HLMs for salivary cortisol and sAA were specified at three levels where individuals were level 3, days of collection level 2 and mean cortisol/sAA values across the day were level 1. Time was centered at waking so that the model intercept represented the mean cortisol/sAA levels at waking, the morning slope represents the mean change from waking to 30 minutes after and the afternoon slope represents the mean decline to bedtime. HLMs models for inflammatory markers were specified at two levels with individuals at level 2 and occasions at level 1. Time was centered at pregnancy so that the model intercept reflected the mean prenatal CRP/IL-6 level and the slope represents the mean change from pregnancy to delivery. In the first stage of the analysis, for all markers, unconditional mean models were computed to evaluate how much variance in biological levels can be attributed to between-subject, between-days (only for sAA and cortisol) and between-occasions variations. Then, the effect of time on each biomarker value was tested. Models initially included random intercepts to allow between-person variability at the intercept. Subsequently, the effect of including random slopes (both for morning and afternoon decline for sAA and cortisol) for time was evaluated. The error term was allowed to vary randomly in each model.

The explanatory variables were centered around the grand mean and entered in the model one-by-one. Confounding variables that were found to be significantly related to biological levels in preliminary analyses were examined as potential predictors. The fixed effect of maternal depressive symptoms on the intercept and time slopes was tested sequentially. Model fit was tested with likelihood deviance difference test for nested models. Specifically, variables were kept in the model when their presence resulted in a significant ($p<.05$) reduction of the likelihood ratio statistic. To test the statistical significance of the regression coefficients, the Wald's chi-square test was performed. To aid interpretation, ln transformed model estimates were interpreted as the percent change in the biological marker level per unit change in the independent variable through the following transformation: $\beta_{\%change}=100 \times [\exp(\beta \ln)]-1$.

Given the high correlation between depression and anxiety, comparable analyses with anxiety scores as individual-level predictor were run and are reported in the supplementary results section.

Graphical analyses were used to explore different patterns of correlations between sAA or cortisol AUCg and inflammatory markers between women scoring above versus under the median of the EPDS. The relationship trends were further investigated through multiple linear regressions. In these models, levels of inflammation were the outcomes variables and the difference in the relationship patterns between stress and inflammatory markers were evaluated by testing the significance of the interaction term between EPDS and cortisol or sAA AUCg on levels of inflammation, while controlling for covariates.

Statistical analyses were performed using SPSS 24 and MLWiN. All statistical tests were two-sided and a $p<.05$ was considered statistically significant.

3.4 Results

3.4.1 Preliminary analyses

Descriptive statistics for all biological markers examined are presented in Table 3.1 using raw values, while ln transformed values are employed in all analyses.

Several potential confounding factors that could impact the assay of biological markers, such as demographic, pregnancy and delivery-related variables as well as situational factors (e.g. sampling time, hours of sleep, spontaneous awakening etc.), were examined. Univariate ANOVA indicated a significant effect of foetal sex on waking cortisol levels ($F(105,1)=5.45$, $p=.021$). Specifically, relative to males, women carrying female foetuses had lower cortisol levels at awakening. Thus, foetal sex was included as a covariate in subsequent cortisol analyses. In addition, time from delivery to saliva collection was significantly related with postnatal cortisol levels at awakening ($r=-.32$, $p<.05$), 30 minutes after ($r=-.26$, $p<.05$) and with cortisol AUCg ($r=-.31$, $p<.05$) on postnatal day 1 of collection, thus was included as control in subsequent analyses. No significant associations were found among demographic and pregnancy-related variables and sAA levels (all ps>.05).

Age was significantly related to levels of CRP during pregnancy ($r=-.29$ $p=.004$), while pre-pregnancy BMI was significantly associated with both CRP ($r=.43$, $p<.001$) and IL-6 ($r=.24$, $p=.02$) levels during pregnancy. Based on these analyses, pre-pregnancy BMI and age were included as covariates in subsequent analyses of psychosocial correlates of inflammation. Furthermore, length of gestation was significantly related to IL-6 levels after delivery ($r=.25$, $p<.05$) and was included as control in subsequent analyses.

Table 3.1 - Descriptive statistics for maternal biological levels during pregnancy and after delivery

	Pregnancy		**Delivery**	
	Mean	SD	Mean	SD
Cortisol (µg/dl)				
Waking	0.381	0.129	0.435	0.182
Waking +30'	0.504	0.151	0.575	0.255
Bedtime	0.177	0.063	0.200	0.089
sAA (U/ml)				
Waking	68.683	63.748	--	--
Waking +30'	47.605	37.974	--	--
Bedtime	97.171	79.583	--	--
CRP (ng/ml)	3786.741	2772.981	11660.282	3541.091
IL-6 (pg/ml)	1.674	1.019	7.025	2.856

3.4.2 Diurnal cortisol

Diurnal variation in cortisol during pregnancy

The unconditional means model for prenatal cortisol showed significant within-person variability in cortisol levels over occasions (level-1; σ^2_{e0}=0.018, p<.001), while variability at the individual level (level-3, σ^2_{v0}=0.001, p=0.62) and between-days (level-2, σ^2_{u0}=0.000, p=0.99) were not significant. Before fitting explanatory models including level-3 predictors, a baseline model of diurnal cortisol was fit to obtain estimates of cortisol levels at awakening (intercept) and the slopes for both the cortisol response to awakening (i.e. morning slope) and diurnal decline (i.e. afternoon slope). Model parameters for ln-transformed cortisol values are presented in Table 3.2 while results in the text and figure are presented using anti-logged values to facilitate interpretation.

Table 3.2 - Preliminary and full prediction models for prenatal diurnal cortisol

Prenatal Cortisol	Model 1			Model 2		
	Estimate	SE	p	Estimate	SE	p
Fixed effects						
Intercept	0.329	0.008	<.001	0.327	0.008	<.001
Morning	0.086	0.010	<.001	0.092	0.024	<.001
Afternoon	-0.152	0.007	<.001	-0.188	0.017	<.001
Fetal sex	**-0.029**	**0.010**	**0.005**	**-0.026**	**0.010**	**0.009**
EPDS				**-0.026**	**0.008**	**0.002**
EPDS X Morning				-0.004	0.014	0.778
EPDS X Afternoon				**0.022**	**0.009**	**0.019**
Random effects						
Level 3 (individual)						
Intercept variance	0.002	0.001	<.001	0.002	0.001	<.001
Morning slope variance	0.005	0.001	<.001	0.005	0.001	<.001
Intercept/morning slope covariance	0.000	0.001	0.668	0.000	0.001	0.871
Level 2 (day)						
Intercept variance	0.005	0.000	<.001	0.005	0.000	<.001
Level 1 (times)						
Intercept variance	0.000	0.000	0.998	0.000	0.000	0.998

The estimated grand mean level of cortisol at awakening was 0.37 ug/dl and there was significant between-person variability in this value (p<.001). In line with previous studies, cortisol diurnal pattern was preserved during pregnancy, with a significant increase 30 minutes after awakening, as indexed by the morning slope (p<.001), and a significant decrease over the rest of the day, as indicated by the afternoon slope (p<.001). This model resulted in a significant improvement of the fit over the unconditional means model (deviance difference (2)=542.13, p<.001). The inclusion of a random morning slope term (as well as the random intercept and morning slope covariance terms) additionally significantly improved the model fit (deviance difference (2)=47.56, p<.001), suggesting significant variability between-

person in the cortisol response to awakening. There was no significant effect of day of collection on mean cortisol values or on cortisol daytime trajectory (all ps>.05), suggesting no systematic differences between measurement on day 1 and day 2. As previously shown, fetal sex was significantly associated with cortisol mean waking level (p=.005), with 2.9% lower values in women carrying a female fetus as compared to male, but not with the overall daytime trajectory (all ps>.05). Thus, the effect of fetal sex was included as a control in all subsequent explanatory models. No other significant associations between cortisol diurnal levels and demographic, pregnancy-related variables, wake time and hours of sleep were found (all ps>.05).

Prenatal depressive symptoms were associated with lower cortisol levels at waking (2.6% lower with every 1-point increase on the EPDS, p=.002) and 30 minutes after waking (3% lower with every 1-point increase on the EPDS, p=.002), after adjusting for covariates. Additionally, prenatal depressive symptoms were related to a flatter afternoon cortisol decline (2.2% smaller slope with every 1-point increase on the EPDS, p=.019). This effect is illustrated in Figure 3.1. Inclusion of depressive symptoms resulted in a significantly improvement of model fit over the baseline model (deviance difference (3)=12.44, p=.006). Additionally, analyses (not reported in table) indicated that the effect of prenatal depression on cortisol diurnal pattern was not moderated by fetal sex.

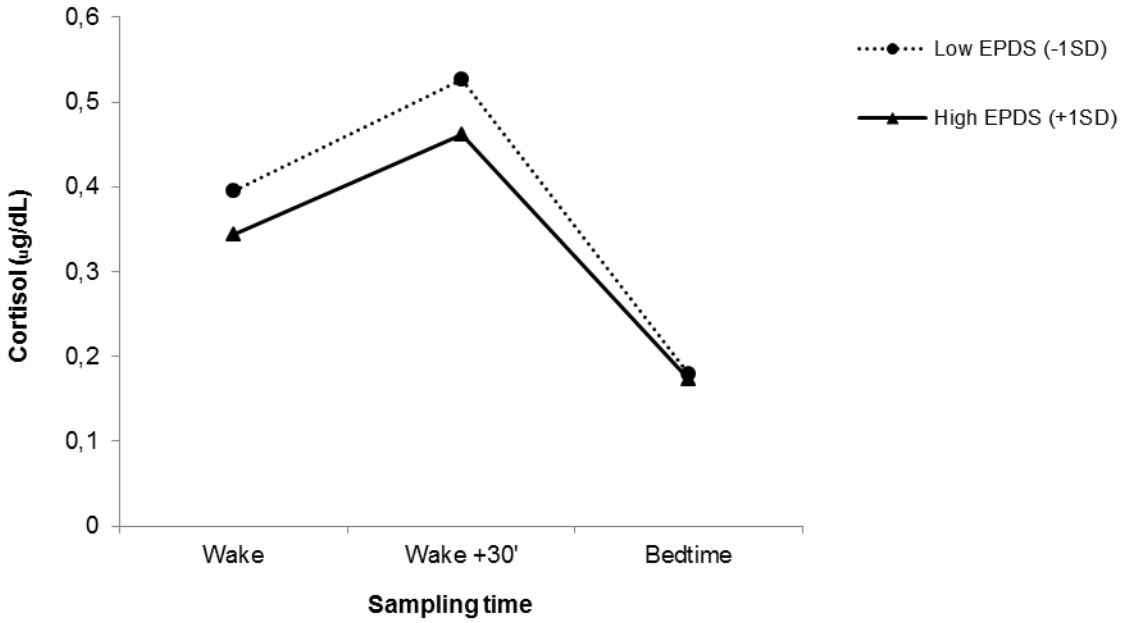

Figure 3.1 - Diurnal cortisol values for women with higher (+1 SD) and lower (-1SD) depressive symptoms during pregnancy, after adjusting for covariates.

Diurnal variation in cortisol after delivery

The base model for postnatal cortisol (Table 3.3) indicated significant between-day (level-2, σ^2_{u0}=0.017, p<.001) and between-person (level-3, σ^2_{v0}=0.004, p<.001) variation in mean waking cortisol levels and suggested that the cortisol daytime trajectory is substantially maintained soon after delivery (p<.001). The inclusion of a random morning slope term (as well as the random intercept and morning slope covariance terms) significantly improved the model fit (deviance difference (2)=16.17, p<.001). The significant positive association between the random effects of intercept at level 3 and morning slope indicates a greater cortisol increase 30 minutes after waking in individuals with higher cortisol levels at waking. There was no significant effect of day of collection on cortisol values (all ps>.05). However, there was a marginally significant effect of time from delivery (p=.072). Specifically, cortisol levels at waking were around 0.2% lower for every additional hour between delivery and sample collection. Thus, time from delivery was included as a covariate in subsequent models. All other demographic, pregnancy- and delivery-related variables were not significantly

associated with cortisol values (all ps>.05). No significant effects of postnatal depressive symptoms, as measured by the EPDS, on mean cortisol waking levels as well as on the diurnal trajectory were found.

Table 3.3 - Preliminary and full prediction models for postnatal diurnal cortisol

Postnatal Cortisol	Model 1			Model 2		
	Estimate	SE	p	Estimate	SE	p
Fixed effects						
Intercept	0.353	0.012	<.001	0.350	0.012	<.001
Morning	0.088	0.017	<.001	0.084	0.016	<.001
Afternoon	-0.170	0.016	<.001	-0.166	0.016	<.001
Time from delivery	-0.002	0.001	0.072	-0.002	0.001	0.078
EPDS				-0.020	0.014	0.155
EPDS X Morning				-0.024	0.019	0.215
EPDS X Afternoon				0.025	0.019	0.176
Random effects						
Level 3 (individual)						
Intercept variance	0.001	0.001	0.297	0.001	0.001	0.191
Morning slope variance	0.002	0.003	0.459	0.001	0.003	0.674
Intercept/morning slope covariance	0.004	0.001	<.001	0.004	0.001	<.001
Level 2 (day)						
Intercept variance	0.017	0.001	<.001	0.017	0.001	<.001
Level 1 (times)						
Intercept variance	0.000	0.000	0.999	0.000	0.000	0.999

Diurnal variation in cortisol from pregnancy to delivery

The unconditional means model for cortisol revealed significant variability between occasions (i.e. pregnancy versus delivery; level-3; σ^2_{v0}=0.024, p<.001). As

expected, there was a significant effect of occasion on mean cortisol waking values (p<.001), with 3.25% higher cortisol values at delivery as compared to prenatal levels, while no effect of occasion on cortisol morning or afternoon slope (p>.05). Both foetal sex and time from delivery were retained in the model as covariates as they were previously found to be related to cortisol levels. Prenatal depressive symptoms were associated with lower cortisol levels at waking during pregnancy (p=.012), but not after delivery (p=.315). Additionally, prenatal depressive symptoms were related to a greater increase in cortisol waking levels from pregnancy to delivery (estimate=0.046, SE=0.017; p=.006). However, the inclusion of prenatal depressive symptoms and of the interactions with time and occasions did not yield to a significant improvement of the fit over the baseline model (deviance difference (6) =9.16, p=0.165).

3.4.3 Diurnal sAA

The unconditional means model for sAA levels during pregnancy showed significant variability in sAA values between person (level-3, σ^2_{v0}=0.412, p<.001) and between days (level-2; σ^2_{u0}=0.446, p<.001). The estimated grand mean level of sAA at awakening was 43.70 U/ml and there was significant variability in this value between persons and days (p<.001). Pregnant women showed the expected sAA diurnal trajectory with a significant decrease from awakening to 30 minutes after (p<.001) and a significant increase over the rest of the day (p<.001) (Table 3.4). This model resulted in a significant improvement of the fit over the unconditional model (deviance difference (2)= 94.25, p<.001). In addition, a random afternoon slope term significantly improve the fit of the model (deviance difference (2)=20.34, p<.001). The negative association between the random effects of intercept at level 3 and afternoon slope indicates that a higher sAA increased from 30 minutes after awakening to bedtime was seen in individuals with lower sAA levels at awakening. There was a significant effect of day of collection on mean sAA waking values (p=.002), with higher levels on day 2 as compared to day 1, while no effect on sAA diurnal trajectory. Thus, day of collection

was retained as a covariate in the following analyses. Demographic, pregnancy-related variables, wake time and hours of sleep were not significantly associated with diurnal sAA levels (all ps>.05).

There was no effect of depressive symptoms on mean sAA waking levels, while there was a marginally significant effect of depression on sAA morning slope (p=.06), with depressive symptoms being related to a flatter morning sAA decline. However, inclusion of depressive symptoms did not yield a significant improvement of model fit over the baseline model (deviance difference (3)=3.78, p=.286).

Table 3.4 – Preliminary and full prediction models for prenatal diurnal sAA

Prenatal sAA	Model 1			Model 2		
	Estimate	SE	p	Estimate	SE	p
Fixed effects						
Intercept	3.731	0.079	<.001	3.731	0.079	<.001
Morning	-0.242	0.052	<.001	-0.241	0.051	<.001
Afternoon	0.460	0.066	<.001	0.461	0.066	<.001
Sampling day	**0.140**	**0.042**	**0.002**	**0.140**	**0.042**	**0.002**
EPDS				-0.126	0.104	0.223
EPDS X Morning				0.128	0.069	0.065
EPDS X Afternoon				0.083	0.090	0.354
Random effects						
Level 3 (individual)						
Intercept variance	0.498	0.077	<.001	0.497	0.077	<.001
Afternoon slope variance	0.193	0.058	<.001	0.194	0.057	<.001
Intercept/afternoon slope covariance	-0.127	0.050	0.011	-0.127	0.050	0.011
Level 2 (day)						
Intercept variance	0.287	0.020	<.001	0.285	0.019	<.001
Level 1 (times)						
Intercept variance	0.000	0.000	0.999	0.000	0.000	0.999

3.4.4 Inflammatory markers

CRP and IL-6 levels during pregnancy

The estimated grand mean level of inflammatory markers during pregnancy was 2880.30 for CRP and 1.54 for IL-6 with significant between-persons variability around these values (p<.001). Age and maternal pre-pregnancy BMI were significantly related to prenatal CRP levels with 6% lower CRP levels per year older (estimate=-0.056, SE=0.018; p=.002) and 9.2% higher CRP with each point on the BMI (estimate=0.086, SE=0.018; p<.001). Only maternal pre-pregnancy BMI was found to significantly predict IL-6 levels during pregnancy (estimate=0.018, SE=0.008; p=.018), with 1.8% higher IL-6 concentrations with each BMI point. All other demographic and pregnancy-related variables were not significantly associated with CRP and IL-6 values (all ps>.05).

There was a significant effect of depressive symptoms on IL-6 levels during pregnancy with around 8.1% higher IL-6 concentrations with every point increase on the EPDS (estimate=0.078, SE=0.039; p=.044). The inclusion of depressive symptoms significantly improved the model fit over the baseline model (deviance difference (1) = 3.95, p=0.044). In contrast, prenatal depressive symptoms were not significantly related to mean CRP levels (estimate=0.075, SE=0.094; p=.429).

CRP and IL-6 levels after delivery

The estimated grand mean level of CRP and IL-6 after delivery were, respectively, 11202 and 6.50 with a significant between person variation around these values (p<.001). Neither CRP nor IL-6 levels after delivery were significantly related to any demographic or pregnancy-related variables (all ps>.05). Duration of pregnancy was significantly associated with IL-6 levels after delivery (estimate=0.084, SE=0.040; p=0.037), with 8.7% higher IL-6 levels per additional gestational week, and was thus included as a covariate in subsequent analyses. No effects of postnatal depressive symptoms on mean CRP or IL-6 levels after delivery were found (all ps>.05).

CRP and IL-6 trajectory from pregnancy to delivery

The unconditional means models for CRP and IL-6 showed significant between-occasion variability (level-1, respectively σ^2_{e0}=0.866 and σ^2_{e0}=0.393, p<.001), while variability at the individual level was not significant (both ps>.05). As expected, both CRP and IL-6 levels were significantly higher soon after delivery as compared to late pregnancy (both ps<.001, Table 3.5).

Table 3.5 – Full prediction models for inflammatory markers trajectory from pregnancy to delivery

	IL-6			CRP		
	Estimate	SE	p	Estimate	SE	P
Fixed effects						
Intercept	0.935	0.029	<.001	7.968	0.070	<.001
Time	1.089	0.050	<.001	1.355	0.074	<.001
Age				**-0.054**	**0.018**	**0.003**
BMI	**1.699**	**0.709**	**0.016**	**0.086**	**0.018**	**<.001**
EPDS	**0.077**	**0.039**	**0.045**	0.074	0.094	0.436
Age x Time				**0.039**	**0.019**	**0.046**
BMI x Time				**-0.081**	**0.019**	**<.001**
EPDS X Time	-0.023	0.064	0.724	-0.155	0.098	0.114
Random effects						
Level 2 (individual)						
Intercept variance	0.081	0.012	<.001	0.473	0.068	<.001
Time variance	0.166	0.030	.001	0.485	0.075	<.001
Intercept/time covariance	-0.051	0.015	<.001	-0.445	0.068	<.001
Level 1 (times)						
Intercept variance	0.000	0.000	1.000	0.000	0.000	1.000

Additionally, there was significant between-person variability in both CRP and IL-6 trajectory from late pregnancy to delivery, as indicated by the significant random slope for time (both ps<.001). The negative associations between the random effects of intercept at level 2 and time slope, both for CRP and IL-6, indicate that a greater increase from pregnancy to delivery was seen in individuals with lower inflammatory markers levels in pregnancy. All demographic, pregnancy- and delivery related variables examined were not significantly related to IL-6 change from pregnancy to delivery. However, there was a significant effect of maternal pre-pregnancy BMI (p<.001) and age (p=0.003) on CRP slope of time, with a higher BMI and a younger age being related to a flatter increase of CRP levels from pregnancy to delivery (in addition to the main effect on mean CRP prenatal levels previously shown; deviance difference (4)=30.3, p<.001). Thus, both maternal age and BMI were included in the subsequent analyses.

No significant effects of prenatal depressive symptoms on CRP and IL-6 slope from pregnancy to delivery were found (all ps>.05).

3.4.5 Correlations between systems

Table 3.6 shows bivariate correlations between depressive symptoms and levels of biological markers in the whole sample. Graphical analyses were employed in order to explore relationship patterns between cortisol/sAA AUCg and IRS markers among women who scored above or below the median at the EPDS both during pregnancy and after delivery. As shown in Figure 3.2, these patterns were quite different for cortisol and IL-6 and for sAA and IL-6 during pregnancy. Specifically, in women scoring above the median at the EPDS, cortisol AUCg and sAA AUCg increased with increasing levels of IL-6 (respectively, p=.03 and p=.002), while this was not true in women scoring below the median (both ps>.05). No differences in relationship trends were apparent after delivery.

Table 3.6 - Bivariate correlations for primary study variables

		1	2	3	4	5	6	7	8
1.	Prenatal EPDS								
2.	Prenatal Cortisol AUCg	-.15							
3.	Prenatal sAA AUCg	.05	**.25****						
4.	Prenatal IL-6	**.19***	.10	.17					
5.	Prenatal CRP	.11	.08	-.01	**.31****				
6.	Postnatal EPDS	**.44****	-.13	.07	.14	.19			
7.	Postnatal Cortisol AUCg	-.13	.09	-.16	-.09	-.03	-.20		
8.	Postnatal IL-6	.12	.01	.06	**.27***	.08	.005	.09	
9.	Postnatal CRP	-.19	.001	-.15	**-.33****	.22	-.02	-.09	-.18

* p<.05; **p<.01

To further explore these relationship patterns during pregnancy, separate models were fitted to evaluate whether average levels of stress hormones predict the average levels of inflammatory markers, and whether depressive symptoms influence these associations, while adjusting for covariates. There was no main effect of cortisol or sAA AUCg on mean IL-6 or CRP levels during pregnancy (all ps>.05). However, there was a significant interaction between prenatal depressive symptoms and sAA (p>.01) and a marginally significant interaction between prenatal depressive symptoms and cortisol (p=.06) on IL-6 levels during pregnancy. Specifically, higher sAA or cortisol AUCg were associated with higher IL-6 levels only in women with higher EPDS scores (+1SD; p<.05), while the association was non-significant in women with lower EPDS scores (p>.05). Both sAA and cortisol models yielded a significant improvement

in the model fit as compared to the IL-6 baseline model (deviance difference for sAA (2)=12.97, p<.01; deviance difference for cortisol (2)=12.03, p<.01).

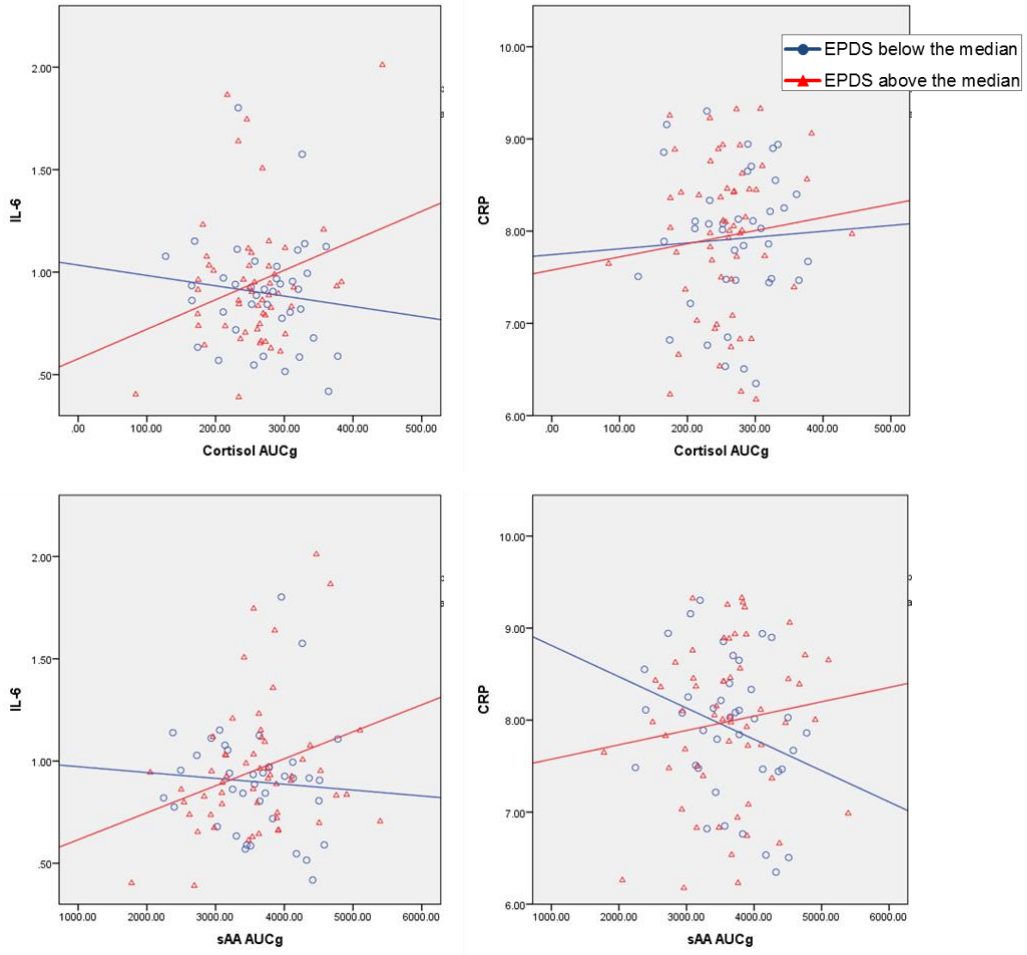

Figure 3.2 - Relationship between antenatal diurnal cortisol or sAA AUCg and inflammatory markers in women scoring above (red) or under (blue) the median at the EPDS during pregnancy.

3.4.6 Supplementary analyses

Given the considerable overlap between depression and anxiety (r=.65 antenatally and r=.46 postnatally, p<.001), we performed an additional set of analyses examining maternal anxiety rather than depression as an individual-level predictor in the explanatory models in order to test the robustness of the effects found for maternal depression. There were no significant effects of anxiety on cortisol diurnal levels, IL-6 and CRP both prenatally and postnatally (all ps>.05). However, the model indicated a

significant main effect of anxiety on mean waking sAA levels (estimate=-0.663, SE=0.312; p=.033) as well as on the sAA morning slope (estimate=0.594, SE=0.209; p=.004) during pregnancy. Specifically, higher levels of anxiety were related to lower sAA concentrations at waking and to a flatter decline from waking to 30 minutes after. This model significantly improved the model fit over the baseline model (deviance difference (3)=19.71, p<.001). Thus, in contrast with findings for depression, antenatal anxiety symptoms were not significantly associated with diurnal cortisol or IL-6 levels, although they were associated with aspects of the sAA diurnal profile.

3.5 Discussion

The present results confirm and extend major findings regarding the relationship between depressive symptoms and dysregulation of the stress and immune response systems during pregnancy studied individually, while providing a more comprehensive picture of these relationships by assessing multiple biological markers simultaneously and examining their change from pre- to post-pregnancy.

3.5.1 Depressive symptoms and SRS and IRS functioning during pregnancy

As hypothesized, results indicated an association between depressive symptoms and both diurnal cortisol variations, as detected through multilevel models, and heightened inflammation in a community sample of pregnant women. Specifically, higher depressive symptoms in late gestation were associated with lower levels of cortisol at waking and 30 minutes after, as well as a flatter diurnal decline, after controlling for covariates. These findings are in line with previous studies on high psychosocial risk samples providing evidence of a flattened diurnal pattern in depressed women (e.g. O'Connor et al., 2014; Suglia et al., 2010). However, when the overall diurnal cortisol output, as indexed by the AUCg, was examined, we were not

able to detect any significant association with depressive symptoms. This result is consistent with previous evidence (e.g. Hellgren et al., 2013; Cheng & Pickler, 2010) and underlines the need for a thorough characterization of cortisol diurnal pattern in order to identify associations between HPA axis functioning and depressive symptoms during pregnancy, especially in low-risk samples.

While cortisol awakening response (CAR) has attracted considerable attention as a promising biomarker of HPA axis function, being associated with several health outcomes (Clow, et al., 2010), including depression in non-pregnant individuals (Huber et al., 2006; Vreeburg, Sophie; Hoogendijk, 2009), we found no evidence of an association between maternal depressive symptoms and CAR in late pregnancy. Although our assessment does not allow us to properly evaluate the CAR (Stalder et al., 2016), as samples were collected only at waking and 30 minutes after, our results converge with previous reports using either composite scores (C.-Y. Cheng & Pickler, 2010; Shea et al., 2007) or mixed models (Hellgren et al., 2013; O'Connor, Tang, et al., 2014). Despite the fact that further investigations are required, it can be hypothesized that while the CAR is still present in late pregnancy, due to the pregnancy-related HPA axis physiological resetting, including elevations in absolute cortisol levels and dampened reactivity, it may dissociate to a certain extent from environmental factors. Alternatively, the current findings converge with a few prior studies to suggest that evening cortisol measures or measures of diurnal decline might be more sensitive to the effect of stress during pregnancy (Harville et al., 2009; O'Connor et al., 2014; Obel et al., 2005).

Furthermore, consistently with previous reports (Cassidy-Bushrow et al., 2012; Christian et al., 2009; Coussons-Read, Okun, & Nettles, 2007), higher depressive symptoms were associated with increased IL-6 levels during pregnancy, after adjusting for relevant covariates. This result is in line with accumulating evidence of a role for inflammation in vulnerability to depression in non-pregnant samples. In addition, it is noteworthy that inflammatory processes are particularly relevant during

pregnancy. Indeed, although circulating levels of pro-inflammatory cytokines have been found to be increased at the end of healthy gestation (Fransson et al., 2012), higher than typical elevations have been associated with adverse obstetric and birth outcomes such as preeclampsia and preterm birth (e.g. Vannuccini et al., 2016; Rusterholz et al., 2007). Accumulating evidence indicates an association between pregnancy morbidities such as preeclampsia and preterm labour and depressive symptoms (reviewed in Alder et al., 2007). Thus, our findings of a link between depressive symptomatology and increased inflammation in a low risk sample of healthy pregnant women, might speak in favour of a possible inflammatory pathway from depression to adverse pregnancy outcomes that would deserve further consideration in future studies.

Importantly, despite the strong association between prenatal depression and anxiety, our exploratory analyses indicated that findings of an altered HPA axis and IRS functioning were specific to depression, as weak and non-significant effects were found for anxiety symptoms. This is particularly relevant as the lack of consistency in the literature emphasizes the need for a clear characterization of the risk phenotypes that may ultimately impact fetal development, both in terms of psychiatric construct and biological processes.

Beside the above mentioned findings, some non-significant findings from our study might be relevant in the context of existing literature. First of all, in contrast to our expectations and to results from previous studies (e.g. Azar & Mercer, 2013; Scrandis et al., 2008), CRP levels were not related to depressive symptomatology. However, a lack of an association between antenatal CRP levels and depressive symptoms has been already reported in some studies (e.g. Cassidy-Bushrow et al., 2012; Catov et al., 2014). It is important to note that CRP is generally considered a non-specific marker of systemic inflammation, being primarily synthesized in the liver in response to pro-inflammatory cytokines, and reflecting levels of circulating interleukins, such as IL-6 (Dantzer & Kelley, 2007). Hence, as expected, prenatal IL-6

and CRP levels were highly correlated in the current sample. However, different trajectories of change over time for IL-6 and CRP have been observed during pregnancy (Christian et al., 2014) and it has been shown that an increase in IL-6 is not always paralleled by CRP production (Czarkowska-Paczek et al., 2005). In addition, CRP levels increase and fall more rapidly and markedly than other acute-phase proteins. Thus, evaluating "basal" levels of CRP might be challenging and it is not possible to rule out a wide range of factors influencing circulating levels of CRP, such as pro-inflammatory cytokines, corticosteroids etc. (Du Clos & Mold, 2004). Therefore, the lack of an association between CRP and depression may be due to methodological issues, especially since CRP, as compared to IL-6, has been found to be particularly sensitive to processing and storage conditions (Skogstrand et al., 2008). On the other hand, it still remains plausible that IL-6, rather than CRP, is a more adequate biomarker for depression, driving the association between inflammation and depressive symptoms.

Secondly, we did not find any substantial evidence of altered SNS functioning, as indexed by sAA diurnal levels, in women at risk for depression during pregnancy. Specifically, only a marginally significant effect of depressive symptoms in flattening the sAA morning decline during pregnancy was detected, but depression was not retained as a significant predictor in the final model. To date, only two studies have examined the link between sAA and depression during pregnancy and these have yielded mixed results. Giesbrecht and colleagues (2013) failed to detect any significant association between depression and sAA daytime trajectory, whereas, more recently, Braithwaite and colleagues (2015) reported higher sAA levels at waking in women with depressive symptoms compared with non-depressed controls. However, in this latter study, sAA levels were averaged across two days of collection and compared between two small groups scoring above and under the cut-off of the EPDS. It is possible that the significant variability between days in sAA mean waking values, as well as the use of the EPDS score as a continuous variable in our analyses, might have limited our

ability to detect a significant effect. Additionally, as sAA levels have been observed to decrease as gestation advances (Giesbrecht et al., 2013; Nierop et al., 2006), pregnancy-related adjustments might further impair the possibility to identify a significant effect. Alternatively, it can be hypothesized that a dysregulation of the SNS might be more closely involved in the occurrence of anxiety rather than depressive symptoms. Indeed, we provided evidence of a significant association between higher levels of prenatal anxiety and lower sAA waking levels as well as a flatter decline from waking to 30 minutes after. This result is in line with previous evidence of an association between sAA or catecholamines concentrations and anxiety during pregnancy (e.g. Giesbrecht et al., 2013; Field et al., 2004; Field et al., 2006).

3.5.2 Depressive symptoms and SRS and IRS functioning after delivery

While we reported a moderate correlation between prenatal and postnatal depressive symptoms, contrary to what we expected, no cross-sectional associations between postnatal depressive symptoms and either diurnal cortisol or inflammatory markers levels were found. This finding is in line with a number of studies which failed to detect any significant association between serum levels of IL-6 evaluated early in labor or soon after delivery and postpartum depression at 1-5 days, 6 weeks and 6 months after delivery (e.g. (Corwin et al., 2015; Fransson et al., 2012; Groer et al., 2015; Skalkidou et al., 2009). However, it is important to note that a small number of studies have found evidence of altered IRS functioning in the early puerperium period and depressive symptoms (e.g. Maes et al., 2000). Interestingly, Boufidou and colleagues (2009) found higher cerebrospinal fluid IL-6 levels, but not serum levels, at delivery to be related to risk for postnatal depression. As far as we know, only Corwin and colleagues (2015) investigated the association between depressive symptoms and HPA axis functioning, as indexed by cortisol diurnal pattern, in the early puerperium (i.e. seven days after delivery); consistent with our results, the authors found no significant association between cortisol diurnal AUCg one week after delivery

and depressive symptoms.

There are a number of possible explanations for these null findings. First of all, despite the strong continuity between prenatal and postnatal depression, it has been argued that antenatal and postpartum depression represent two separate disorders (Kammerer et al., 2006), thus possibly reflecting distinct endocrine and inflammatory states. Additionally, it is also possible that the association between depressive symptoms and biomarkers during the first few weeks after childbirth might be masked by the strong hormonal and inflammatory response to parturition. Indeed, in line with previous reports (e.g. Corwin et al., 2015), we observed a significant activation of both the stress and the immune response systems in the first days after childbirth, as suggested by the general increase over the prenatal levels in all markers. It is possible that, as the biological response to delivery normalizes, significant associations between depression and cortisol or cytokines might emerge. Indeed, in the study by Corwin and colleagues (2015) no significant differences were observed between postnatally depressed women and controls in cortisol AUCg one week after delivery, but significant differences were highlighted two weeks after delivery. Consistently, a small number of studies have examined the cortisol diurnal pattern postnatally and found evidence of a link between depressive symptoms and suppression of the CAR (De Rezende et al., 2016; Taylor et al., 2009) as well as a flatter diurnal pattern (De Rezende et al., 2016) from 6 weeks to 6 months postpartum. Additionally, it can be hypothesized that a follow up evaluation of depressive symptoms could have revealed significant differences in the HPA axis and IRS functioning after delivery in women who later developed postpartum depression. Consistently with this, Liu and colleagues (2016) recently reported higher CRP and IL-6 levels after delivery in women with postpartum depression 6 months later, as compared to controls.

3.5.3 Prenatal depressive symptoms and change in SRS and IRS functioning from pregnancy to delivery

The current study was also designed to explore whether prenatal depressive symptoms were associated with a different trajectory of change in cortisol and inflammatory markers concentrations from pregnancy to the early puerperium.

Cortisol is known to increase through gestation and advancing labor and to peak during delivery. The physiological changes in the HPA axis functioning, mainly driven by the secretion of corticotropin-releasing hormone (CRH) from the placenta (Mastorakos & Ilias, 2003), are thought to play a role in maternal and fetal wellbeing and to promote normal labor progression (Allolio et al., 1990; Paula J. Brunton & Russell, 2008; Brian Harris et al., 1994). Although we provided marginal evidence of a greater increase in cortisol waking levels from pregnancy to the first days after childbirth in women with higher antenatal depressive symptoms, the final model did not yield to a significant improvement of the model fit over the baseline model. This null finding might be an issue of statistical power. Alternatively, it can be hypothesized that due to the substantial elevations in cortisol levels in response to labor and delivery, significant associations with mild variations in antenatal depressive symptoms might be difficult to detect. To our knowledge, no previous study has evaluated the association between prenatal depressive symptoms and changes in cortisol from pregnancy to the early puerperium. Yim and colleagues (2009) evaluated the longitudinal trajectory of pCRH across gestation and found a significant increase in pCRH levels until delivery in women who later developed postpartum depressive symptoms. Additionally, O'Keane and colleagues (2011) reported a smaller drop in CRH levels from pregnancy to 1-6 days after delivery in women showing higher symptoms of postpartum blues. While cortisol and CRH at 37 weeks of gestation have been shown to be significantly associated (Sandman et al., 2006), considerable methodological differences between the current findings and the above mentioned studies limit possible comparisons. Future studies in larger and different samples,

particularly those containing more clinical depressed women, could be able to better address the role of antenatal depressive symptoms in influencing cortisol trajectory from pregnancy to the early puerperium.

While we observed a marked increase in concentrations of inflammatory markers from pregnancy to the early puerperium, prenatal depressive symptoms were not found to significantly affect the longitudinal trajectory of the markers we examined. Our findings are in contrast with results from Maes and colleagues (2000) reporting higher IL-6 levels soon after delivery in women whose depression scores significantly increased from pregnancy to delivery. However, the unusual way of combining prenatal and postnatal mood symptoms in this study makes it hard to compare results. To date, only a handful of studies have evaluated the relationship between mood and IRS functioning antenatally and postnatally (e.g. Corwin et al., 2015; Scrandis et al., 2008; Maes et al., 2000) and these have mainly focused on postpartum depression. However, as prenatal depression is considered one of the strongest predictors of postnatal depression (Robertson et al., 2004) and a dysregulation of stress hormones and cytokines is thought to be involved in the development of postnatal depression, the role of prenatal depressive symptoms in influencing the longitudinal trajectory of endocrine and immune biomarkers from pregnancy to the early postpartum warrants further consideration in future studies.

3.5.4 Correlations between SRS and IRS

Our findings of differences in both the IRS and HPA axis in association with depression during pregnancy fit well within the existing literature. However, it has been suggested that alterations in any one system, studied individually, may offer only a partial picture of the processes involved in vulnerability for psychopathology. Nonetheless, the bidirectional interactions between the stress and the immune systems in the perinatal period and whether they differ according to symptomatology remain largely unclear and unexplored.

Our exploratory analyses indicate that prenatal depressive symptoms moderated the association between IL-6 and sAA and, marginally, between IL-6 and cortisol. In particular, as hypothesized, higher diurnal levels of sAA or cortisol were associated with higher inflammation only in women with higher depressive symptoms during pregnancy. In healthy non-pregnant adults, a rise in pro-inflammatory cytokines, as a consequence of an infection or stressor, activates the central components of the SRS, increasing cortisol and catecholamines secretion (Elenkov, 2008). Glucocorticoids and catecholamines, in turn, are thought to act as anti-inflammatory agents, systemically mediating a suppression of the inflammatory response and up-regulating the production of anti-inflammatory cytokines (Elenkov & Chrousos, 2006), in order to protect organism from the detrimental effects of a prolonged inflammatory response. In the last decade, significant advances have been made in understanding the neuroendocrine-immune interface and there is now reasonable evidence to suggest that the hormonal control of the pro- versus anti-inflammatory cytokine balance may represent a key mechanism by which stress gets 'under the skin' (Elenkov & Chrousos 2006). However, to date, only a limited number of studies have explored the HPA axis-immune interface during pregnancy (e.g. Corwin et al., 2013; Walsh et al., 2016; Shelton et al., 2015) and, to our knowledge, no studies investigated the role of the SNS in these mechanisms.

Increasing evidence, particularly from the observation of the course of autoimmune disease during pregnancy, suggests that increased levels of cortisol and norepinephrine in the third trimester of gestation might inhibit pro-inflammatory cytokine production and potentiate anti-inflammatory cytokine production (Elenkov et al., 2001; Elenkov & Chrousos, 2002; Mizokami et al., 2004). Tentatively, our findings, among the first evaluating this mechanism, could be interpreted to indicate that the ability of stress hormones to limit inflammation may be impaired in pregnant women with higher depressive symptoms, as suggested by the positive association between sAA or cortisol diurnal levels and IL-6 concentrations. As tight regulation of

inflammatory responses during pregnancy is crucial for a successful gestation and delivery (Mor et al., 2011), a disruption of the regulatory stress hormones-inflammatory circuit might lead to an over-activation of the IRS, with potential adverse consequence for mother and baby. Reduced regulation of the inflammatory response in non-pregnant adults has been related to chronic stress and has been associated to glucocorticoid resistance (Cohen et al., 2012; Miller et al., 2009; Rohleder, 2012). Despite the preliminary nature of our findings, they are in line with few existing evidence of a disruption of the negative feedback relationship between cortisol and pro-inflammatory cytokines during pregnancy, considered suggestive of glucocorticoid resistance, in high risk samples (Corwin et al., 2013; Walsh et al., 2016). Nevertheless, it is important to emphasize that the current findings are small-scale and exploratory and need replication in larger longitudinal studies. Although antenatal depressive symptoms were not associated with averaged diurnal cortisol or sAA levels, as indexed by the AUCg, in the whole sample, they were significantly associated with an altered diurnal cortisol profile and, marginally, with sAA pattern, possibly reflecting subtle disturbances in the HPA axis and SNS functioning. We might speculate that a dysfunctional SRS might be less capable of inhibiting the inflammatory response, as it normally does, thus leading to increased inflammation. Additionally, it has been recently proposed that the well-documented anti-inflammatory effects of glucocorticoids might occur at high dosages, while at low or intermediate concentrations, glucocorticoids might lose their immunosuppressive properties and even stimulate the inflammatory response (Horowitz et al., 2013; Sorrells, JR, Munhoz, & Sapolsky, 2016).

Contrary to our predictions and to results obtained by Corwin and colleagues (2013), we found no significant association between stress hormones and inflammatory markers in women with lower depressive symptoms. Although this result could be due to limited statistical power to detect a small effect, it could also suggest that the anti-inflammatory response to the physiological pregnancy-related increase in

cortisol is dampened or lacking in the third trimester of healthy pregnancy. This is in line with findings from Corwin and colleagues (2015) reporting a significant positive association between postnatal diurnal cortisol and pro-inflammatory cytokines in postnatally depressed women, while no association in euthymic women. However, we are at the very beginning of our understanding of these mechanisms during the perinatal period. In order to better capture and understand deviations from typical functioning, the well-documented bidirectional stress-immune interactions occurring outside the perinatal period needs to be fully investigated during pregnancy and in the early puerperium.

3.5.5 Limitations

Some limitations of the present study are noteworthy. First, it is important to note that the current findings come from a sample of healthy women from a middle-high socioeconomic background and that, as expected in a community sample, levels of depressive symptoms were relatively low, thus limiting generalizability to populations at high psychosocial risk or clinical psychiatric populations. It is possible that evaluating women with clinically significant depression could reveal more profound alterations of the SRS and IRS, although this hypothesis needs to be directly tested. Second, a larger sample size would have allowed more power to detect small effects, although the longitudinal design, the exclusion of complicated pregnancies or women taking any chronic medication and a multidimensional approach strengthens our results. Third, despite the current findings are in line with evidence of a role of dysregulation in the HPA axis and IRS functioning in increasing vulnerability for depression (Horowitz et al., 2013), it is important to underline that, due to the correlational nature of this study, it is not possible to establish whether the observed associations reflect causal processes. In addition, we cannot rule out unmeasured confounds or third variables that might explain the associations we reported, including genetic variants that might account for both inflammation or cortisol dysregulation and

occurrence of depressive symptoms (reviewed in Barnes, Mondelli, & Pariante, 2017; Spijker & van Rossum, 2009). Fourth, we collected data at one time only during pregnancy and after delivery, thus limiting the generalizability of our results at other gestational and postpartum time-windows. It is our aim to follow-up this cohort to determine if the observed alterations could be predictive of later postpartum symptoms as well as their impact on infant development. Fifth, our assessment of the HPA axis and SNS activity was quite thorough, measuring cortisol and sAA three times a day over two days as recommended in the existing literature (Harville et al., 2009) and including diaries. However, salivary samples were collected at home and despite considerable effort to ensure compliance with the protocol and fidelity of the diurnal collection, these were not objectively measured. Lastly, although it is acknowledged that the parasympathetic branch of the autonomic nervous system (PNS) is involved in stress regulation, we did not include any measure of PNS functioning and we did not evaluate SNS functioning soon after delivery. However, we believe that our study could lead the way to future research adopting a multidimensional approach to the investigation of perinatal depression.

3.6 Conclusions

To conclude, the current study adds to the growing literature suggesting a dysregulation of the HPA axis and IRS in perinatal depression and is among the first to report initial evidence suggestive of a disruption in the feedback relationship between pro-inflammatory cytokines and stress hormones during the perinatal period in women with higher depressive symptoms, emphasizing the need for an integrated multisystem approach to the understanding of the biological underpinnings of perinatal depression. Confirming our findings in larger samples may help to identify early biomarkers associated with perinatal depression as well as new treatment targets, improving the quality of life in women and their children.

Chapter 4: Beyond the HPA-axis: exploring maternal prenatal influences on birth outcomes and stress reactivity.

4.1 Introduction

Mounting evidence indicates that maternal depression during pregnancy is associated with an increased risk of altered physiological, behavioral, emotional and cognitive outcomes in offspring (reviewed in Gentile, 2017). Additionally, antenatal depression has been linked with a modest but statistically significant increase risk for shortened gestation and smaller size at birth (for a meta-analyses see Grote et al., 2010), which in turn, are known to constitute risk factors for the subsequent cognitive and socio-emotional development (Wadhwa, 2005). These early perturbations to child development might confer heightened vulnerability for the development of later psychopathology (Schlotz & Phillips, 2009). Notably, associations between prenatal stress and some indices of child development were found when using an in vitro fertilization prenatal cross fostering design which controls for maternal heritable factors (Rice et al., 2010). Effects have also been found to remain strong and significant when controlling for measured confounding factors, such as smoking or socioeconomic status (e.g. O'Connor et al., 2013), or postnatal effects, such as postnatal symptomatology (e.g. O'Donnell et al., 2013, though not in all studies (e.g. Rice et al., 2010). Thus, it has been proposed that biological mechanisms occurring in the intrauterine environment might mediate, at least in part, the link between antenatal maternal depression and adverse developmental outcomes, in a process often described as "fetal programming" (e.g. Barker, 2004). In particular, several effects of the in utero exposure to maternal depression are thought to arise from an alteration of

the foetal stress response systems, such as the Hypothalamic-Pituitary-Adrenal (HPA) axis (see Glover, O'Connor, & O'Donnell, 2010), carrying possibly long-lasting effects on how the brain will respond physiologically and behaviourally to stress and enhancing the risk of adverse behavioral and physiological outcomes (Megan R. Gunnar & Vazquez, 2001; Tarullo & Gunnar, 2006). In the last decades, an increasing number of reports have sought to elucidate the biological mechanisms underlying fetal programming of offspring's stress reactivity. Based on animal studies, scholars have focused primarily on the mediating role of the maternal HPA axis. However, the weight of supporting evidence in humans is not yet convincing (Rakers et al., 2017) and it is now clear that complementary or alternative mechanisms of stress involving, for example, the maternal sympathetic nervous system (SNS) or the inflammatory response systems (IRS) warrant further consideration. In the following sections, we will examine available evidence on a possible role of the HPA axis, SNS and IRS in fetal programming.

4.1.1 Prenatal HPA axis functioning and offspring's stress reactivity

Glucocorticoids such as cortisol, play a crucial role in promoting fetal brain development and organ maturation (McGowan & Matthews, 2018; Reynolds, 2013b) during pregnancy. However, excessive fetal exposure to glucocorticoids, both synthetic and endogenous, can have detrimental effects in later life (Seckl & Holmes, 2007; Waffarn & Davis, 2012). Indeed, while the fetus is largely protected from cortisol exposure through the activity of the placental enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), a proportion of maternal cortisol crosses the placenta and influences fetal development (Gitau et al., 1998). Thus, it has been hypothesized that distress-linked elevations in maternal cortisol or a distress-linked impairment of the activity of placental 11β-HSD2, might lead to more cortisol passing through the placenta and directly affecting fetal development and stress physiology, with potentially long-lasting consequences (O'Donnell et al., 2009). Additionally, a

dysregulation of maternal HPA axis has been proposed as a potential pathway through which antenatal depression might lead to adverse birth outcomes. In particular, it has been suggested that stress-related increases in maternal cortisol might alter the blood flow to placenta, resulting in restriction of oxygen and nutrients to the fetus, which in turn might lead to fetal growth restriction and/or to premature delivery.

In humans, only partial support for a link between antenatal maternal cortisol and offspring development has been found (reviewed in Zijlmans et al., 2015). Preliminary findings relate higher maternal cortisol during pregnancy with lower offspring birthweight and/or shorter gestational age (reviewed in Reynolds, 2013), although results are dependent on the gestational timing and method of cortisol assessment. Notably, a large prospective study by Goedhart and colleagues (2010) showed that the negative association between maternal cortisol levels in early pregnancy and birth outcomes became statistically insignificant when potential confounders where controlled for in the analyses.

A handful of prospective studies have investigated the association between antenatal maternal cortisol and offspring stress reactivity, and these have provided mixed results. High quality and methodologically rigorous research from Davis and colleagues (2011) showed higher maternal afternoon cortisol in late pregnancy to predict greater newborns' cortisol reactivity to the heel-stick at birth. Additionally, increased levels of maternal cortisol in early gestation were associated with newborns' slower behavioral recovery to the painful stressor. In line with these findings, higher maternal cortisol have been associated with greater cortisol reactivity to the inoculation at 4 years old (Gutteling et al., 2004) and to the first day of school at 5 years (Gutteling et al., 2005) in two small samples. However, an association between higher amniotic fluid cortisol, which is though to provide a more direct index of fetal cortisol exposure, and 17-month-olds' lower cortisol response to maternal separation in a sample of 125 infants (O'Connor et al., 2013) has also been reported. Additionally, a number of studies failed to find any significant association between maternal cortisol levels during

pregnancy and infant's cortisol reactivity to either painful (e.g. Braithwaite, Murphy, & Ramchandani, 2016) or social (e.g. Tollenaar et al., 2011) stressors over the first year of age. Interestingly, a recent study by Giesbrecth and colleagues (2017) on a large sample of 236 mother-infant dyads highlighted sex differences in the effects of prenatal maternal cortisol and distress on 3-month-olds' cortisol response to the heel-stick, with a blunted cortisol response being associated with exposure to high maternal distress and flattened diurnal cortisol trajectory in females, whereas with a steeper maternal diurnal cortisol profile in males.

4.1.2 Prenatal SNS functioning and offspring stress reactivity

During pregnancy, the SNS is increasingly involved to support maternal and fetal energetic requests, with a rise in blood volume and heart rate as well as a reduction in vascular resistance (Silversides & Colman, 2007). Although catecholamines, produced by the SNS under stress conditions, do not cross the placental barrier in biologically relevant concentrations (Giannakoulopoulos et al., 1999), stress-related activation of the SNS might affect utero-placental perfusion, indirectly influencing fetal growth and, in turn, activating the fetal HPA axis (Rakers et al., 2017). Furthermore, catecholamines might down-regulate 11β-HSD2 gene expression (Sarkar et al., 2001), thus possibly increasing fetal glucocorticoid exposure. Despite evidence suggests that elevations in maternal catecholamines, as a result of stress-induced activation of the SNS, could be an alternative mechanism by which antenatal depression might impact offspring's outcomes, a SNS-mediated mechanism of fetal programming have been very poorly explored in humans. Recently, the opportunity to evaluate maternal SNS functioning through the non-invasive assessment of levels of salivary alpha amylase (sAA), an enzyme produced by salivary glands (Nater & Rohleder, 2009), leads the way for more research into the role of maternal SNS in fetal programming. However, to our knowledge, only a very small number of studies explored the association between sAA levels during

pregnancy and child outcomes. Specifically, Giesbrecht and colleagues (2015) found evidence of an association between maternal prenatal levels of sAA and newborns' birth weight in a large sample of dyads (N=291), with women showing a blunted sAA awakening response having lower birth weight infants. Additionally, an altered diurnal sAA pattern was found to relate to previous history of miscarriage in a smaller sample of women (N=93; Giesbrecht et al., 2013). Furthermore, Rash and colleagues (2016) found that maternal prenatal sAA awakening response distinguished among 6-month-olds stress reactivity profiles using discriminant function analyses on a large sample of mothers and infants (N=254). Lastly, Braithwaithe and colleagues (2017) found a significant interaction between maternal prenatal sAA diurnal levels and infants' sex in predicting 2-month-olds' distress to limit in a sample of 88 infants. However, the study was underpowered and the separate effect in males and females, although in the opposite direction (with higher maternal prenatal sAA diurnal levels being associated with lower levels of distress to limits in boys, while higher in females), were not significant. To our knowledge, no previous study has directly examined how maternal antenatal SNS functioning, as indexed by sAA diurnal levels, relate to early cortisol and behavioral reactivity to stress in offspring.

4.1.3 Prenatal IRS functioning and offsprings' stress reactivity

Emerging data support a role of inflammatory pathways in the pathophysiology of depression (Miller & Raison, 2016) and, consistently with this hypothesis, inflammatory markers, such as Interleukine-6 (IL-6) and C-reactive protein (CRP) have been generally found to be increased in depressed pregnant women (Osborne & Monk, 2013). However, knowledge about fetal programming by maternal inflammation is limited. During healthy pregnancy cytokines, produced by cells of the immune system in response to stress and infections, play a crucial role in maintaining a fine balance between pro-inflammatory and anti-inflammatory influences. While circulating levels of pro-inflammatory cytokines, such as Interleukine-6 (IL-6), are increased at the

end of healthy pregnancy (Curry et al., 2008), higher than typical elevations have been linked with adverse pregnancy and birth outcomes such as rejection of the fetus, preeclampsia, spontaneous preterm delivery and lower birth weight (e.g. Vannuccini et al., 2016; Rusterholz et al., 2007; Gillespie, Porter, & Christian, 2016; Sykes et al., 2012). In addition, preliminary evidence exists for a link between maternal antenatal distress, maternal inflammation and adverse birth outcomes. Specifically, Okun and colleagues (2013) reported an association between maternal increased IL-6 levels and smaller birth weights in a small sample of depressed women (N=32), although the effect became non-significant once the model was adjusted for several covariates (i.e. race, parity, education, BMI and medications). Furthermore, Coussons-Read and colleagues (2012) found increased psychological distress and higher levels of inflammatory markers, such as IL-6 or TNF-α, in women who delivered prematurely (N=17) as compared to women who delivered at term (N=156). Furthermore, antenatal levels of IL-6 partially mediate the effects of maternal distress on gestational length. However, the direction of the effect was unexpected (i.e. with higher levels of distress being associated with lower IL-6 and, in turn, less impact on gestational length) and it was no longer significant after correction for multiple comparisons.

Besides affecting maternal health and birth outcomes, animal models suggest that maternal antenatal inflammation could influence fetal development and stress-related physiology with long-lasting effects (e.g. Rounioja et al., 2005; Samuelsson et al., 2004). Consistently with animal data, preliminary studies on very preterm babies showed a link between prenatal exposure to inflammation and altered infants' HPA activity (e.g. Gover et al., 2013; Soliman et al., 2004). In addition, maternal IRS activation during pregnancy have been consistently associated with a range of neuronal dysfunctions and related behavioral phenotypes, such as autism and schizophrenia (for a review see Knuesel et al., 2014) and initial neuroimaging evidence in human suggests that maternal IL-6 levels during pregnancy can affect fetal brain development, leading to altered newborns' amygdala volume and

connectivity (Graham et al., 2017) and reduced functional anisotropy in the central portion of the uncinate fasciculus of newborns' brains (Rasmussen et al., 2018). Nevertheless, the actual mechanisms of fetal programming by maternal inflammation are still mostly unknown. Interestingly, it has been shown that placental transfer of IL-6 occurs in human healthy pregnancy (Zarestky et al., 2004). In addition, indirect effects mediated by placental inflammation have been hypothesized and require further exploration (reviewed in Rakers et al., 2017).

To the best of our knowledge, only two studies in human have investigated the association between maternal antenatal cytokines levels and child behavioral and stress-related outcomes. Specifically, Osborne and colleagues (2018) showed that higher levels of maternal pro-inflammatory cytokines in late pregnancy were associated with less optimal neurobehavioral function soon after birth and greater cortisol reactivity to the immunization at 12 months of age in a sample of 49 depressed women and 57 healthy controls. However, results are limited to unadjusted correlations and require replication while accounting for potential confounders of the association. Furthermore, a pilot study by Gustaffson and colleagues (2018)showed that maternal antenatal inflammation, indexed using a latent variable that included il-6, TNF-α and monocyte chemoattractant protein-1 concentrations, mediate the link between prenatal depressive symptoms and 6-month-olds negative affect. However, the sample was relatively small (N=68) and consisted of women at risk for attention-deficit/hyperactivity disorder, thus findings might not generalize to larger and different populations.

4.2 Summary and study hypotheses

There is mounting evidence suggesting that in utero exposure to maternal depression is a risk factor for subsequent child development. One mechanism through which maternal antenatal depression is thought to affect offspring development is by altering the fetal development of the stress response systems with long term effects.

However, the biological “mediators” involved in the embedding of such adverse experience into the individual stress physiology remains unknown. The most studied candidate has been cortisol, the end product of the HPA axis, however, the evidence in humans for an association between maternal antenatal cortisol levels and offspring outcomes, such as birth outcomes and stress reactivity, is inconsistent (reviewed in Zjlmans et al., 2015). This is mainly because results are beset by large variability in cortisol measures (i.e. sampling times, gestational weeks and biological material from which cortisol is measured), infant’s age and inconsistent inclusion of possible confounders in the analyses as well as inconsistent reporting and inappropriate handling of missing data, thus making hard to disentangle the effects of maternal cortisol from the consequences of variability in study design and methodology. Salivary cortisol is considered a reliable measure of total free plasma cortisol and, compared to blood cortisol measures, allow to obtain ‘stress-free’ repeated samples over the day to picture the circadian rhythmicity of cortisol secretion. Furthermore multiple diurnal cortisol measures are thought to better capture individual variability in the HPA-axis functioning and to obtain more reliable estimates of global cortisol production (Adam & Kumari, 2009); however, only few studies have studied the role of variations in maternal diurnal cortisol pattern in fetal programming and none have examined possible associations with newborns’ stress reactivity soon after birth. Alternatives to an HPA-axis pathway in the link between antenatal maternal depression and offspring outcomes have not yet received considerable empirical support. While initial evidence exists for an association between an altered maternal SNS or IRS functioning during pregnancy and birth outcomes, very little is known about the potential role of maternal inflammation or diurnal variation in sAA levels in shaping infants stress reactivity.

The current study sought to investigate the influence of naturally occurring variations in maternal depressive symptoms, stress hormones and inflammatory markers in late pregnancy on birth outcomes and early stress regulation in a sample of

healthy women and infants. To our knowledge, the present study is unique in the assessment of multiple biological markers of maternal stress and immune systems in association with infant outcomes. We evaluated newborns soon after birth in order to limit postnatal influences and we focused on late pregnancy, as exposure to maternal stress signals in late gestation is thought to influence the developing fetal stress response systems (Davis et al., 2011) and is associated with later risk for emotional problems (Rice et al., 2007). Furthermore, we tested the robustness of the effects of antenatal maternal depression on infant early outcomes, by investigating associations with possible overlapping predictors, such as maternal anxiety. Based on available literature, we hypothesized that higher maternal depressive symptoms during pregnancy would be associated with more negative birth outcomes and with greater stress reactivity in offspring. Furthermore, we predicted that variations in maternal diurnal cortisol pattern would be associated with newborns' birth outcomes and stress resactivity. Conversely, analyses on possible associations between maternal antenatal inflammation or sAA levels and newborns' birth outcomes and stress reactivity were exploratory and we made no a priori hypothesis due to limited available literature.

4.3 Material and methods

4.3.1 Participants

Study participants included mother-infant dyads from the Effects of Depression on Infants (EDI) Study, an ongoing longitudinal investigation into the effects of maternal depression on infants' bio-behavioral development. Women at 30-33 gestational weeks were consecutively recruited in 3 Italian hospitals and followed longitudinally. Prenatal inclusion criteria were: aged 18-45 years, normotensive, with singleton uncomplicated pregnancy, non-smoker, not afflicted by any disease, not taking any chronic medications, and with no known substance/alcohol abuse problems or chronic psychiatric disorders (except for depression and anxiety). From the initial

sample of 110 women, 6 were excluded for reasons related to intrauterine death and newborn health problems (N= 2), delivery in a different hospital (N= 1) and lack of behavioral data (N= 3). Most women (mean age=33.04, SD=3.83) were Italian (97.1%), middle-high class (94.8%) and primiparous (89.4%). Infants (51.9% males) were mostly born by vaginal delivery (82.7%) and were full term, except for two born, respectively, at 35 and 36 gestational weeks in good health. Women who were excluded did not differ from participants on any demographic variables, depression or anxiety scores.

4.3.2 Procedure

All pregnant women filled in two questionnaires on anxiety and depression and a demographic and pregnancy information form between 30-33 gestational weeks (mean gestational age=31.45; SD=1.40) and provided biological samples between 34-36 gestational weeks (mean gestational age=34.76; SD=1.12). Between 48-72 hours after delivery, infants' behavioral and cortisol response to the heel-stick was assessed and women filled in a form on delivery and new-born's health. The Ethics Committee of the Scientific Institute Eugenio Medea, of University College London, and of the hospitals involved approved the study protocol.

4.3.3 Maternal assessment

Psychological assessment. Maternal antenatal depressive symptoms were evaluated through the Italian version (Benvenuti et al., 1999) of the 10-item Edinburgh Postnatal Depression Scale (EPDS; Cox et al.,1987), a self-report questionnaires widely used to screen for perinatal depression. Maternal anxiety symptoms were assessed through the Italian version (Pedrabissi & Santinello, 1989) of the 20-item trait anxiety subscale of the State-Trait Anxiety Inventory (STAI-T; Spielberger et al., 1970), a well-validated self-report measure of trait anxiety, widely employed during pregnancy (e.g. Goedhart et al., 2010).

Health status. Both during pregnancy and after delivery, participants were asked to fill in an ad-hoc form to collect data on health, pregnancy (e.g. any complication/risk/medical disorder etc.) and delivery (e.g. length of labor, analgesia etc.).

Biological assessment. Ninety-seven women out of 104 consented to blood draw. Blood was drawn by venipuncture in the morning and kept refrigerated at +4° until it reached the Biological Lab of Medea Institute where serum was centrifuged, aliquoted, and stored at −80°. Biological assays for IL-6 and CRP levels were run in duplicate by using Quantikine High Sensitivity ELISA kits (R&D Systems Europe, LTD) at LaboSpace in Milan according to the manufacturer's instructions. Intra-assay coefficient of variation (CV) was <6% for IL-6 and <3% for CRP, inter-assay CV was <10% for both markers.

Saliva was collected at home on two consecutive days immediately upon awakening, 30 minutes post-awakening and before going to bed to provide a general index of the diurnal pattern (O'Donnell et al., 2013). Participants were instructed to collect whole unstimulated saliva samples by passive drool, to record time of collection on a diary and to avoid eating, tooth-brushing and exercising 30 minutes before collection and eating a meal in the hour before the evening sample. The mean time from the awakening collection and the 30-minutes post-waking collection was 30.82 minutes on day 1 (SD=3.64, range: 20.00-60.00) and 31.44 minutes on day 2 (SD=6.81, range: 20.00-90.00). As 1 pregnant woman collected the second sample more than one hour from awakening, this sample was excluded from analyses (e.g. O'Donnell, 2013). All saliva samples were stored frozen at -80° until assayed for salivary cortisol at the Biological Lab of Medea Institute, using a competitive high sensitivity enzyme immunoassay kit (Expanded Range High Sensitivity Cortisol EIA Kit, Salimetrics), and for sAA at the Salimetrics Centre of Excellence testing lab at Anglia Ruskin University, using a kinetic enzyme assay kit (Salimetrics α-Amylase Kinetic Enzyme Assay Kit). All samples for each woman were run in the same assay to

minimize method variability. Salivary cortisol assays were run in duplicate, except for 2 samples with minimal volume. Average intra- and inter-assay coefficients were <6% and <8%, respectively. A random 10% of the sAA assays were run in duplicate to confirm reliability. The intra-assay coefficient of variation was < 3%.

4.3.4 Neonatal assessment

Health and birth outcomes. Information on newborns' health (i.e. gestational age, weight, body length and head circumference at birth, Apgar scores, any complication/abnormal sign, any medication/treatment, breast or formula feeding, time of discharge) was extracted from medical records.

Behavioral assessment. Newborns' behaviour was videotaped during 10-minute baseline period, followed by the heel-stick and by 5-minutes recovery between 48 and 72 hours after birth (mean=57.06; SD=12.15). The average length of the heel stick was 3.99 minutes (SD=2.32). Newborns' videotaped behavior was evaluated every 20-seconds on a 5-point scale (i.e. sleep, drowsy, awake and alert, awake and fussy, and crying), according to a modified version of a coding system employed by Davis et al. (2011). The highest state observed during each epoch was coded. As the length of the heel-stick was variable, the first 2 minutes from the beginning of the blood draw were coded for all infants to ensure complete data for the whole sample. The average state score for each of the three phases (10-minutes baseline, 2-minutes response, 5-minutes recovery) was calculated. For approximately 10% of cases (N=10), two observers, independently coded newborn's behaviour. Intra-class correlation was, respectively, equal to 1.0 for baseline, 0.99 for response and 0.99 for recovery. Coders were blinded to all prenatal or postnatal data.

Cortisol collection and assay. Salivary cortisol samples were collected after the baseline period before the beginning of the heel-stick, and after 20 and 40 minutes through specifically designed swab (SalivaBio Infant's Swab, Salimetrics). Infants were not fed in the 30 minutes before the heel-stick (mean time from last feeding=68.50

minutes, SD=50.05) and were not handled during the study protocol besides that which was strictly required for the examination. Complete cortisol data were available for 49 infants while one or two sample were missing for 30 infants and 25 infants had no data due to insufficient saliva volumes. Infants with complete, partial or missing data did not differ on any sociodemographic/maternal variables, infants-related variables or situational factors. Saliva samples were stored at -80º until assayed for cortisol according to the same procedure described for maternal cortisol. All samples from any individual infant were run on the same assay and in duplicate, excepted for 10 samples with minimal volume. The average intra- and inter-assay coefficients of variance were below 7% and 10%, respectively.

4.3.5 Statistical analyses

Variables were first examined for outliers and skewness. Distributions of both maternal and infants' biological markers were positively skewed even after removing samples greater than 3 SD from the mean (N=7 for maternal cortisol, N=4 for sAA, N=3 for IL-6, n=4 for infants' cortisol), thus variables were natural log transformed to approximate normal distributions. Four summary parameters were calculated to index different aspects of maternal HPA and SNS diurnal functioning. Specifically, cortisol and sAA values were averaged across the 2 days for each time point and mean values were used to compute: awakening values, response to awakening (calculated by subtracting waking values from the 30min post-waking levels) and diurnal slope (calculated by subtracting the bedtime values from the waking values), as done in prior work (e.g. de Weerth et al., 2013). Additionally, daily average cortisol and sAA were calculated as the area under the curve (AUCg) using the trapezoid method with respect to the ground (Pruessner et al., 2003) for each day separately and, as the two values were highly correlated ($r=.58$, $p<.001$, for cortisol, $r=.74$, $p<.001$ for sAA), the mean of the 2 days was used. To evaluate the potential effect of variables known to affect stress and immune physiology, preliminary Pearson correlations and univariate

analysis of variance (ANOVA) were employed. All variables found to be significantly associated with the outcomes examined where included as covariates in all subsequent analyses.

Separate hierarchical regression analyses were performed to evaluate the effects of maternal prenatal depression and biological markers on infant birth outcomes (i.e. birth weight, body length, and head circumference), while adjusting for covariates. Hierarchical Linear Models (HLMs) were estimated to investigate the influence of maternal depression and biological functioning during pregnancy on the trajectories of infants' cortisol and behavioral reactivity, while accounting for the hierarchical structure of the data (three time-points nested within individuals). HLMs were specified at two levels where individuals were level 2 and time was level 1. Time was centered at baseline so that the model intercept represents the mean cortisol/behavioral state at baseline. Before fitting explanatory models including level-2 predictors, a baseline model of cortisol and behavioral response was fitted to describe the trajectory of infants' response, including a linear and quadratic slope for time. A random intercept and a random linear slope were included to allow between-person variability. The explanatory variables were centered around the grand mean and entered in the model one-by-one. Gender was centered at males. Model fit was tested with likelihood deviance difference tests for nested models.

Given the high correlation between maternal depression and anxiety ($r=.60$, $p<.001$), an additional set of analyses were performed to evaluate the effects of maternal anxiety, rather than depression and are reported in Supplementary Section.

Statistical analyses were performed using SPSS 24 and MLWiN 3.02.

4.4 Results

4.4.1 Descriptive analyses and confounders

Descriptive characteristics for all study variables are presented in Table 4.1, whereas correlations between prenatal variables, birth outcomes and stress reactivity are shown in Table 4.2.

In a series of univariate correlation analyses we evaluated the associations between sociodemographic factors (maternal age, marital status, education and SES), pregnancy- and delivery-related factors (parity, mode of delivery, assisted delivery, length of labor), infant factors (gestational age, birth weight, gender, Apgar scores and postnatal age), situational factors (length of the heel-stick procedure, time of the day and time from last feeding) and newborn outcomes. Gestational age was associated with 20-min post-stressor cortisol levels ($r=-.31$, $p=.02$) and length of the heel-stick was positively related to both 20-min post-stressor cortisol levels ($r=.30$, $p=.02$) and averaged behavioral state during the response period ($r=.20$, $p=.04$). Thus, they were included as covariates in HLMs.

Additionally, while infant's sex was not related to cortisol or behavior, there was a significant association between fetal sex and maternal waking cortisol levels ($F(101,1)=5.02$, $p=.03$). As sex-differences in the association between prenatal distress and infant cortisol have also been reported (e.g. Giesbrecth et al., 2017), infant's gender was included as a covariate in subsequent analyses. Furthermore, gestational age, sex and maternal pre-pregnancy BMI were associated with birth outcomes, with infants with higher gestational ages, males and born from mothers with higher BMI, having greater anthropometric measures (all $ps<.05$), and were included as covariates in hierarchical regression analyses.

Table 4.1 - Descriptive statistics for study variables at the prenatal and postnatal assessment

Study Variable	Mean	SD	Range
Prenatal			
Maternal cortisol (µg/dl)			
Waking	0.38	0.13	0.13-0.83
Waking +30’	0.50	0.15	0.10-0.91
Bedtime	0.18	0.06	0.01-0.41
Response to waking	0.12	0.16	-0.52-0.52
Diurnal slope	0.20	0.13	-0.12-.70
AUCg	262.63	58.59	83.76-442.71
Maternal sAA (U/ml)			
Waking	69.84	64.75	3.00-463.84
Waking +30’	47.74	37.90	2.80-190.10
Bedtime	99.14	80.95	3.28-562.71
Response to waking	-21.07	52.65	-350.94-119.97
Diurnal slope	-28.48	85.67	-405.11-298.32
AUCg	3601.07	690.76	1777.74-5394.20
Maternal CRP (ng/ml)	3730.16	2766.02	480.04-11244.10
Maternal IL-6 (pg/ml)	1.68	1.04	0.48-6.47
Maternal depression (EPDS)	5.41	4.44	0-19
Maternal anxiety (STAI-T)	36.52	9.64	21-71
Postnatal			
Gestational age (weeks)	39.48	1.25	35-42
Birth weight (grams)	3296.83	443.27	2170-4440
Body Length (cm)	50.08	1.88	45-54
Head Circumference (cm)	34.48	1.30	31.50-39.50
Newborns’ cortisol (µg/dl)			
Baseline	0.66	0.45	0.06-2.27
20-min post-stressor	0.83	0.63	0.15-3.00
40-min post-stressor	0.82	0.73	0.17-3.04
Newborns’ behavior			
Baseline	2.15	1.16	1.00-4.63
Response	3.69	1.10	1.00-5.00
Recovery	2.47	1.17	1.00-4.80

Table 4.2 – Bivariate correlations among prenatal variables, birth outcomes and infant stress reactivity

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. Prenatal EPDS													
2. Prenatal Cortisol AUCg	-.17												
3. Prenatal sAA AUCg	.07	**.24***											
4. Prenatal IL-6	**.20***	.13	.19										
5. Prenatal CRP	.13	.11	-.01	**.31****									
6. Birth weight	.11	-.03	**.18**^{t}	.03	**.24***								
7. Body length	.06	-.05	.08	-.03	.14	**.90****							
8. Head Circumference	.16	.02	.06	-.18	.09	**.63****	**.59****						
9. Cortisol baseline	**-.22**^{t}	.01	-.00	-.10	.06	.14	.11	.05					
10. Cortisol post 20’	-.08	-.03	.04	-.14	-.14	-.02	-.06	.03	.27				
11. Cortisol post 40’	-.08	.04	-.02	-.07	.04	-.06	-.08	.00	.01	**.80****			
12. Behavioral baseline	-.00	.09	.01	-.01	.02	-.01	.00	-.13	**.31****	.21	.17		
13. Behavioral response	-.10	-.05	.11	-.06	-.09	.13	.07	.08	.00	.21	.03	.01	
14. Behavioral recovery	-.16	.12	.10	.02	-.07	.11	.13	.08	.12	**.30***	.11	.14	**.51****

Note: AUCg, area under the curve with respect to the ground; sAA, salivary alpha amylase; CRP, C-Reactive Protein; IL-6, Interleukine-6.
* p<.05; **p<.01, ^{t} p=.06, p>.05 was considered non-significant.

4.4.2 Association between maternal prenatal variables and birth outcomes

As shown in Table 4.3, multiple hierarchical regression analyses revealed significant associations between both higher maternal sAA waking and AUCg levels and higher infant weight at birth, as well as between higher IL-6 levels and smaller head circumference at birth, after controlling for pre-pregnancy BMI, infant's sex, and gestational age. No significant associations between maternal cortisol or maternal depression and any birth outcomes were found.

4.4.3 Association between maternal prenatal variables and newborns' cortisol reactivity

The unconditional means model for newborns cortisol showed significant variability at the individual level (level-2; σ^2_{u0}=0.042, p<.001) and between-occasions (level-1; σ^2_{e0}=0.057 p<.001). Infants displayed the expected cortisol response characterized by a significant linear slope of time (p=.03), and a marginally significant quadratic slope (p=.07). Also the random linear slope term was statistically significant (p<.001), suggesting significant between-person variability in the cortisol linear increase. Overall, this model resulted in a significant improvement in fit over the unconditional means model (deviance difference (4)=37.50, p<.001).

Fixed independent effects of prenatal variables (i.e. depression, cortisol, sAA, CRP and IL-6) on mean cortisol baseline levels and on the linear and quadratic slopes of time were tested separately, while controlling for gestational age, infant's sex and length of the heel-stick.

As shown in Table 4.4, maternal cortisol response to awakening (CAR) was significantly associated with both the linear (p=0.012) and quadratic (p=.013) slopes of newborn's cortisol response. Specifically, as shown in Figure 4.1a, higher maternal CAR during pregnancy was related to a flatter cortisol response to the heel-stick, while lower maternal CAR was associated with greater infants' cortisol reactivity.

Table 4.3 – Hierarchical linear regression analyses predicting weight and head circumference at birth

	Birth Weight										**Head Circumference**							
	Cortisol AUCg		sAA AUCg		sAA waking		IL-6		CRP		Cortisol AUCg		sAA AUCg		IL-6		CRP	
	β	p	β	p	β	p	β	p	β	p	β	p	β	p	β	p	β	p
Step 1:																		
Gender	**-.19**	**.03**	**-.18**	**.04**	**-.18**	**.04**	-.13	.17	-.14	.14	-.17	.08	**-.17**	**.08**	-.10	.34	-.14	.19
Pre-pregnancy BMI	**.23**	**.01**	**.22**	**.01**	**.22**	**.01**	.16	.09	**.19**	**.05**	**.20**	**.04**	**.19**	**.06**	.11	.30	.12	.27
Gestational Age	**.41**	**.00**	**.40**	**.00**	**.40**	**000**	**.43**	**.00**	**.42**	**.00**	**.24**	**.01**	**.23**	**.02**	**.27**	**.01**	**.25**	**.02**
ΔR^2 for step 1	.28	.00	.26	.00	.26	.00	.25	.00	.24	.00	.15	.00	.14	.00	.11	.03	.10	.03
F_{model}	13.14	.00	12.50	.00	12.50	.00	9.70	.00	9.64	.00	5.28	.00	5.03	.00	3.26	.03	3.25	.03
Step 2:																		
EPDS	.12	.14	.14	.10	.14	.10	.13	.16	.15	.11	.12	.20	.15	.12	.15	.16	.16	.12
ΔR^2 for step 2	.01	.14	.02	.10	.02	.10	.02	.16	.02	.11	.01	.20	.02	.12	.02	.16	.02	.12
F_{model}	10.51	.00	10.21	.00	10.21	.00	7.85	.00	8.01	.00	4.41	.00	4.44	.00	2.97	.02	3.08	.02
Step 3:																		
Biological predictors	-.06	.46	**.18**	**.03**	**.17**	**.05**	-.06	.52	.09	.36	-.09	.35	.02	.82	**-.33**	**.00**	-.01	.96
ΔR^2 for step 3	.00	.46	**.03**	**.03**	**.03**	**.05**	.00	.52	.01	.36	.01	.35	.00	.82	.10	.00	.00	.96
F_{model}	8.48	.00	9.36	.00	9.18	.00	6.32	.00	6.56	.00	3.70	.00	3.53	.01	4.62	.00	2.43	.04

Note: AUCg, area under the curve with respect to the ground; sAA, salivary alpha amylase; BMI, Body Mass Index;

Table 4.4 – Full prediction models for the effects of maternal depression (EPDS) and cortisol diurnal indices on infants' cortisol response

	Model 1 Waking		**Model 2 CAR**		**Model 3 Diurnal slope**		**Model 4 AUCg**	
	Estimate (SE)	p	Estimate (SE)	p	Estimate (SE)	p	Estimate (SE)	p
Fixed effects								
Intercept	0.508 (0.039)	<.001	0.508 (0.038)	<.001	0.504 (0.038)	<.001	0.505 (0.046)	<.001
Gender	-0.053 (0.052)	0.31	-0.050 (0.051)	0.32	-0.046 (0.050)	0.36	-0.051 (0.051)	0.32
Gestational Age	-0.022 (0.019)	0.25	-0.025 (0.020)	0.21	-0.027 (0.019)	0.16	-0.021 (0.020)	0.29
Heel-stick length	0.001 (0.011)	0.98	0.001 (0.010)	0.93	0.004 (0.011)	0.69	0.001 (0.011)	0.91
EPDS	-0.075 (0.040)	0.06	-0.068 (0.038)	0.08	-0.063 (0.039)	0.11	-0.071 (0.040)	0.08
Cortisol	-0.135 (0.315)	0.66	0.245 (0.253)	0.33	0.337 (0.286)	0.24	0.000 (0.001)	0.94
Linear	-0.005 (0.008)	0.54	0.008 (0.003)	<.001	0.013 (0.005)	0.01	0.003 (0.003)	0.27
EPDS	0.002 (0.003)	0.47	0.001 (0.003)	0.59	0.001 (0.003)	0.77	0.001 (0.003)	0.73
Cortisol	0.029 (0.023)	0.20	**-0.042 (0.017)**	**0.01**	0.035 (0.020)	0.08	-0.000 (0.000)	0.48
Quadratic	0.000 (0.000)	0.41	-0.000 (0.000)	<.001	-0.000 (0.000)	0.02	-0.000 (0.000)	0.51
EPDS	-0.000 (0.000)	0.65	-0.000 (0.000)	0.79	-0.000 (0.000)	0.94	-0.000 (0.000)	0.95
Cortisol	-0.001 (0.000)	0.17	**0.001 (0.000)**	**0.01**	0.001 (0.000)	0.09	0.000 (0.000)	0.32
Random effects								
Level 2 (individual)								
Intercept variance	0.034(0.010)	<.001	0.036(0.010)	<.001	0.034(0.010)	<.001	0.034(0.010)	<.001
Linear slope variance	0.000(0.000)	<.001	0.000(0.000)	<.001	0.000(0.000)	<.001	0.000(0.000)	<.001
Intercept/Linear slope covariance	-0.000(0.000)	0.09	-0.001(0.000)	0.07	-0.000(0.000)	0.07	-0.000(0.000)	0.09
Level 1 (occasions)								
Intercept variance	0.022(0.005)	<.001	0.020(0.004)	<.001	0.022(0.005)	<.001	0.022(0.005)	<.001

Note: Cortisol diurnal indices are examined separately in Model 1-4. CAR, cortisol awakening response, AUCg, area under the curve with respect to the ground;

The inclusion of maternal CAR resulted in a significant improvement in the model fit (deviance difference (3)=9.173, p=.02). In contrast, there was no significant association between newborns' cortisol response and maternal sAA, IL-6 or CRP levels.

4.4.4 Association between maternal prenatal variables and newborns' behavioral reactivity

The unconditional means model for newborns behavioral regulation showed significant variability between-occasions (level-1; σ^2_{e0}=0.136, p<.001), while variability at the individual level was not significant (level-2; σ^2_{u0}=0.006, p=.47). There was a significant behavioral change in response to the heel-stick, as indexed by the significant linear and quadratic slopes of time (both ps<.001). The random linear slope term was statistically significant (p<.001), suggesting significant between-person variability in the behavioral linear increase. Additionally, the significant positive association (p=.013) between the random effects of intercept at level-2 and the linear slope indicates that there was a greater increased in the behavioral state in response to the heel-stick in individuals with higher behavioral states at baseline. Overall, this model results in a significant improve of the fit over the unconditional means model (deviance difference (4)=106.46, p<.001).

Fixed independent effects of prenatal variables on mean behavioral state at baseline and change over time were tested separately, while controlling for covariates. Maternal diurnal slope was significantly associated with both the linear (p=.02) and quadratic (p=.05) slopes of newborns' behavioral response (Table 4.5). Specifically, as shown in Figure 4.1b, a lower maternal cortisol diurnal slope was associated with greater behavioral reactivity in the infant, while a higher maternal diurnal slope was related to a less marked behavioral reactivity. The inclusion of maternal diurnal slope results in a significant improvement of the model fit (deviance difference (3)=7.8, p=.05).

Table 4.5 – Full prediction models for the effects of maternal depression (EPDS) and cortisol diurnal indices on infants' behavioural response

	Model 1 Waking		**Model 2 CAR**		**Model 3 Diurnal slope**		**Model 4 AUCg**	
	Estimate (SE)	p	Estimate (SE)	p	Estimate (SE)	p	Estimate (SE)	p
Fixed effects								
Intercept	1.086 (0.040)	<.001	1.090 (0.040)	<.001	1.086 (0.040)	<.001	1.086 (0.040)	<.001
Gender	0.001 (0.046)	0.99	0.001 (0.046)	0.99	0.004 (0.046)	0.98	0.008 (0.046)	0.86
Gestational Age	0.020 (0.018)	0.28	0.014 (0.019)	0.46	0.020 (0.018)	0.23	0.022 (0.019)	0.22
Heel-stick length	0.009 (0.010)	0.39	0.011 (0.010)	0.24	0.009 (0.010)	0.36	0.012 (0.010)	0.21
EPDS	0.002 (0.046)	0.96	0.003 (0.046)	0.95	0.003 (0.046)	0.95	0.008 (0.047)	0.86
Cortisol	0.180 (0.380)	0.63	0.264 (0.322)	0.41	0.274 (0.353)	0.44	0.001 (0.001)	0.35
Linear	0.047 (0.008)	<.001	0.029 (0.003)	<.001	0.038 (0.004)	<.001	0.048 (0.011)	<.001
EPDS	-0.002 (0.003)	0.50	-0.001 (0.003)	0.79	-0.002 (0.003)	0.56	-0.002 (0.003)	0.56
Cortisol	**-0.055 (0.025)**	**0.03**	-0.003 (0.022)	0.88	**-0.056 (0.024)**	**0.02**	-0.000 (0.000)	0.08
Quadratic	-0.000 (0.000)	<.001	-0.000 (0.000)	<.001	-0.001 (0.000)	<.001	-0.001 (0.000)	<.001
EPDS	0.000 (0.000)	0.83	-0.000 (0.000)	0.92	0.000 (0.000)	0.90	0.000 (0.000)	0.86
Cortisol	0.001 (0.000)	0.07	0.000 (0.000)	0.92	**0.001 (0.000)**	**0.05**	0.000 (0.000)	0.07
Random effects								
Level 2 (individual)								
Intercept variance	0.058(0.017)	<.001	0.056(0.017)	<.001	0.058(0.017)	<.001	0.058(0.017)	<.001
Linear slope variance	0.000(0.000)	<.001	0.000(0.000)	<.001	0.000(0.000)	<.001	0.000(0.000)	<.001
Intercept/Linear slope covariance	-0.001(0.000)	.01	-0.001(0.000)	.01	-0.001(0.000)	.01	-0.001(0.000)	.01
Level 1 (occasions)								
Intercept variance	0.055(0.008)	<.001	0.057(0.008)	<.001	0.055(0.008)	<.001	0.056(0.008)	<.001

Note: Cortisol diurnal indices are examined separately in Model 1-4. CAR, cortisol awakening response, AUCg, area under the curve with respect to the ground;

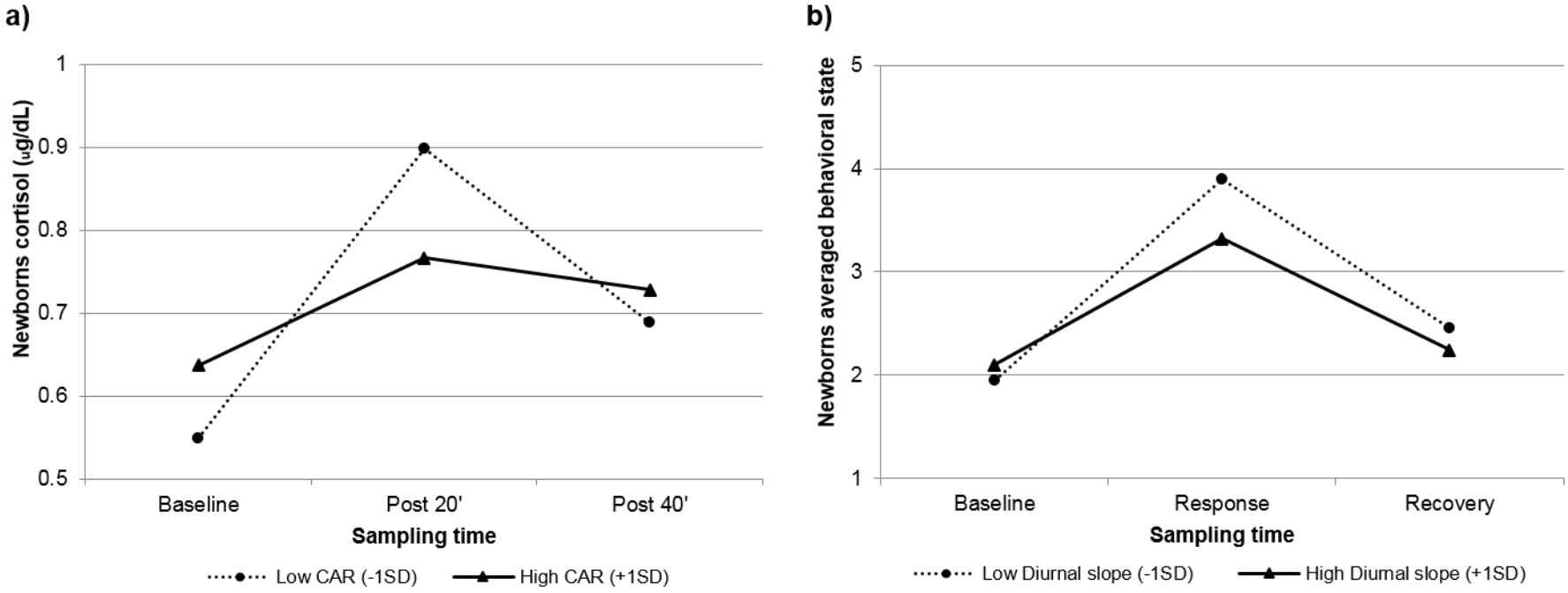

Figure 4.1 – a) Cortisol values before and after the heel-stick for newborns whose mothers had higher (+1 SD) and lower (-1SD) cortisol awakening response (CAR), after adjusting for covariates; b) Averaged behavioural state before, during and after the heel-stick for newborns exposed prenatally to higher (+1 SD) and lower (-1SD) maternal cortisol diurnal slope, after adjusting for covariates

Additionally, maternal cortisol at awakening was significantly associated with the linear slope of newborns' response (p=.03), with lower levels of cortisol being associated with a steeper increase in behavioral response to the heel-stick, while higher levels of cortisol were related to a flatter increase. However, the inclusion of maternal cortisol at awakening did not significantly improve the model fit (deviance difference (3)=6.34, p=.10). Maternal depressive symptoms, diurnal sAA or inflammatory markers were not significantly associated with infants' behavioral regulation.

4.4.5 Supplementary analyses

The set of analyses run to test the effects of maternal anxiety on newborns' cortisol and behavioral reactivity yielded comparable results to those found for maternal depression. Specifically, no significant main effect for maternal anxiety on newborns' cortisol and behavioral response was found (all ps>.05). In addition, including maternal anxiety, rather than depression, did not change the direction and significance of the effects of maternal diurnal cortisol measures on newborns' cortisol and behavioral response.

4.5 Discussion

Findings from the present study extend available literature by showing that variations in maternal diurnal cortisol in the last trimester of pregnancy are related to specific newborns' behavioural and physiological patterns of stress reactivity soon after birth, in line with our hypothesis. Furthermore, the current results add to the limited literature on alternative biological mechanisms involved in fetal programming, by highlighting significant associations between variations in markers of maternal SNS and IRS functioning and birth outcomes in a low risk sample of mother-infant dyads. In

contrast with our predictions, maternal self-reported depressive or anxiety symptoms during pregnancy were not related with newborn's birth outcomes or stress reactivity.

4.5.1 Prenatal maternal factors and birth outcomes

Markers of maternal IRS and SNS functioning during pregnancy were significantly associated with newborns' anthropometric measures, such as head circumference and birth weight. Unexpectedly, however,maternal diurnal cortisol levels and self-reported distress symptoms were not related to any birth outcomes.

The significant association between higher prenatal maternal IL-6 levels and smaller head circumference at birth is a novel finding. While observational epidemiological studies report that maternal infections during pregnancy – which activate IRS - are associated with increased risk of neuropsychiatric disorders, such as schizophrenia, in the resulting offspring (Estes & Mcallister, 2016), the extent to which there is a programming effect of maternal cytokines is unknown. Head circumference is considered a marker for intrauterine brain development and is associated with later cognitive function (Broekman et al., 2009a). Thus, our findings are consistent with results from animal studies (e.g. Meyer et al., 2006) and preliminary brain imaging studies in human infants (Grahman et al., 2017; Rasmussen et al., 2018) suggesting that maternal inflammation can affect fetal growth and neural development. Whether directly transferred through the placenta or indirectly affecting the fetal development through placental inflammation, elevated cytokines are hypothesized to act at multiple levels of the fetal brain, affecting neurogenesis, gliogenesis and the neurotransmitter systems (reviewed in Rakers et al., 2017). An alternative mechanism is that epigenetic mechanisms could mediate the impact of prenatal maternal inflammation on fetal brain development with potentially long-lasting effects as suggested by results of animal studies (Kundakovic & Jaric, 2017). Future efforts are needed to elucidate the role of maternal IL-6 levels in influencing rates of fetal growth and brain development in humans.

Interestingly, consistently with previous findings (reviewed in Osborne & Monk, 2013), we reported a positive association between IL-6 levels and depressive symptoms in a low risk sample of pregnant women free from any medication or medical conditions. This result is in line with accumulating evidence for a role of inflammation in the vulnerability for depression in non-pregnant samples (Miller & Raison, 2016) and encourages further research into a possible inflammatory pathway from depression to altered offspring outcomes. However, it is important to note that, contrary to our initial hypothesis, depressive symptoms were not associated with newborns' birth outcomes. This is consistent with studies on low risk samples showing null or weak associations between prenatal continuous depression measures and adverse birth outcomes (reviewed in Grote et al., 2010). Furthermore, a systematic review showed that antenatal depression is rarely associated with newborn's gestational age and birth weight in larger studies and in studies where potential confounders of the association, such as sociodemographic variables, timing of delivery and pre-pregnancy maternal BMI, are taken into account (Accort et al., 2015). Thus, we might speculate that the association between antenatal depression and birth outcomes is spurious and might arise from the combination of multiple risk factors. Research on high-risk samples are needed to further elucidate the link between prenatal depression, IRS functioning and birth outcomes.

Exploratory analyses showed that higher maternal prenatal sAA levels at awakening and throughout the day were associated with higher infant birth weight. Our findings are partially in line with the only published study on the link between maternal sAA and birth outcomes (Giesbrecht et al., 2015), which showed an association between greater birth weight and higher maternal sAA waking levels and decrease after waking. Replication in larger cohorts is needed before the significance of these preliminary findings can be discussed. We might speculate that mild elevations in maternal sAA indicate an increased involvement of the SNS to ensure maternal and fetal energetic resources for growth during late normative pregnancy. This hypothesis

is in line with evidence (e.g. DiPietro et al., 2011), suggesting that adjustments in maternal neuroendocrine functioning over gestation are mainly generated and supported by the feto-placental unit, within a bi-directional communication.

Lastly, in contrast with previous studies (e.g. Buss et al.,2009, Bolten et al., 2011, Kivlighan et al., 2008) and with our initial hypothesis, maternal diurnal cortisol levels in late pregnancy were not associated with infants' birth outcomes. Notably, in the majority of studies reporting a significant link between maternal cortisol and lower birthweight, cortisol was assessed in early-mid pregnancy (reviewed in Reynolds et al., 2013). Thus, it is possible that a critical window of susceptibility for setting foetal growth trajectory, and for a role of HPA axis in influencing it, occurs early in gestation. Additionally, while animal studies provided strong support for a role of maternal HPA axis in determining fetal growth (see Cottrell & Seckl, 2009 for a review), a number of large studies in humans failed to detect a significant association between maternal cortisol and birth outcomes, once the model was adjusted for significant covariates, such as gestational age (e.g. Goedhart et al., 2010; Li et al., 2012; Gilles et al., 2018). Likewise, even among those studies detecting significant associations between maternal antenatal cortisol and birth weight, correlations were relatively weak, suggesting that maternal cortisol is only one factor contributing to infant birth weight among many others including gestational age, pregnancy complication and placental conditions. Further research is needed to disentangle the complex interplay of factors that contribute to low birth weight infants.

4.5.2 Prenatal maternal factors and newborns' stress reactivity

Infants displayed the expected increase in cortisol levels and behavioural distress in response to the heel-stick in the neonatal period. In line with our hypotheses, findings support a role of variations in maternal diurnal cortisol rhythm, and in particular in maternal CAR and diurnal slope, during late pregnancy in influencing offspring's stress reactivity soon after birth. However, maternal self-

reported depressive symptoms, sAA diurnal levels and inflammatory markers were not significantly associated with newborn's stress response at the heel-stick.

Maternal CAR was related to newborns' cortisol reactivity, with infants prenatally exposed to a higher maternal CAR showing a flatter cortisol response to stress. Only two studies have examined the role of maternal prenatal CAR on infant stress reactivity. In line with our findings, Giesbrecht et al. (2017) reported a significant association between a higher maternal CAR and blunted cortisol reactivity in 3-month old boys, whereas De Weerth et al. (2013) related a higher maternal CAR to impaired infants' cortisol habituation, as shown by a stable cortisol response over repeated maternal separations. Despite methodological differences, these preliminary findings might suggest that prenatal exposure to higher maternal CAR might impair the infants' capacity to flexibly react to stress.

Maternal cortisol diurnal slope during pregnancy was significantly associated with newborns' behavioral response to the heel-prick, with a smaller decline in maternal cortisol levels throughout the day being associated with greater infant behavioral reactivity. These findings are novel; while previous studies have shown greater behavioral stress reactivity in infants prenatally exposed to higher maternal cortisol (e.g. Davis et al., 2011; Werner et al., 2013), none of these studies included diurnal cortisol measures.

Both the CAR and diurnal slope are key components of the diurnal cortisol rhythm that are relatively preserved and sensitive to psychological distress during pregnancy (Kivlighan et al., 2008). However, their role in fetal programming is still largely unknown. Thus, it is unclear why different measures of maternal diurnal cortisol relate to different aspects of infants stress reactivity (i.e. physiological or behavioral). Interestingly, in line with previous reports (e.g. De Weerth et al., 2013) maternal CAR and cortisol decline were moderately correlated, with higher CARs being related to flatter cortisol declines. Therefore, it would seem that an alteration of maternal diurnal cortisol pattern is, to a certain extent, associated with both a dampened cortisol

reactivity and an amplified behavioral response to stress in the infants soon after birth. As a lack of coordination between adrenocortical and behavioral responses has been associated with later psychopathology (e.g. Quas et al. 2000), it would be important to evaluate whether neonatal profiles of reactivity persist and are predictive of later patterns of stress reactivity and later emotional or behavioral dysregulation.

Questions remain about the mechanisms underlying the associations between diurnal variation in maternal cortisol diurnal measures and patterns of infants' stress reactivity. On the one hand, we might speculate that both a higher than typical CAR or a flatter cortisol diurnal decline might result in higher fetal cortisol exposure and potentially alter the set-point of fetal stress response systems. Consistently with this, animal studies suggest that the rise in glucocorticoids at the circadian peak saturates the 11β-HSD2 enzyme, leading to greater placental transfer of maternal glucocorticoids to the fetus (Venihaki et al., 2000). Alternatively, while the stress reactivity patterns observed a few hours after birth can be considered largely independent of postnatal influences, hereditary transmission might still explain the observed associations, with mother-infant shared genes accounting for variation in both maternal diurnal cortisol patterns and newborns' stress response (e.g. Van Hulle et al., 2012).

Some non-significant results are worth mentioning. First of all, differently from what we hypothesized, we did not find any effect of maternal depressive symptoms on infants' stress regulation. This result, however, is in line with several studies (e.g. Stroud et al., 2016; Braithwaithe et al., 2016; Thomas et al., 2017) and with the results of a recent systematic review (Bleker et al., 2013). In particular, the majority of studies included in the review (N=10 among 13) failed to detect any independent association between maternal antenatal depression and children's stress reactivity (Bleker et al., 2018). In contrast, a considerable amount of studies do find an association when moderating or mediating factors involving especially the quality of postnatal environment were taken into account (e.g. Sharp et al., 2016; Laurent et al., 2011),

thus suggesting that the fetal stress response system are not affected by prenatal exposure to maternal depression per se but rather by a complex combination of prenatal and postnatal exposures. We might speculate that the effects of prenatal maternal depression on offspring's stress reactivity might emerge later in development (O'Donnell et al., 2013) and these might be the result of the interplay between prenatal and postnatal maternal influences. However, methodological reasons might also explain this null finding, including the limited sample size whichmight have reduced the chance to detect a true effect, and the possibility that variations in depressive symptoms in a low-risk sample have little to no effect on the developing foetus, as compared to stronger exposures (e.g. Vedhara et al., 2012). Secondly, consistent with prior work (e.g. Davis et al., 2011; Baibazarova et al., 2013) maternal prenatal cortisol and depressive symptoms were not significantly associated, thus calling into question the role of cortisol as a mediator of maternal distress on fetal development. It is possible that the HPA axis-resetting occurring during pregnancy might overcome our ability to identify significant associations between endocrine and self-reports stress measures. Or, it is also plausible that relatively low levels of depression do not significantly affect the HPA axis regulation.

Our exploratory analyses do not support alternatives to an HPA-axis mediated mechanism, involving the SNS and the IRS in influencing newborns' stress reactivity. While animal studies consistently showed that prenatal exposure to maternal inflammation could impact the fetal stress response systems, leading to altered stress reactivity in adulthood (e.g. Samuelsson et al., 2004), to our knowledge, only Osborne and colleagues (2018) have investigated the role of maternal prenatal inflammation in influencing offspring's stress reactivity. The authors reported unadjusted positive correlations between higher levels of maternal inflammatory markers in late pregnancy and infant higher cortisol levels in response to the immunization at 12 months of age. However, it is important to note that, although the sample size was comparable to the current one, findings were based on a sample of clinically depressed women. It is

possible that levels of maternal inflammation during healthy pregnancy in a low risk sample might not have been high enough to significantly influence fetal development, thus explaining the lack of significant findings in the current study. Alternatively, differences in infant's age at the assessment might account for discrepancies in findings. Noteworthy, Osborne and colleagues (2018) did not find any difference in infant's cortisol reactivity at 2 months of age between cases and controls, however they did not report data on possible associations among maternal antenatal inflammation and offspring's stress reactivity at 2 months of age. Further replication of the current findings in different cohorts is needed.

Similarly, the current study did not provide evidence of an association among prenatal maternal sAA levels and newborns' stress reactivity. Only two published studies have thus far examined this association, reporting significant links between maternal sAA levels in early-mid gestation and, respectively, 2-month-olds' negative emotionality (Braithwaite, Murphy, et al., 2017) and 6-month-olds stress-reactivity profiles (Rash et al., 2016), while no significant links were found in late pregnancy (Rash et al., 2016). Future research should investigate whether, as a consequence of changes in placental physiology and in plasticity of physiological systems, distinct biological mechanisms might become more prominent in influencing the developing fetus across gestation.

4.5.3 Limitations

Despite the strengths of the present study, including a prospective design, the assessment of multiple biological markers, the inclusion of confounders and the neonatal assessment soon after birth, the interpretation of findings should be cautious due to several limitations. First, data are based on a relatively small middle-high SES community sample evaluated in late pregnancy and levels of self-reported depressive symptoms were relatively low, thus limiting generalizability of results to high-risk populations and different gestational windows. Secondly, maternal salivary samples

were collected at home and compliance with the protocol was not objectively measured. Third, as previously reported (Egliston, McMahon, & Austin, 2007), obtaining sufficient saliva volumes from newborns was a challenge, thus leading to limited sample size for cortisol analyses. HLMs were employed in order to maximize the number of infants included in the final analyses and obtain reliable estimates of effects despite missing values for one or more time-points. Additionally, it cannot be excluded that newborns' cortisol reactivity might be masked by a still-unresolved cortisol response to delivery although duration of labor and mode of delivery were unrelated to infants' cortisol levels.

Lastly, as we did not use a relevant genetically informative design (Rice et al., in press) and the study is observational, we cannot rule out possible pleiotropic effects of shared genes which both influence maternal stress levels and infants' outcomes, as well as unmeasured confounding and causal inferences cannot be drawn.

4.6 Conclusions

Taken together, the current results suggest that antenatal factors, related to alterations in maternal stress and immune response systems, are involved in supporting fetal growth and development with possible implications for later outcomes. However, the weak or null association between self-reported depressive symptoms and biological measures underlines the incomplete picture of the mechanisms through which prenatal depression may be transmitted to the foetus, affecting later developmental outcomes, and highlights the needs for further research into alternative stress mechanisms largely neglected until now. We believe that a better understanding of the complex pathways underlying fetal programming will not only improve our knowledge of typical and atypical fetal development but will also have important implications for the development of effective targeted intervention.

Chapter 5: Prenatal maternal influences on infant stress reactivity, temperament and psychomotor development at 3 months of age.

5.1 Introduction

The potential for events experienced early in life to impact the long-term developmental trajectories has long been known. Evidence from animal studies indicate that induced stress during pregnancy can adversely affect fetal development, in a process often described as fetal programming (Seckl & Holmes, 2007), resulting in altered behavioral and physiological outcomes, including impaired stress regulation and delay in motor and cognitive development (reviewed in Weinstock, 2008), that persist into adulthood. In humans, there is also initial evidence of a link between antenatal exposure to maternal stress, in particular maternal depression, and heightened risk for a wide range of negative neurodevelopmental outcomes (for a review see Van den Bergh et al., 2017). More specifically, a number of reports have found poorer performances on the Neonatal Behavioral Assessment Scale soon after birth (e.g. Osborne et al., 2018), lower cognitive performance at later assessments (e.g. Bergman et al., 2007), increased fearfulness (e.g. Bergman et al., 2007) and negative affectivity (e.g. Glynn et al., 2018; Rouse & Goodman, 2014) in infants of prenatally depressed mothers. Additionally, some prospective studies relate prenatal exposure to maternal depression to an altered HPA-axis regulation in offspring (e.g. Laurent et al., 2013; Waters et al., 2013; Brennan et al., 2008), although a number of reports have failed to detect any significant association (e.g. Davis et al., 2011; Braithwaite et al., 2016; Tollenaar et al., 2011). The association between prenatal depression and a wide range of child developmental outcomes, including stress reactivity, temperament and cognition, appears to be independent of postnatal

maternal mood or relevant demographic characteristics (e.g. Davis et al., 2004; Connor et al., 2002; Glynn et al., 2018). In addition, although literature on possible genetic basis of the association among antenatal stress and child development is still limited, preliminary studies using genetically informed design have begun to disentangle environmental and genetic contributions and have provided initial evidence suggesting that some of the reported associations, though not all, are at least partially independent from genetics (e.g. Rice et al., 2010). Furthermore, although the correlational nature of the majority of the above mentioned findings preclude causal inferences, results concur with experimental animal models showing that prenatal stress exposure is associated with altered physiological, behavioral and cognitive outcomes (Frasch et al., 2018; Marta Weinstock, 2017). Given the relevance of these milestones for later health and development, early difficulties in stress regulation and neuromotor delays can lay the foundations for subsequent behavioral and cognitive problems (e.g. Dougherty et al., 2009; Gunnar & Vazquez, 2001; Olson et al., 2005).

Despite a growing body of evidence linking antenatal depression to negative outcomes in offspring, little is known about the biological mechanisms that underlie these effects in humans. In the last decades, the attention of scholars has been mainly directed toward the mediating role of the hypothalamic–pituitary–adrenal (HPA) axis in programming fetal brain and behavior. More specifically, as extensively described in Chapter 1, it is hypothesized that mood-related increased levels of maternal cortisol might cross the placenta in a quantity sufficient to affect fetal development with long-lasting consequences on offspring's behavior and physiology (Seckl & Meaney, 2004). Well-established evidence from animal models indicates that prenatal over-exposure to glucocorticoids can affect most regions of the developing fetal brain, including regions involved in the regulation of emotion, stress and cognitive functions, resulting in enduring alterations in offspring stress regulation (e.g. Abe et al., 2007), increased fearful or inhibited behaviors in response to challenges (e.g. Dickerson et al., 2005)

and neuromotor deficits (e.g. Schneide et al., 1999). Consistent with the hypothesized role of glucocorticoids in these associations is the observation, in humans, that prenatal exposure to synthetic glucocorticoids is associated with impaired infant HPA axis regulation (e.g. Davis et al., 2011; Erni et al., 2012), temperamental difficulties, such as behavioral inhibition or fearful behaviors in response to novelty (Trautman, Meyer-Bahlburg, Postelnek, & New, 1995), and neurodevelopmental delays (Spinillo et al., 2004; Wapner et al., 2007). However, only a handful of studies in humans has explored the link between antenatal maternal endogenous cortisol and child's neurodevelopmental outcomes such as temperament, stress reactivity and cognition, yielding to mixed findings (reviewed in Zijlmans, Riksen-Walraven, & de Weerth, 2015).

Specifically, evidence for an association between maternal stress hormones during pregnancy and infants' temperamental traits is scarce. Two large high quality studies demonstrated that maternal afternoon cortisol (Davis et al., 2007) and corticotropin-releasing hormone (CRH; Davis et al., 2005) during late pregnancy, but not earlier, were associated with maternal reports of increased negative reactivity in 2-month-olds (Davis et al., 2005). Likewise, Werner and colleagues (2013) showed that maternal morning cortisol in late pregnancy was positively associated with observer-rated reactivity to novelty in 4-month-old infants. However, a fair number of studies have failed to find any significant association between maternal prenatal cortisol both in early and late pregnancy and infant temperament as assessed either through maternal or observer reports (Rouse & Goodman, 2014; Buitelaar et al., 2003; Braithwaite, Murphy, Ramchandani, & Hill, 2017; Rothenberger et al., 2011). Similarly, amniotic fluid cortisol was not found to be related to fear reactivity at 17 months (Bergman et al., 2010) or to fear and distress to limitations at 3 months (Baibazarova et al., 2013). However, the majority of these studies rely on single maternal cortisol sampling (e.g. Davis et al., 2007; Werner et al., 2013) and a few studies did not control

for possible confounders of the association (e.g. Rothenberger et al., 2011; Buitelaar et al., 2003).

Likewise, a limited number of prospective studies have investigated the association between antenatal maternal cortisol and offspring's cortisol reactivity with inconsistent results. Higher maternal cortisol during pregnancy has been associated with greater cortisol and behavioral reactivity from early after birth through the preschool years in a number of studies (Davis et al., 2011; Gutteling, De Weerth, & Buitelaar, 2005; Werner et al., 2013b), but not all (e.g. Tollenaar et al., 2011; Braithwaite et al., 2016). Additionally, a link between elevated cortisol levels during pregnancy and a blunted cortisol response to stress in the offspring has been reported in a few studies (O'Connor et al., 2014; Giesbrecht, et al., 2017).

Even preliminary evidence for a link between maternal cortisol during pregnancy and infant motor and mental development in humans is inconclusive. Higher maternal cortisol in late pregnancy, but not in early or mid-gestation, has been found to predict lower cognitive development at 3 months (Huizink et al., 2003; Buitelaar et al., 2003), 17 months (Bergman et al., 2010) and 7-year-olds (Lewinn et al., 2009), but not at 8 months (Huiznik et al., 2003; Buitelaar et al., 2003). Remarkably, methodological rigorous work from Davis and Sandman (2010) on a low risk sample of 125 full-term infants, reported an opposite effect of prenatal maternal cortisol on infants' cognitive development based upon the timing of exposure. More specifically, higher maternal cortisol early in gestation was related to lower mental development at 12 months, while elevated maternal cortisol in late pregnancy predicted higher mental development. In line with these findings, more recently, the authors found higher maternal cortisol in late pregnancy, but not earlier, to predict greater cortical thickness in frontal regions and better cognitive functioning in 6-9 year-olds children (Davis, Head, Buss, & Sandman, 2017). Additionally, while Huizinik and colleagues (2003) reported a significant association between higher maternal cortisol in late pregnancy and infants' lower motor development at 3 and 8 months, other

studies with similar sample sizes failed to find any significant association between maternal cortisol and motor outcomes (Bergman et al., 2010; Davis & Sandman, 2010).

Collectively, weak evidence exists for an association between maternal prenatal cortisol and physiological, behavioral and cognitive outcomes in the child (reviewed in Zijlmans et al., 2015), thus encouraging further research into complementary or competing stress-related mechanisms, involving for example the Sympathetic Nervous System (SNS) and the Inflammatory Response System (IRS). However, to date, such alternative pathways have been largely neglected.

As extensively described in chapter 1, despite the fact that chatecholamines, produced by the SNS under stress conditions, do not cross the placental barrier in humans (Giannakoulopoulos et al., 1999), stress-related activation of the SNS might affect the utero-placental blood flow (Merlot et al., 2008), thus influencing indirectly foetal growth and, in turn, activating the foetal HPA axis (Roelfsema et al., 2005). Salivary alpha amylase (sAA) represents a non-invasive marker of SNS activity (Nater & Rohleder, 2009) and has only recently been employed to explore an SNS-mediating pathway in fetal programming. In particular, to date, maternal sAA levels during pregnancy have been linked to pregnancy and birth outcomes in two published studies (Giesbrecht et al., 2013; 2015), while only two works examined prenatal maternal sAA in relation to later infants' neurodevelopmental outcomes (Braithwaite et al., 2017; Rash et al., 2016). Specifically, Braithwaite and colleagues (2017) found a significant interaction between maternal prenatal sAA levels and infant's sex in predicting maternal reports of 2-month-olds' distress to limitations in a sample of 88 mother-infant dyads. However, this study has limited statistical power and the effect was non-significant once females and males were examined separately. Additionally, in a study by Rash and colleagues (2016) maternal sAA, together with other stress markers, discriminated between 6-month-olds' stress reactivity patterns. However, to our

knowledge, no published studies have investigated the association between maternal sAA levels during pregnancy and infants' cognitive or motor development.

Similarly, the IRS represents another potential candidate mediator for fetal programming of later neurodevelopment. Nevertheless, to date, it has not yet received considerable empirical support in humans. Maternal inflammation during pregnancy has been shown to be associated with adverse pregnancy, birth and later child health outcomes (e.g. Vannuccini et al., 2016; Rusterholz et al., 2007). Furthermore maternal immune activation during pregnancy have been associated with an increased risk for offspring neuropsychiatric disorders (Estes & Mcallister, 2016). Additionally, animal models suggest that pro-inflammatory cytokines, secreted by the IRS, play a crucial role in influencing the developing fetal brain, including HPA axis regulation (e.g. Samuelsson et al., 2004). In humans, preliminary studies have highlighted a role of maternal inflammation during pregnancy in mediating the effects of antenatal distress on birth outcomes, such as birth weight and gestational length (Okun et al., 2013; Coussons-read et al., 2012; Miller et al., 2017). Research on the role of maternal inflammation during pregnancy on later child outcomes is only at the beginning. To date, the group led by Claudia Buss has conducted the largest study on the influence of antenatal maternal inflammation on offspring longitudinal brain development. The authors showed that higher maternal IL-6 levels during pregnancy were associated with an alteration of newborns' amygdala volume and connectivity, leading to lower impulse control at 2 years of age (Graham et al., 2017), and with both reduced functional anisotropy in the central portion of the uncinate fasciculus of newborns' brains and poorer cognitive development at 12 months of age (Rasmussen et al., 2018). Furthermore, recent work by Osborne and colleagues (2018) showed an association between higher levels of maternal proinflammatory cytokines in late pregnancy and both offspring's poorer performance on the Neonatal Behavioral Assessment Scale 6 days after birth and greater cortisol reactivity at 12 months of age, although results were not adjusted for potential confounders. Gustaffson and

colleagues (2018) showed that maternal antenatal inflammation mediated the link between prenatal depressive symptoms and 6-month-olds negative affect (Gustafsson et al., 2018). However, findings were based on a relatively small sample (N=62) of women at risk for attention-deficit/hyperactivity disorder and may not generalize to different samples.

Taken together, these very preliminary results encourage more research into the link between maternal depression, IRS functioning during pregnancy and later neurodevelopmental in humans.

5.2 Summary and study hypotheses

Despite some evidence for a role of maternal cortisol in shaping fetal brain development and for the long-lasting effects of such exposures, several grey areas remain about the direction and nature of these effects. For example, a number of studies did not include potential confounders such as maternal postnatal symptomatology, child's gender and age (Rothenberger et al., 2011; De Weerth et al., 2003), thus limiting possible inferences about the actual effects of fetal programming. Furthermore, the majority of studies rely on single maternal cortisol sampling (e.g. Davis et al., 2007; Werner et al., 2013) and did not take into account the possible role of variations in the diurnal cortisol pattern in influencing fetal development. Lastly, the majority of studies failed to detect an association between maternal endocrine stress measures and measures of psychological distress (e.g., Davis et al., 2011; Davis & Sandman, 2010; Bergman, et al., 2010; Davis & Sandman, 2012), thus questioning the role of the HPA-axis as a mediating mechanism of the effects of antenatal depression on child outcomes. Alternative biological mechanisms, involving maternal IRS or SNS, underlying fetal programming of later physiological and behavioral outcomes are just beginning to be empirically examined. In chapter 4, we first investigated the impact of naturally occurring variations in the functioning of maternal stress and immune response systems in late pregnancy on birth outcomes and stress reactivity soon after

birth. In the current work, we now turn to investigating whether the effects of maternal prenatal influences on infant physiological and behavioral outcomes persist in the postpartum period and whether effects that were not evident soon after birth might be revealed later in development. To this aim, we followed-up the same sample of healthy mother and infants 3 months after delivery and we combined self-report assessment of maternal depression during pregnancy with multiple biological markers of the stress and inflammation to explore the independent effects of prenatal maternal variables on 3-month-olds' development. We investigated a broad range of infant outcomes, including physiological stress reactivity, behavioral adaptation, temperament, and mental and motor development, which are considered important markers of later development, in an effort to better capture the effects of fetal programming as well as the extent to which distinct maternal biological systems might be differentially involved in predicting risk for adverse outcomes. Additionally, we controlled for several potential prenatal, perinatal and postnatal confounders and we investigated the extent to which the observed associations were specific for maternal antenatal depression, as compared to anxiety.

Based on most of the findings in the literature, we hypothesized that infants born to mothers with higher depressive symptoms during pregnancy would have a more difficult temperament, be more reactive to stress and show poorer cognitive performances than infants of mothers with lower depressive symptoms. Furthermore, we predicted that alterations in maternal diurnal cortisol pattern during pregnancy would be related to infant's temperament, stress reactivity and cognitive outcomes. In contrast, we made no a priori predictions for an association between markers of maternal SNS and IRS functioning and infants' outcomes, due to limited available literature.

5.3 Material and methods

5.3.1 Participants

The study sample consists of mother-infants dyads recruited as part of a larger ongoing longitudinal study named the Effects of Depression on Infants (EDI) Study based at the Medea Scientific Institute in Italy. Women, mostly Italian (97.2%), middle-high class (94.9%) during their first pregnancy (90.7%), were recruited serially between 30th and 33rd weeks of gestation and followed longitudinally. Women with multiple and/or complicated gestation, smoking or drug using, afflicted by any chronic disease or taking any chronic medications were excluded. Additionally, only healthy full term (or late preterm >35 weeks) infants were included in the current study. Thus, 1 intrauterine death after the prenatal assessment and 2 newborns with serious health problems after birth were excluded from the initial sample of 110 women, resulting in 107 mother-infant dyads been included in the current study. Infants (52.3% males and 47.7% females) were mostly delivered vaginally (82.2%) at 39.47 mean gestational age (SD=1.25). Women who were excluded from the 12-weeks postnatal assessment did not differ from participants on any demographic variables, depression or anxiety scores.

5.3.2 Procedure

Maternal depressive and anxiety symptoms were assessed between 30th and 33rd weeks of gestation and 12 weeks after delivery. Maternal salivary and blood samples were collected between 34th and 36th weeks of gestation, as described below, and maternal cognitive function was assessed on the same occasion. At 12 weeks of age, infants' behavioral and cortisol response to the routine first inoculation visit as well as cognitive and motor development were assessed in two separate sessions. Additionally, at the prenatal assessment women were asked to fill in a form on pregnancy-related data, while at the postnatal assessment they were asked to

complete a form on infants' health and two questionnaires on infants' temperament, daily sleep and crying behaviour.

The Ethics Committee of University College London, of Scientific Institute Eugenio Medea and of the hospitals and pediatric health centers involved approved the study protocol.

5.3.3 Maternal assessment

Psychological assessment. Prenatal and postnatal depressive symptoms were evaluated through the Italian version (Benvenuti et al., 1999) of the 10-item Edinburgh Postnatal Depression Scale (EPDS; Cox et al., 1987), the most widely used self-report questionnaire to assess perinatal depressive symptoms on a 4-point Likert scale. Prenatal and postnatal anxiety symptoms were evaluated on a 4-point Likert scale through the Italian version (Pedrabissi & Santinello, 1989) of the 20-item trait anxiety subscale of the State-Trait Anxiety Inventory (STAI) (Spielberger et al., 1970).

Maternal cognitive function was evaluated during pregnancy through the Raven Standard Progressive Matrices (RSPM, Raven et al., 1998), a widely employed test that evaluate non-verbal general cognitive ability through 60 multiple choice items. The test time was limited to 30 minutes and the RSPM raw scores, as a measure of individual general cognitive ability, were employed in the analyses.

Health status. During pregnancy, women were asked to fill in an ad-hoc form to collect data concerning prenatal and perinatal medical history (e.g. any complication/risk/medical disorder, parity, actual and pre-pregnancy weight and height, medication etc.)

Biological assessment. Ninety-four women out of 107 consented to a blood draw between 34-36 gestational weeks. Blood samples were drawn in the morning by venipuncture and kept refrigerated at +4° until they reached the Biological Lab of Medea Institute were serum was centrifuged, aliquoted, and stored at −80 °C until analyzed for C-Reactive Protein (CRP) and IL-6 levels. Biological assay were run in

duplicate by using Quantikine High Sensitivity ELISA kits (R&D Systems Europe, LTD) at LaboSpace in Milan according to the instructions of the manufacturer. Intra-assay coefficient of variation (CV) was <6% for IL-6 and <3% for CRP, inter-assay CV was <10% for both markers.

Saliva samples were collected at home on two consecutive days immediately upon awakening, 30 minutes post-awakening and before going to bed by passive drool (Granger et al., 2007). Participants were asked to record time of collection on a diary and avoid eating, tooth-brushing and exercising in the 30 minutes before collection and eating a major meal in the hour before the evening sample. All women provided completed saliva samples. The mean time from the awakening collection and the 30-minutes post-waking collection was, respectively, 30.84 minutes on day 1 (SD=3.69, range: 20.00-60.00) and 31.48 minutes on day 2 (SD=6.90, range: 20.00-90.00). As 1 woman collected the second sample on day 2 more than one hour from awakening, and the decline in cortisol usually occurs one hour after waking up, this sample was excluded from analyses as done in prior work (O'Donnell, 2013; O'Connor, 2014). All saliva samples were stored frozen at -80° until assayed for salivary cortisol at the Biological Lab of Medea Institute, using a competitive high sensitivity enzyme immunoassay kit (Expanded Range High Sensitivity Cortisol EIA Kit, Salimetrics), and for sAA at the Salimetrics Centre of Excellence testing lab at Anglia Ruskin University, using a kinetic enzyme assay kit (Salimetrics α-Amylase Kinetic Enzyme Assay Kit). All samples from one woman were run in the same assay to minimize method variability. Salivary cortisol assays were run in duplicate and averaged, except for 2 samples with minimal volume. Average intra- and inter-assay coefficients were <6% and <8%, respectively. Results were computed in µg/dl. A random 10% of the sAA assays were run in duplicate to confirm reliability. The intra-assay coefficient of variation was < 3%. Results were computed in U/mL.

5.3.4 Infant assessment

Temperament was assessed using the 37-items very short form of the Infant Behavior Questionnaire (IBQ-R-VSF), a well-established standardized measure designed to evaluate temperament in infancy aged from 3 to 12 months by caregiver report on a 7-point scale (Putnam et al., 2014). The items measured three broad, empirically derived, temperamental dimensions, namely negative affectivity, positive affectivity/surgency, and orienting/regulatory capacity, with levels of reliability and stability similar to those obtained with the longer versions of the scale and other temperament measures (Putnam et al., 2014).

Crying at 12 weeks of age was assessed through the Baby's Day Diary (Barr, 1985), a 24-hour record of infant behaviors (i.e. awake and alert, awake and fussing, awake and crying, awake and inconsolable crying, feeding, sleeping) to be completed at home by the caregiver. Total minutes of infant distress (fussing, crying, and inconsolable crying) per day and minutes of inconsolable crying per day were abstracted from the diary. The assessment of frequency and duration of crying, as evaluated through the Baby's Day Diary, has been shown to strongly correlate with audiotape recordings (Barr et al., 1988; St James Roberts et al., 1993) and be independent of postnatal maternal depressive symptoms (Miller et al., 1993).

Mental and motor development at 12 weeks was assessed through the Bayley Scales of Infant and Toddler Development – Third Edition (Bayley III, Bayley 2006) in a session at the Medea Institute by a trained clinical psychologist blind to prenatal data. The Bayley III are a well-standardized and widely employed developmental assessment, providing three standardized composite scores for cognitive, language, and motor domains as well as 5 subtests (Cognitive, Expressive Communication, Receptive Communication, Fine Motor, Gross Motor). In the current study we focused on the Cognitive and Motor scales. Age-standardized composite scores for each scale were calculated by using test norms (mean=100; SD=15). Data for the Bayley III were available for 104 infants out of 107, as the remaining infants did not attend the session

at the Medea Institute due to logistic reasons (i.e. living too far from the Institute).

Health status. Data on physical health (e.g. weight, body length, any medication, any disease/hospitalization from birth to date), feeding and sleep were collected at 12 weeks of age, jointly with situational data concerning the inoculation day (e.g. duration of trip to the health centres, time of last feeding, time of last sleeping etc.) through an ad hoc form that the researcher filled out with the mother. Additionally, data concerning infants' health at birth (e.g. gestational age, weight, etc.) were extracted from medical records at birth.

Cortisol collection and assay. Infants' cortisol and behavioural responses to the inoculation were evaluated at approximately 12 weeks of age (SD=1.84) during the routine first inoculation visit. Specifically, the infant was undressed and laid down and two injections, respectively, for the hexavalent vaccine and pneumococcal vaccine, were administered in the infants' thigh. Then the infant was given to the mother, who was free to soothe the baby. The whole procedure was videotaped. Mean duration of the shot administration was 1.06 minutes (SD=00.30). Data concerning the inoculation were available for 94 infants out of 107, as for 2 infants' parents did not consent to the inoculation, while 11 infants were vaccinated in a district outside the province of Lecco and Como that were involved in the project and for which we gained the approval of their ethics committees'. Three infant saliva samples were collected in the waiting room, respectively right before entering in the medical room (baseline) and 20 and 40 minutes after the administration of the shot, using a specifically designed swab (SalivaBio Infant's Swab, Salimetrics). All the inoculations were performed in the morning between 09.00 and 12.00, except for 4 infants who were examined at 2 pm. Time of the day was examined as a covariate in the analyses. With the exception of 1 infant who was fed 11 minutes before the first saliva collection, babies were not fed in the 30 minutes before the saliva collection (mean time from last feeding=102.99 minutes, SD=54.60). Saliva samples were stored at -80º until assayed for cortisol according to the same procedure described for maternal cortisol assay. All samples

were run in duplicates, with the exception of 8 samples that were run in singlet due to minimal saliva volume. All samples from one infant were run on the same assay. The average intra- and inter-assay coefficients of variance were below 7% and 10%, respectively. Out of the 94 infants taking part to the inoculation, complete cortisol data were available for 78 infants, while one or two samples were missing for 16 infants due to insufficient saliva volume. Infants with complete or partial data did not differ on any sociodemographic/maternal or infants' variables or situational factors (i.e. time of the day, length of the inoculation, behavioral score).

Behavioral assessment. Coding of infant distress began once the last needle was retracted and continued for the subsequent 2 minutes at 5-seconds intervals according to a 4-point scale by Jahromi and colleagues (2004) indicating an increasing in the intensity of the negative affect (i.e. no vocalization; fussing, whining or whimpering; low-intensity crying; very intense loud crying). The predominant (>2.5 seconds) level of intensity during each 5-seconds interval was scored. A measure of overall crying intensity was obtained by averaging scores across the intervals, while a measure of overall cry duration was calculated by adding the number of intervals during which the infant was distressed (i.e. received a rating >0) and multiplying by 5. The videotapes were coded by a trained graduate student with an MSc in Developmental Psychology, blinded to all prenatal and postnatal data. Twenty-one percent of the observations were coded by an independent trained coder. Intra-class correlation was 0.998, $p<.001$.

5.3.5 Statistical analyses

First, we examined variables for outliers and skewness. As distributions of maternal and infants' biological concentrations were positively skewed even after removing samples greater than 3 SD from the mean (n=7 for prenatal cortisol, n=4 for sAA, n=3 for prenatal IL-6, n=4 for infants' cortisol), variables were natural log (ln) transformed to approximate normal distributions. Model parameters for ln-transformed

values are presented in tables while non-transformed values are employed in the descriptive statistics table and figures to facilitate interpretation. Secondly, as extensively described in Chapter 2 and 4, four summaries measures were calculated from maternal daytime salivary samples to index different aspects of maternal HPA and SNS prenatal diurnal functioning, namely, awakening values, response to awakening, diurnal slope and total diurnal output as indexed by the area under the curve with respect to the ground (AUCg; Pruessner et al., 2003). As the number of women at risk for depression according to the EPDS cut-off of 10 was fairly low (17.3%), the total score of the EPDS was employed as a continuous predictor in the analyses.

Preliminary analyses were run to investigate the potential effect of variables known to affect infant's outcomes and included Pearson bivariate correlations and univariate analysis of variance (ANOVA). Only confounders which were significantly related to the dependent variables were included in subsequent analyses as covariates.

In order to evaluate the independent effects of maternal prenatal variables on infants' neurodevelopmental outcomes, hierarchical regression analyses and multilevel models were run as appropriate. Specifically, separate hierarchical regression analyses were performed to evaluate the impact of prenatal maternal variables on infant's outcomes (i.e. IBQ-R-VSF scales scores, Bayley III scales scores, Baby's Day Diary measures and behavioral response to stress), while adjusting for covariates. Hierarchical Linear Models (HLMs) were estimated to explore the influence of antenatal maternal variables on the trajectories of infants' cortisol response to the inoculation, while accounting for the hierarchical structure of the data (three time-points nested within individuals). Specifically, HLMs were specified at two levels where individuals were level 2 and time was level 1. Time was centered at baseline so that the model intercept represents the mean cortisol level at baseline. Unconditional means models were computed to evaluate how much variance in cortisol levels can be

explained by between-subject and between-occasions variations. Afterwards, we fitted a baseline model of cortisol response that describes the trajectory of infants' response and includes a linear and quadratic slope for time. Additionally, models included a random intercept and a random linear slope to allow between-person variability, while the error term was allowed to vary randomly in each model. The level-2 predictors were centered around the grand mean and entered in the model one-by-one. Gender was centered at males. Model fit was tested with likelihood deviance difference test for nested models. Specifically, variables were kept in the model when their presence resulted in a significant ($p<.05$) reduction of the likelihood ratio statistic. To test the statistical significance of the regression coefficients, the Wald's chi-square test was performed. Statistical analyses were performed using SPSS 24 and MLWiN. All statistical tests were two-sided and a $p<.05$ was considered statistically significant.

Given the high correlation between maternal depression and anxiety during pregnancy ($r=.67$, $p<.001$), an additional set of analyses investigating the effects of maternal anxiety, rather than depression, as individual-level predictor in the exploratory models were run and are reported in the supplementary results section.

5.3.6 Covariates.

Variables examined as potential confounders of the relationship between prenatal maternal variables and infants' outcomes in preliminary analyses included sociodemographic factors (i.e. maternal age, education, SES, parity, infants' age and gender) and health-related factors (i.e. mode of delivery, length of labor, gestational age, birth weight, head circumference, actual weight, postnatal smoke exposure, breastfeeding versus formula-feeding). In addition, the effects of factors related to the day of inoculation on infant stress response were examined (i.e. time of the day, time from last feeding and last sleeping, length of the inoculation procedure, mode of transport, duration of the journey, being distressed or quiet before the inoculation). Furthermore, as general cognitive ability is a genetically based and substantially

heritable trait (e.g. Kirkpatrick et al., 2014) and maternal cognitive functioning is known to have a direct influence on children's intellectual development, we examined the association between maternal IQ as assessed by the RPSM, and infant's Bayley III scores.

Maternal age was significantly associated with infants' 20-min post-stressor cortisol levels ($r=.24$, $p<.05$). Additionally, females were found to show marginally higher levels of cortisol at the 40-min post stressor collection than males ($F(1, 83)=3.69$, $p=.06$). Thus, maternal age and infant's gender were included as covariate in subsequent analyses on stress reactivity.

Infant's gestational age at birth was significantly related to infants' IBQ positive affectivity scores ($r=.28$, $p=.007$), while females showed more minutes of inconsolable cry during the day as compared to males ($F(1, 102)=3.80$, $p=.05$). Both infant's gender and gestational age were included in the subsequent models of infant's temperament and cry.

Both gestational age at birth and maternal IQ were significantly associated with infants' cognitive development (respectively, $r=.23$, $p=.01$ and $r=.24$, $p=.01$). In addition, females tended to perform higher on the Motor scale, as compared to males ($F(1, 101)=3.53$, $p=.06$). Thus gestational age, gender and maternal IQ were retained as covariate in the hierarchical linear regressions models on infants' psychomotor development.

Lastly, given that evidence exists of an association between maternal postnatal depression and infant's outcomes (e.g., Pauli-Pott et al., 2004; Keenan, Grace, & Gunthorpe, 2003), we examined this relation in our sample. Maternal postnatal depressive symptoms were significantly related to infant's IBQ negative affectivity scores ($r=.24$, $p=.02$) and with infants' 20-min post-stressor cortisol levels ($r=-.22$, $p=.04$). Thus, maternal postnatal depression was included as possible confounder in all regression and multilevel models. Models were performed both with and without including postnatal depression. Adjusting for maternal depressive symptoms at 3

months did not affect the statistical significance and direction of the association between prenatal maternal variables and infants' outcomes in any of the analyses presented below.

5.4 Results

5.4.1 Descriptive analyses

Descriptive statistics for all study variables are presented in Table 5.1. Preliminary bivariate correlations showed a marginally significant association between maternal depression and IL-6 levels during pregnancy (r=.19, p=.06). Additionally, there was a significant association between maternal prenatal depressive symptoms and infants' cortisol concentrations 40-minutes post-stressor (r=.22, p=.04). In contrast, intensity and duration of newborns' behavioural distress at the inoculation were not correlated with any maternal variables (all ps>.05). Infant's negative affectivity scores were positively related with maternal prenatal depressive symptoms (r=.45, p<.001), and negatively associated with maternal cortisol AUCg levels and sAA response to awakening during pregnancy (respectively r=-.22, p=.04; r=-.21, p=.05). Both positive affectivity and orienting/regulatory capacity IBQ scores were negatively related with maternal prenatal cortisol diurnal slope (respectively, r=-.22, p=.03 and r=-.26, p=.01). Duration of total infant distress during the day, as assessed on the Baby's Day Diary, was negatively related to maternal prenatal CAR (r=-.22, p=.02), while minutes of inconsolable crying were related to sAA response to awakening (r=-.29, p=.003). Infants' cognitive development was negatively related to maternal cortisol AUCg during pregnancy (r=-.26, p=.01) and marginally related to cortisol levels at awakening (r=-.18, p=.07).

Table 5.1 – Descriptive statistics for study variables at the prenatal and postnatal assessment

Study Variable	Mean	SD	Range
Prenatal			
Maternal cortisol (µg/dl)			
Waking	0.38	0.13	0.13-0.83
Waking +30’	0.50	0.15	0.10-0.91
Bedtime	0.18	0.06	0.01-0.41
Maternal sAA (U/ml)			
Waking	69.78	64.27	3.00-463.84
Waking +30’	47.90	38.30	2.80-190.10
Bedtime	97.44	80.09	3.28-562.71
Maternal CRP (ng/ml)	3720.98	2705.67	480.04-11179.80
Maternal IL-6 (pg/ml)	1.68	1.03	0.48-6.47
Maternal depression (EPDS)	5.36	4.46	0-19
Maternal anxiety (STAI-T)	36.52	9.60	21-71
Postnatal			
Cognitive composite score	102.36	10.81	75-125
Motor composite score	99.73	8.14	85-121
Negative affectivity	3.46	0.87	1.58-5.42
Positive affectivity	3.87	0.79	2.00-5.54
Orienting/regulatory capacity	5.62	0.57	4.30-6.75
Daily Total Distress (min)	114.23	74.41	00-335.00
Daily Inconsolable cry (min)	7.50	18.35	0-110
Behavioral response to stress	1.41	0.79	0.25-3.00
Infants’ cortisol (µg/dl)			
Baseline	0.31	0.23	0.03-1.10
20-min post-stressor	0.78	0.35	0.01-1.86
40-min post-stressor	0.47	0.25	0.11-1.37

5.4.2 Prenatal maternal variables and infant's psychomotor development

As shown in Table 5.2, multiple hierarchical regression analyses revealed significant associations between higher maternal cortisol AUCg and lower infant cognitive development at 3 months (β=-.37, t= -3.99, p<.001), as well as between higher maternal CRP and lower cognitive scores (β=-.23, t= -2.21, p=.03), after controlling for infant's gender, gestational age, maternal IQ and both prenatal and postnatal depression (β=-.37, t= -3.99, p<.001). No significant effect of maternal prenatal variables on infant's motor development was found.

Table 5.2 – Hierarchical linear regression analyses predicting 3-month-olds' cognitive scores

	Cortisol AUCg		**sAA AUCg**		**IL-6**		**CRP**	
	β	p	β	p	β	p	β	p
Step 1:								
Gender	.12	.20	.11	.25	.08	.45	.08	.44
Gestational Age	**.21**	**.03**	**.22**	**.02**	.20	.07	.20	.06
Maternal IQ	**.23**	**.02**	**.22**	**.02**	.20	.07	.18	.10
Postnatal EPDS	.05	.58	.05	.61	.05	.62	.06	57
ΔR^2 for step 1	**.12**	**.01**	**.12**	**.01**	.09	.09	.09	.10
F_{model}	**3.26**	**.01**	**3.30**	**.01**	2.07	.09	2.03	.10
Step 2:								
Prenatal EPDS	-.13	.28	-.12	.30	-.13	.33	-.13	.32
ΔR^2 for step 2	.13	.28	.01	.30	.01	.33	.01	.32
F_{model}	**2.85**	**.02**	2.86	.02	1.85	.11	1.82	.12
Step 3:								
Biological predictors	**-.37**	**<.001**	-.12	.21	-.07	.50	**-.23**	**.03**
ΔR^2 for step 3	**.26**	**<.001**	.01	.21	.00	.50	**.05**	**.03**
F_{model}	**5.40**	**<.001**	**2.66**	**.02**	1.61	.15	**2.40**	**.03**

Concerning the covariates, gestational age was significantly positively associated with both motor and cognitive development, while maternal IQ was significantly related to infant's cognitive scores. Additionally, infant's gender was related to motor development, with females scoring higher than males. Results in Table 5.2 are reported only for cortisol AUCg, as all other diurnal indices were not significantly associated with infant's psychomotor development.

5.4.3 Prenatal maternal variables and infant's temperament

As shown in Table 5.3, multiple hierarchical regression analyses revealed a positive association between maternal prenatal depressive symptoms and infants' IBQ negative affectivity scores (β=-.38, t= 3.08, p=.003), after controlling for infant's gender, gestational age, and postnatal depression. Additionally, maternal sAA response to awakening was related to infants' NA scores (β=-.28, t= -3.02, p=.003), after controlling for covariates. Specifically, a greater morning sAA decline after awakening was associated with infants' higher negative affectivity scores. Moreover, there was a marginal association between higher maternal cortisol levels during pregnancy, as indexed by AUCg, and lower scores on infant's negative affectivity at 3 months, after adjusting for covariates (β=-.19, t= -1.8, p=.07). No significant effect of maternal prenatal variables on infant's IBQ positive affectivity and orienting/regulatory capacity scores, as well as infant's daily cry and sleep behavior, as reported at the Baby's Day Diary, was found. Concerning the covariates, postnatal depressive symptoms were significantly positively related with infant's negative affectivity scores (β=.30, t=2.95, p=.004), while gestational age was significantly related with infant's positive affectivity scores (β=.28, t=2.81, p=.006).

Table 5.3 – Hierarchical linear regression analyses predicting 3-month-olds' NA scores

	Cortisol AUCg		sAA response to waking		IL-6		CRP	
	β	p	β	p	β	p	β	p
Step 1:								
Gender	.17	.10	.19	.06	.17	.13	.14	.21
Gestational Age	.07	.47	.05	.62	.10	.37	.09	.40
Postnatal EPDS	**.31**	**.004**	**.30**	**.004**	**.33**	**.004**	**.34**	**.003**
ΔR^2 for step 1	**.13**	**.01**	**.13**	**.01**	**.15**	**.01**	**.15**	**.01**
F_{model}	**4.02**	**.01**	**4.14**	**.01**	**4.12**	**.01**	**4.12**	**.01**
Step 2:								
Prenatal EPDS	**.38**	**.004**	**.38**	**.003**	**.35**	**.01**	**.36**	**.01**
ΔR^2 for step 2	**.08**	**.004**	**.09**	**.003**	**.07**	**.01**	**.08**	**.01**
F_{model}	**5.49**	**.001**	**5.78**	**>.001**	**4.97**	**.001**	**5.11**	**.001**
Step 3:								
Biological predictors	-.19	.07	**-.28**	**.003**	-.17	.11	-.18	.10
ΔR^2 for step 3	.03	.07	**.08**	**.003**	.03	.12	.03	.10
F_{model}	**5.26**	**.001**	**6.91**	**>.001**	**4.57**	**.001**	**4.74**	**.001**

5.4.4 Prenatal maternal variables and infant's behavioural response to stress

Multiple hierarchical regression analyses revealed no significant association between maternal prenatal variables and either intensity or duration of infant's behavioural distress at the inoculation, after adjusting for covariates (all ps>.05).

5.4.5 Prenatal maternal variables and infant's cortisol stress reactivity

The unconditional means model for infants' cortisol showed significant variability between-occasions (level-1; p<.001), but not at the individual level (level-2;

p=.78). Three-month-olds showed the expected cortisol response to the inoculation as indexed by the significant linear and quadratic slopes of time (both ps<.001). Also, the random linear slope term was statistically significant (p<.001), indicating significant between-person variability in the cortisol linear increase. Overall, this model results in a significant improvement of the fit over the unconditional means model (deviance difference (4)=131.07, p<.001). Fixed independent effects of prenatal variables (i.e. depressive symptoms, cortisol and sAA diurnal indices, CRP and IL-6 levels) on mean cortisol levels at baseline and on cortisol response over time were tested separately, while controlling for maternal age, infant's sex and postnatal depression.

Table 5.4 – Full prediction model for the independent effects of maternal depression (EPDS) on infants' cortisol response

	Infant cortisol response	
	Estimate (SE)	p
Fixed effects		
Intercept	0.260 (0.021)	<.001
Gender	-0.009 (0.024)	.71
Maternal Age	0.006 (0.003)	.07
Postnatal EPDS	-0.030 (0.019)	.12
Prenatal EPDS	0.026 (0.026)	.32
Linear	0.022 (0.002)	<.001
Prenatal EPDS	-0.003 (0.002)	.14
Quadratic	-0.000 (0.000)	<.001
Prenatal EPDS	**-0.000 (0.000)**	**.04**
Random effects		
Level 2 (individual)		
Intercept variance	0.008(0.004)	.06
Linear slope variance	0.000 (0.000)	.03
Intercept/Linear slope covariance	-0.000(0.000)	.24
Level 1 (occasions)		
Intercept variance	0.017(0.003)	<.001

As shown in Table 5.4, there was a significant main effect of maternal prenatal depressive symptoms on the quadratic slope of infants' cortisol response (p=.04).

Specifically, as shown in Figure 5.1, higher depressive symptoms were associated with a flatter quadratic slope, indicating a slower recovery to pre-stressor cortisol levels. However, maternal depression was not significantly associated with cortisol baseline levels or with the linear slope of the response (all ps>.05). The inclusion of maternal prenatal depression marginally improved the model fit (deviance difference (3)=7.80, p=.05).

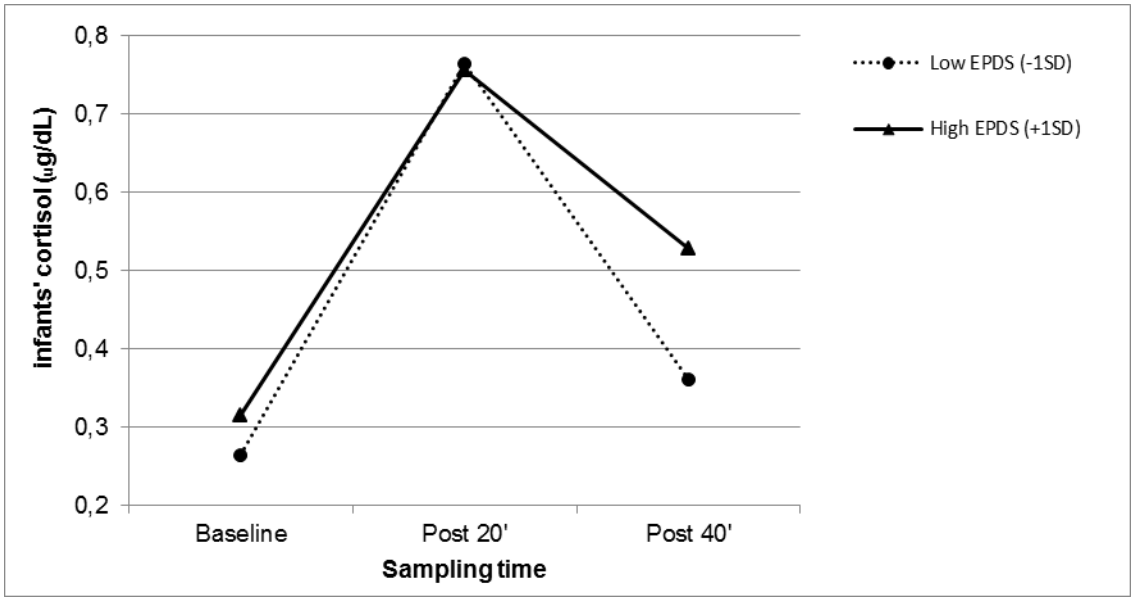

Figure 5.1 – Cortisol values before and after the inoculation for infants exposed prenatally to higher (+1 SD) and lower (-1SD) maternal depression, as indexed by the antenatal EPDS score, after adjusting for covariates

Furthermore, maternal overall diurnal level of sAA, as indexed by AUCg, was significantly related to infant's cortisol levels at baseline (estimate=-0.00, SE=.00, p=.03), but not in response to stress, with higher levels of maternal sAA being associated to infant's lower cortisol baseline levels. However, the inclusion of maternal sAA AUCg did not significantly improve the model fit over the baseline model (deviance difference (3)=5.3, p=.15).

Lastly, there were no significant association between maternal IL-6, CRP or cortisol levels during pregnancy and infants' cortisol concentrations at baseline or in response to stress.

5.4.6 Supplementary analyses

A supplementary set of analyses was run to test the independent effect of maternal anxiety, rather than depression, on infant outcomes. For what concerns temperament, maternal anxiety was not significantly associated with infants' IBQ scale scores (p=.17). The association between maternal sAA response to awakening and negative affectivity scores remained significant (β=-.30, t=-2.89, p=.005), even after controlling for maternal anxiety rather than depression. With regards to psychomotor development, differently from depression, maternal anxiety was significantly associated with infants' cognitive scores, with higher anxiety symptoms during pregnancy being related to lower cognitive development (β=-.31, t=-2.43, p=.016), after adjusting for covariates. Nevertheless, the association between maternal prenatal cortisol and infant's cognitive scores remained significant (β=-.29, t=-2.99, p=.004), even after controlling for maternal anxiety rather than depression, while the association between prenatal CRP levels and cognitive scores was marginally significant (β=-.19, t=-1.86, p=.06) when adjusting for anxiety. Regarding infants' stress response, no significant or marginally significant effect of maternal anxiety on newborns' intensity or duration of behavioral distress as well as on cortisol baseline level or response to the inoculation was found.

5.5 Discussion

Findings from the present study provide further evidence of an association between maternal antenatal depression and infants' cortisol stress reactivity and extend prior work highlighting significant associations between multiple biological measures of stress and inflammation during pregnancy and the trajectory of infant development, including cognitive, temperamental and stress-related outcomes, in a low-risk sample of healthy 3-month-old infants.

5.5.1 Prenatal maternal factors and infants' psychomotor development

The current study is among a small number of other very recent reports that suggest that maternal stress and immune signals in utero predict infant early cognitive development, independently from prenatal and postnatal maternal depressive symptoms. In contrast, no effect of prenatal maternal influences on motor development was found. Specifically, in line with our predictions, we observed lower cognitive scores in infants prenatally exposed to higher levels of maternal diurnal cortisol, after controlling for several potential confounders. Our findings converge with animal evidence indicating that prenatal maternal stress can lead to attentional, neuro-motor and learning deficits in offspring (reviewed in Owen, Andrews, & Matthews, 2005) and with studies linking prenatal glucocorticoids administration and reduced cognitive abilities (e.g. Coe & Lubach, 2005) or cortical thinning (Davis et al., 2013). While glucocorticoids play a critical role in promoting fetal growth and neuronal maturation, excessive levels are considered neurotoxic (Uno et al., 1994). Our findings, though essentially correlational in nature, are consistent with the hypothesis that higher than average elevations in maternal diurnal cortisol within normative pregnancy can impact fetal brain development and influence later cognitive development. These results are in line with the limited available literature in humans linking fetal overexposure to endogenous cortisol in late pregnancy with poor cognitive performances from 3 months to 7 years of age (Huiznik et al., 2003; Buitelaar et al., 2003; Bergman et al., 2010; LeWinn et al., 2009). However, it is important to note that Davis and Sandman (2010) found higher maternal cortisol early in gestation but lower cortisol in late pregnancy to be related to lower mental development at 12 months. Despite the similar populations, there are a few methodological differences between that study and ours, including that the former relied on cortisol assessments based on a single occasion and from plasma and that the evaluation of infant development was undertaken later, at 12 months, which might partially explain the inconsistencies in results.

Although the exact mechanisms underlying the association between maternal cortisol and infant cognitive outcomes are still unclear, some hypotheses have been proposed. First of all, although the fetal brain is protected from elevations in maternal cortisol by the activity of placental 11-βHSD, it has been shown that the expression of this enzyme is reduced in the last stages of gestation, thus allowing more cortisol to cross the placenta and influence fetal brain development (Murphy et al., 2006). In particular, the hippocampus, crucially involved in cognition and behavior (Andrews & Matthews, 2004), is particularly sensitive to excessive levels of glucocorticoids. As it has been shown that glucocorticoid receptors in the fetal hippocampus are already expressed at 24 weeks of gestation (Noorlander et al., 2006), it can be hypothesized that cortisol overexposure in late pregnancy could influence hippocampal development and adversely impact brain and behavioral development. Secondly, maternal cortisol might influence fetal brain development indirectly, by affecting placental blood vessel tone and possibly leading to reduced utero-placental blood flow, with long-term consequences on neurodevelopmental outcomes.

Furthermore, exploratory analyses provided novel evidence for an association between higher levels of maternal CRP during late pregnancy and poorer cognitive development at 3 months. Accumulating evidence from animal models suggests that maternal inflammation can impact the fetal developing brain. For example, in mice, induced maternal IRS activation has been found to affect fetal brain myelination (Cai et al., 2000) and alter hippocampus histology, with negative consequences for later learning and memory (Golan et al., 2005; Samuelsson et al., 2006). However, knowledge on the programming role of maternal inflammation on fetal brain development in normative human pregnancy is limited. The current findings are in line with initial evidence of an association between maternal circulating levels of inflammatory markers during pregnancy and both less optimal neonatal neurobehavioral function at 6 days of age (Osborne et al., 2018) and poorer cognitive development at 12 months of age (Rasmussen et al., 2018), suggesting that naturally

occurring variations in antenatal maternal levels of inflammation could influence fetal brain development, affecting neurodevelopmental and cognitive outcomes early in life. Interestingly, recent neuroimaging evidence showed that maternal inflammation during pregnancy is associated with structural and functional alterations in newborns brain (Graham et al., 2017; Rasmussen et al., 2018). In particular, Rasmussen and colleagues (2018) found that maternal IL-6 levels during pregnancy were inversely associated with newborns' fractional anisotropy of the uncinated fasciculus, a region proximal to the amygdala and hippocampus, while predicted an accelerated postnatal growth of fractional anisotropy of the same region. Importantly, the accelerated postnatal growth in the structural connectivity of the uncinated mediated the association between antenatal Il-6 levels and infants' cognitive outcomes. Despite these findings are only preliminary and observational, they support the notion that maternal antenatal inflammation could exert a programming effects on offspring's cognitive development by affecting fetal structural brain development. It has been hypothesized that maternal inflammation during pregnancy could influence offspring neurogenesis through inflammatory mediators, leading to altered cognitive and behavioral outcomes (Golan et al., 2005). Nonetheless, the mechanisms through which maternal inflammation could actually operate upon the fetal brain are still unknown and need to be investigated in future studies.

Despite CRP and IL-6 being correlated in the current sample, maternal IL-6 concentrations were not found to predict 3-month-olds' mental development. CRP is generally considered a non-specific marker of systemic inflammation, reflecting levels of circulating interleukins (Dantzer & Kelley, 2007). Although future studies are needed into the link between variations in maternal prenatal inflammation and offspring outcomes, our results might suggest that CRP could constitute a valid biomarker for fetal programming effects, indexing general maternal inflammation in the intrauterine environment.

As both maternal and infant factors are known to play a role in shaping infants' mental development, it is worth mentioning that the effects we reported were independent of maternal postnatal symptomatology and were not fully explained by infant characteristics, such as gender and gestational age, or by maternal IQ. These results strengthen the hypothesis that prenatal factors independently predict infant cognitive development. These findings are important because evidence suggests that early performance on the Bayley cognitive scale are associated with IQ in later childhood (Cheng & Pickler, 2010; Laucht, Esser, & Schmidt, 1997), which, in turn, have been related to later academic performance and the occurrence of behavioral problems (Boutwell et al., 2017). Thus, an alteration of the trajectories of fetal brain development, resulting in poorer early mental development, could constitute one possible pathway through which prenatal stress exposures might hamper offspring development, with long-term consequences.

In contrast with our initial hypotheses, we found no evidence of an association between maternal prenatal depressive symptoms and 3-month-olds' cognitive development. As extensively described in Chapter 1, findings of an association between maternal antenatal depression and child mental development are controversial. Although issues related to statistical power and to relatively mildlevels of maternal depression in the current sample might have limited our ability to detect a significant association, it is worth mentioning that null findings have been previously reported both in clinical (e.g. Osborne et al., 2018; Santucci et al., 2014) and non-clinical samples (Tse et al., 2010). However, it is noteworthy that we do report a significant inverse association among maternal antenatal anxiety symptoms and infant cognitive outcomes. While depression and anxiety have both been independently related to poor offspring's cognitive performances (e.g. Brouwers, Van Baar, & Pop, 2001; Evans et al., 2012), few studies have sought to compare their effects and these have yielded to mixed finding with stronger effect being reported either for depression (Barker et al., 2011; Lin et al., 2017) or for anxiety (Ibanez et al., 2015). Further

replication of the current findings in different samples and at different ages is needed before any conclusions can be drawn.

Likewise, our exploratory analyses failed to detect any association between maternal antenatal sAA levels and infants' psychomotor development. Evidence for a role of maternal SNS in fetal programming in humans is scarce and to our knowledge, no published data exists on the link between prenatal maternal sAA levels and fetal brain development. Given the exploratory nature of the current findings, replication is needed in larger and different sample.

Lastly, in line with most studies (e.g. Bergman et al., 2010; Davis & Sandman, 2010), we did not find any significant effects of prenatal maternal factors on infant's motor development at 3 months. It is possible that the regions of the brain more crucially involved in cognitive functioning, such as the hippocampus and prefrontal cortex, are more sensitive to the alterations in the intra-uterine environment, than areas involved in motor development (Davis & Sandman, 2010).

5.5.2 Prenatal maternal factors and infant temperament

As hypothesized, maternal depression during pregnancy was significantly associated with maternal reports of infants' negative affectivity (NA) at 3 months of age. Furthermore, we found preliminary evidence of an association between maternal antenatal sAA response to awakening and infants' NA. These results were independent of infant gender and gestational age, as well as measures of concurrent maternal depression. Contrary to our predictions, maternal antenatal diurnal cortisol was not significantly associated with infants' temperament. Furthermore, we did not find any associations between prenatal maternal variables and infants' positive affectivity and orienting/regulatory capacity or crying and sleep behaviors as recorded on the Baby's Day Diary.

Temperament refers to early biologically-based individual differences in reactivity and self-regulation (Rothbart, 1991) which are thought to be embedded in

the infant's neurophysiology and to be influenced by both genetic and environmental factors (e.g. Silberg et al., 2005). The current results indicate an association between intrauterine environmental factors and aspects of infant temperament, as reported by mothers, related to the tendency to experience negative emotions. As expected, maternal depressive symptoms during pregnancy were significantly associated with 3-month-old infants' negative affectivity; these effects were specific for maternal depression, rather than anxiety. This is in line with a number of studies that identify antenatal depression as a significant predictor of infants' negative affectivity in human (e.g. Austin et al., 2005; Davis et al., 2007; Huot et al., 2004) and with animal models indicating an increase in fearful or reactive behavior in offspring prenatally exposed to maternal stress (Welberg & Seckl, 2001; Weinstock, 2005). Notably, infant NA can be reliably assessed as early as 3 months of age (Gartsein & Rothbarth, 2003) and is a relatively stable temperamental trait over time (Putnam et al., 2008) which has been associated with an increased later risk for emotional and behavioral problems, including depression (Dougherty et al., 2010). Taken together, these lines of evidence converge to suggest that intrauterine environmental factors might influence infant temperament, thus possibly, laying the foundations for later behavioral problems (e.g. Gartstein et al., 2012). However, the mechanisms underlying the link between antenatal maternal depression and infant NA are still to be understood. The current study offers an important extension to existing literature by examining a number of potential biological mediators, involving the stress and immune response systems. Our findings provide evidence that variations in maternal SNS diurnal functioning during pregnancy, as indexed by sAA levels, are associated with maternal report of infant NA 3 months after birth. More specifically, a greater morning sAA decline after awakening was related to higher scores on infant NA, after adjusting for covariates. However, it is important to mention that the observed association was independent of maternal depressive symptoms, thus possibly suggesting that maternal antenatal depressive symptoms and stress signals operate through independent pathways in shaping infant

temperament. To our knowledge, only Braithwaithe and colleagues (2017) evaluated the influence of maternal sAA levels during pregnancy on infant temperament and found that maternal sAA diurnal levels predicted 2-month-olds negative emotionality in a sex-dependent manner. Specifically, high levels of maternal sAA were marginally associated with reduced negative emotionality in males, while in females there was the opposite association, although non-significant. Despite methodological differences limiting possible comparisons, taken together these preliminary findings indicate that maternal sAA diurnal levels during pregnancy might be a sensitive biomarker for understanding how prenatal factors shape temperament in infancy. In contrast to Braithwaithe and colleagues (2017), we did not find any significant association between the overall daytime level of maternal sAA, as indexed by the AUCg, and infants' difficult temperament. On the contrary, our findings seem to suggest that subtle dynamic variations in the sAA diurnal pattern, rather than the total amount of sAA, are more closely implicated in programming behavioral outcomes in a healthy sample of pregnant women and infants. Although it is unclear why maternal sAA response to awakening may be particularly relevant for infant temperament, it has been recently proposed that fluctuations of stress hormones related to the diurnal pattern of the stress response systems might convey more adaptively-relevant information to the foetus concerning the postnatal environment than absolute levels (Giesbrecht et al., 2017). However, the preliminary nature of the current finding limits possible interpretation and requires replication in larger samples.

Despite the focus on HPA-axis mediated mechanisms of fetal programming in the literature, contrary to our hypothesis, in the current study the association between maternal antenatal cortisol and maternal reports of infant temperamental NA did not reach statistical significance. We only observed a marginally significant association and the direction of the association, with higher levels of maternal cortisol predicting lower levels of infant NA was unexpected and requires further investigation in larger sample. It is noteworthy that results from recent studies relate higher prenatal

exposure to concentrations of stress markers such as cortisol and sAA to lower negative emotionality in males, while the opposite association in females (Braithwaite et al., 2017a; Braithwaite et al., 2017b). Although the current sample size does not allow us to investigate possible gender effects in the observed associations, this remains an important area for future research.

In line with prior work (e.g. Bergman et al., 2007; Austin et al., 2005) concurrent maternal depressive symptoms were also significantly associated with infant NA, thus raising a number of issues. First of all, while maternal report, as compared to laboratory assessment, allows us to obtain evaluations of infants' behaviors occurring in naturalistic settings at different times, we cannot rule out a self-report bias with postnatally depressed mothers rating their infants as more difficult. However, parent-report and structured observation have been shown to provide convergent evaluation and, importantly, it has been shown that parental depression generally does not influence IBQ-R ratings (Garstein & Marmion, 2008). Secondly, a bidirectional link between postnatal maternal depression and infant NA is also likely to occur with either postnatal depressive symptoms negatively affecting infant temperament or infant difficult temperament having a negative impact on maternal psychological state, or both. However, it is important to note that the effect of prenatal depression remained strong and significant even after statistically controlling for postnatal mood, thus suggesting that postnatal mood cannot fully account for these results.

Additionally, we cannot rule out other mediating mechanisms underlying the link between prenatal maternal factors and infant NA, including genetics. Indeed, it is possible that shared genetic susceptibility may contribute to the observed associations between maternal depressive symptoms and infants' tendency to experience negative emotions. Future research is needed to disentangle intra-uterine environmental effects and genetic contributions.

5.5.3 Prenatal maternal factors and infants' stress reactivity

In line with our initial hypothesis, maternal depressive symptoms during pregnancy predicted infants' cortisol response to stress at 3 months, thus supporting existing evidence for a role of maternal depression in programming fetal stress reactivity. Differently from what we hypothesized however, maternal antenatal cortisol was not related to infants' HPA axis stress response. Similarly, no associations between prenatal maternal factors and infants' behavioral distress to the inoculation were found.

As a whole, infants from the current sample displayed the expected cortisol response to the inoculation. However, maternal depression during late pregnancy was associated with individual differences in infants' stress regulation, with infants prenatally exposed to higher depressive symptoms showing a flatter recovery of cortisol levels 40 minutes after the inoculation. Notably, this effect remained significant after controlling for maternal age, infant's gender and concurrent depression, suggesting that prenatal exposure uniquely accounts for the association. Additionally, it appears to be specific to maternal depression rather than anxiety. The present findings are consistent with data from animal models that relates exposure to prenatal stress to changes in offspring HPA axis function, including HPA axis greater reactivity to stress (Huiznik, Mulder & Buitelaar, 2004). Additionally, this result is in line with the limited number of prospective studies in humans linking maternal prenatal depression to offspring slower cortisol recovery to experimental challenge at 12 months (Waters et al., 2013a) and to greater cortisol reactivity to stress across the first year of age (Leung et al., 2010; Brennan et al., 2008; Stroud et al., 2016; Fernandes et al., 2014). Interestingly, Davis and colleagues (2011) found that maternal depression during the third trimester of pregnancy was associated with slower newborn behavioral recovery to the heel-stick soon after birth, while no associations were found with newborn's cortisol response. It is important to note that a recent systematic review found only little evidence for an association between prenatal exposure to maternal depression

and stress reactivity in offspring (Bleker et al., 2018). The authors suggested that large heterogeneity in whether the stressor evoke a response at all across studies might partially explain inconsistent results. In particular, studies that fail to detect a significant stress response in general, reported no associations between maternal antenatal depression and infant's stress response (Azar et al., 2007; Laurent et al., 2011; Sharp et al., 2012). Thus, it has been suggested that maternal antenatal depression might affect the shape of the response trajectory rather than the magnitude (Bleker et al., 2018). Our findings speak in favor of this hypothesis, showing a flatter recovery in cortisol levels after the stressor in children antenatally exposed to higher maternal depressive symptoms.

In utero programming of the fetal stress response systems have been proposed as the core mechanism underlying the link between antenatal depression and later offspring outcomes (e.g. Kapoor, Petropoulos, & Matthews, 2008). Our findings support this hypothesis suggesting that prenatal exposure to maternal depression may exert programming effects on the fetus by altering the set points of the fetal HPA axis with later consequences for infant's stress regulation. This result is particularly relevant as a dysregulation of the HPA axis has been suggested as an early marker of vulnerability for later behavioral and emotional problems in children (e.g. Turner-Cobb, Rixon, & Jessop, 2008). Additionally, it is noteworhty that depressed individuals, as compared to controls, have been found to fail to adjust the cortisol response to stress, showing a lack of dynamic changes in cortisol levels following stress exposure across the age span (Lopez-Duran, Kovacs, & George, 2009). For example, it has been reported that depressed preschoolers displayed persistently elevated cortisol levels in response to stressors (Luby et al., 2003; 2004). Although the current findings require replication in larger and different cohorts, we might speculate that a lack of recovery in cortisol levels after a painful stressor in 3-month-old infants of prenatally depressed women might resemble the pattern of response observed in depressed individuals, suggesting that fetal programming of

HPA-axis reactivity could constitute a possible pathway of transmission of depression susceptibility. It would be of interest to determine whether these early differences in stress reactivity pattern persist over time and whether they can predict later outcomes, such as later depressive symptoms.

It is critical to underline the fact that we are not able to rule out the possibility that shared genetic factors (or a combination of genetic factors and environmental exposure) between mother and child may account for infants' early differences in stress reactivity and may be a possible mechanisms of transmission of susceptibility from mother to child (e.g. Dougherty et al., 2010). While it has been shown that the effects of prenatal stress on several infant outcomes is at least partially independent of genetics (Rice et al., 2010) and may be attributed to environmental factors, future studies adopting genetic informative designs are needed to disentangle the unique environmental effect of maternal antenatal depression on offspring's stress reactivity.

The nature of the in utero biological pathways leading to differences in the offspring's HPA axis stress reactivity following exposure to antenatal depression remains to be clarified. We examined the impact of naturally occurring variations in maternal stress and immune functioning in late pregnancy on 3-month-olds stress reactivity. Notably, maternal prenatal cortisol, sAA and inflammatory markers were not significantly associated with infants' cortisol response to stress, after adjusting for maternal age, infant's gender and postnatal depression. While the current study was mostly exploratory with regards to possible effects of maternal antenatal sAA and inflammation on child outcomes, as available literature in humans is strikingly limited, the lack of an effect of maternal antenatal cortisol on infant HPA-axis stress reactivity is unexpected. Indeed, although few reports failed to detect a significant association between maternal antenatal cortisol and infant's cortisol stress reactivity (e.g. Tollenaar et al., 2011; Braithwaite et al., 2016), a number of studies shows evidence for a positive links (e.g. Davis et al., 2011; Gutteling, De Weerth, & Buitelaar, 2004; Gutteling et al., 2005). Methodological differences between studies, including

differences in maternal cortisol assessment and gestational timing, may account for the conflicting evidence. Additionally, it is possible that small variations in maternal cortisol concentrations in a low risk sample of healthy women during the last weeks of pregnancy are not capable to significantly affect the fetal developing HPA axis. Or, it is also possible that unmeasured postnatal environmental factors might alter the unfolding of prenatal processes involved in programming of offspring's stress-related physiology, as initial evidence in humans suggests (e.g. Sharp et al., 2012; Kaplan et al., 2009), thus confounding the observed associations.

Lastly, we did not find any significant association between the maternal prenatal variables examined and 3-month-olds' intensity and duration of behavioral distress to the inoculation. This is in contrast with several sources of evidence showing a link between either maternal depression and infant's behavioral reactivity (e.g. Davis et al., 2011; Werner et al., 2007) or maternal cortisol and infant's behavior in response to stress (e.g. De Weerth et al., 2013; Werner et al., 2013b). Inconsistencies might in part arise from variability in methods used to evaluate both maternal and infant functioning. Additionally, it is possible that little inter-individual variability exists in the behavioral response to the first inoculation, with almost all infants displaying a significant level of behavioral distress following the shot administration. This is in line with findings from Jahromi and colleagues (2004) who reported a decrease in the intensity and duration of infants' distress from 2 to 6 months. While our assessment of infants' behavior was based on observer's ratings, due to logistical issues related to being in the waiting room with other families not involved in the current research, in line with prior work (e.g. Jahromi et al., 2004) we were not able to videotape infants' behavior before the inoculation and several minutes after. While the experimenter rated the infants before the inoculation as being "quiet" or "distressed", we did not have a proper baseline period that could allow us to model the infant's trajectory of response over time, thus limiting the possibility to detect significant associations between prenatal factors and subtle variations in the behavioral stress response.

5.5.4 Limitations

A number of limitations should be taken into account when interpreting the current findings. First, our small sample size limits the statistical power of the findings. In addition, our results are based on a low-risk middle-high SES sample of healthy women and infants, thus limiting generalizability to different populations. Replication of the current findings in different and larger cohorts is needed. Secondly, while the assessment of multiple biological markers is a strength of the current study, our investigation was limited to one time point in late pregnancy and the observed associations might not extend to other gestational times. Prospective studies including different gestational windows of exposure, along with multiple stress markers, are likely to be particularly informative. Third, as we relied on naturally occurring variations in maternal depressive symptoms and associated biological markers, it is difficult to disentangle the role of prenatal factors from the effects of other related factors, including maternal diet or lifestyle or shared genetic factors that are likely to play a role in the "risk" transmission from mother to infant. Nevertheless, a wide range of possible confounders such as sociodemographic factors, health and pregnancy-related factors were controlled in statistical analyses. Additionally, only healthy pregnant women free from any medication, alcohol or smoking use were included. However, genetic factors or factors related to changes in diet or lifestyle remain areas for future investigations. Similarly, while we controlled for the effects of maternal postnatal depressive symptoms, we did not adjust for a number of additional postnatal environmental factors, including the quality of mother-child relationship, which is known to affect infant development (e.g. Raby et al., 2015). As a sensitive rearing environment has been shown to play a role in the link between prenatal adverse exposure and later outcomes in offspring (e.g. Grant et al., 2009; Bergman et al., 2010) and the assessment of postnatal depressive symptoms is not likely to fully reflect the quality of postnatal experience, future studies should explicitly examine the role of maternal caregiving behavior.

5.6 Conclusions

To sum up, the current study provided support for a specific role of maternal antenatal depression in shaping infant temperamental NA and cortisol reactivity to stress. Additionally, maternal cortisol and CRP levels in late pregnancy were found to contribute in a unique way to 3-month-olds' cognitive development, while maternal antenatal sAA levels appear to be related to infant NA. Taken together, the present findings add to growing evidence on the role of prenatal maternal influences on later child development, suggesting that the developing fetal brain is particularly sensitive to the intrauterine environment and adapts to even relatively small variations in maternal stress and inflammatory signals, with consequences that are evident 3 months after birth. Findings from the current study encourage further investigation of SNS and IRS mechanisms in fetal programming that have been largely neglected until now. However, our results do not support the hypothesis that distress-linked variations in cortisol, sAA and inflammatory marker levels during pregnancy mediate the effects of maternal depression on fetal development. Indeed, we reported only a marginally significant association between maternal depressive symptoms and IL-6 levels, that interestingly were not found to be related to any of the outcomes evaluated 3 months after birth, while no associations between maternal distress and cortisol or sAA or CRP levels, in line with several previous reports (e.g. Davis et al., 2011; Bergman et al., 2010). While a number of limitations discussed above may partially account for these null associations, these findings underscore the needs to explore alternative mechanisms of effects, involving for example epigenetics or placental mechanisms of regulation, in order to elucidate the full extent of the in-utero pathways leading to different developmental trajectories. Moreover, it would be of great interest to evaluate whether the observed individual neurodevelopmental differences are stable over time and whether they predict later outcomes. It is our purpose to follow up this sample to evaluate whether the effects of prenatal maternal influences persist to later ages and to examine the role played by postnatal caregiving. A better understanding of the

origins and trajectories of early individual differences will be crucial for the design of effective intervention and prevention strategies.

Chapter 6: The role of postnatal maternal care in the association between maternal prenatal influences and 3-month-olds' bio-behavioral outcomes.

6.1 Introduction

There is mounting evidence that in utero exposure to maternal depression during pregnancy can influence fetal development, leading to negative neurodevelopmental outcomes in the offspring during infancy and beyond, including delayed cognitive development, fearful or reactive temperament and altered stress reactivity (e.g. Gentile, 2017). The process through which fetal development is perturbed as a consequence of exposure to an altered intrauterine environment has often been described as fetal programming (e.g. Barker, 2004; Seckl & Holmes, 2007). In particular, it has been proposed that mood-related alterations in the functioning of some biological systems, such as the Hypothalamic-Pituitary-Adrenal (HPA) axis, the Sympathetic Nervous System (SNS) and the Inflammatory Response Systems (IRS), might mediate the observed effects of fetal programming (reviewed in Rakers et al., 2017). In chapters 4 and 5, we examined the effects of antenatal exposure to variations in maternal depressive symptoms, stress and inflammatory biological markers on infant's developmental outcomes soon after birth and at 3 months of age. However, considerable evidence suggests that fetal programming of later outcomes can be modified by postnatal environmental conditions (O'Donnell & Meaney, 2017), thus underscoring the need to account not only for prenatal effects but also for early postnatal environmental factors in a comprehensive model of developmental programming.

Long-standing evidence within the attachment theory framework and recent neuroscience support the fundamental role of a sensitive and responsive caregiver as one of the leading forces directing infants' psychobiological developmental trajectories (Tottenham, 2017). The quality of early parental care has been found to predict a number of outcomes in children, including socio-emotional and cognitive development (e.g. Austin et al., 2017; De Wolff & Van Ijzendoorn, 1997; Gartstein, Hancock, & Iverson, 2017; Mills-Koonce et al., 2015). Additionally, considerable evidence supports the role of a sensitive and responsive caregiver as an external behavioral and physiological regulator throughout the first year of life (Crockenberg & Leerkes, 2004), influencing infants' developing stress response systems (Gunnar, 2017). In particular, it has been shown that a sensitive caregiver is able to buffer child's HPA axis reactivity (reviewed in Gunnar & Hostinar, 2015), while disturbances in early care can disrupt neuroendocrine stress regulation (Gunnar & Quevedo, 2007), with a non-optimal caregiving environment, characterized by low maternal sensitivity, leading to exaggerated stress reactivity (Albers et al., 2008; Blair et al., 2008; Hane et al., 2010; Blair et al., 2006). The biological embedding of early caregiving experiences within the infant's HPA axis has been proposed as a potential pathway through which the quality of caregiving can alter children's developmental trajectories, reducing or exacerbating the risk for psychopathology (e.g. Gunnar & Quevedo, 2007). However, while several studies investigating the impact of antenatal depression on child development have accounted for the effects of postnatal symptomatology (e.g. O'Donnell et al., 2013), the potential role of parenting style, in mediating or moderating antenatal maternal influences, has been largely neglected.

Substantial continuity has been reported in maternal prenatal and postnatal depressive symptoms and family disharmony, suggesting that continued exposure to adversity might occur (Bekkhus et al., 2011). Additionally, maternal postnatal depression has been found to negatively affect maternal caregiving behaviors (e.g. Bernard et al., 2018). However, the extent to which poor maternal parenting in the

early postnatal period might explain, at least partially, some of the outcomes in the offspring that have been traditionally associated with antenatal depression is largely unknown (Monk et al., 2012).

Furthermore, it is still unclear whether the quality of parenting received during early development may moderate the effects of prenatal stress exposure on infants' physiological and behavioral outcomes. Evidence from rodent and primate models suggest that variations in postnatal caregiving are able to modify the bio-behavioral outcomes associated with prenatal stress exposure, by either counteracting the detrimental influences of prenatal adversities on offspring development or exacerbating the risk of poor outcomes (Francis et al., 2003; Maccari et al., 1995; Sanchez, 2006; Sapolsky, 1997; Wakshlak & Marta, 1990).

For example, it has been shown that postnatal maternal adequate caregiving behaviors, such as handling or "licking and grooming", can reverse offspring increases in emotional reactivity (Wakshlak & Marta, 1990), deficits in hippocampal neurogenesis (Lemaire et al., 2006), impairment in glucocorticoids feedback (Maccari et al., 1995) and morphological and hormonal alterations (Del Cerro et al., 2010) associated with prenatal stress exposure. Additionally, initial evidence suggests that similar effects might operate in humans too, as shown by studies demonstrating that early maternal tactile stimulation, such as maternal stroking, might moderate the effects of prenatal stress on children's physiological and behavioural outcomes (Sharp et al., 2012; Sharp et al., 2015; Pickles et al., 2017). However, only a handful of prospective studies examined the buffering role of sensitive caregiving in the link between prenatal maternal distress, such as depressive or anxiety symptoms, and infants' developmental outcomes, including temperament, cognition and stress reactivity.

A review of the literature yielded only three published studies examining the moderating role of postnatal maternal care in the link between self-reported prenatal stress and cognitive development, and these provided mixed results. More specifically,

Grant and colleagues (2010) found that maternal sensitivity to distress moderated the link between prenatal exposure to maternal anxiety disorder and 7-month-olds cognitive, but not motor, development in a sample of 77 mother-infant dyads, while controlling for postnatal concurrent symptoms. In particular, a positive association between maternal sensitivity and cognitive performance among infants prenatally exposed to maternal anxiety (N=14), but not among controls (N=63), was reported. In contrast, no evidence was found for a moderating role of maternal sensitivity and structuring in the link between maternal antenatal objective and subjective stress and 30-month-olds cognitive development in a sample of 128 mother-infant dyads from the Queensland Flood Study (Austin et al., 2017). Likewise, Bergman and colleagues (2008) found that attachment did not moderate the link between maternal antenatal stress exposure (as indicated by the number of stressful events experienced during pregnancy) and 17-month-olds cognitive development in a sample of 125 mother-infant dyads. However, considerable differences in infants' age and "maternal stress" phenotype under study makes difficult to compare findings from the above mentioned studies.

Similarly, very few studies investigated whether the quality of maternal caregiving was able to moderate the association between prenatal stress and infants' temperament. In particular, Bergman and colleagues (2008) reported that an insecure-ambivalent attachment pattern exacerbates the link between prenatal stress exposure and 17-month-olds' fearfulness as observed during a standardized laboratory assessment. Conversely,Kaplan and collaborators (2009) did not find any significant effect of maternal sensitivity in moderating the link between prenatal psychiatric diagnosis and maternal report of 4-month-olds' temperament, although the small sample size (N=44) limited the statistical power of the study.

Furthermore, to our knowledge, only three human studies explored the buffering role of maternal sensitive caregiving on infants' prenatal-stress associated stress physiology. In a small prospective study, maternal sensitivity moderated the

prenatal effects of maternal psychiatric diagnosis on 4-month-olds' baseline cortisol levels, with maternal sensitivity protecting from elevations in cortisol levels among infants of prenatally depressed/anxious mothers (N=19), while no effects were reported on infants' heart rate variability (Kaplan, Evans & Monk, 2009). Grant and colleagues (2010) found maternal sensitivity to be associated with lower 7-month-olds' behavioral reactivity to the still-face procedure; and the association was more marked in infants of prenatally anxious women (N=16). In contrast, while maternal sensitivity independently predicted infant's cortisol response to the still-face, it did not moderate the association between prenatal maternal anxiety and infants' cortisol reactivity (Grant et al., 2009).

Lastly, despite increasing evidence for a role of maternal antenatal disturbances in the functioning of stress-related biological systems in fetal programming (e.g. Davis et al., 2011; Giesbrecht et al., 2017; Graham et al., 2017), to date only Bergman and colleagues (2010) investigated the extent to which the quality of caregiving might moderate the link between maternal antenatal variations in stress hormones and child outcomes. Specifically, the authors found that a secure infant-mother attachment protected from the detrimental effects of prenatal exposure to higher levels of cortisol on 17-month-olds' cognitive functioning.

6.2 Summary and study hypotheses

While a great deal of research has shown that in utero exposure to maternal depression is associated with an increased risk for altered physiological and behavioral outcomes in the offspring (reviewed in Gentile, 2017), the extent to which prenatal factors and postnatal factors related to the quality of early caregiving interact to influence infants' outcomes remains unknown. In particular, despite the fact that antenatal depression is one of the strongest predictor of postpartum depression (e.g. Heron et al., 2004) which, in turn, has been related to disturbances in early parenting (Easterbrooks, Biesecker, & Lyons-Ruth, 2000; Easterbrooks et al., 2008), studies in

the DOHaD field have rarely accounted for the quality of postnatal care. Thus, it is unclear whether the quality of postnatal care may explain some of the developmental outcomes that have been originally attributed to the intrauterine exposure to antenatal depression. Furthermore, while animal models consistently show that postnatal maternal care moderates the impact of antenatal stress exposure on the development of the offspring (Weinstock et al., 2017), generalizability of these findings to humans is still unknown. A review of the literature yelded only a handful of prospective studies examining the role of caregiving in moderating the impact of prenatal maternal stress on the offspring's development (Austin et al., 2017; Bergman et al., 2008; Kaplan et al., 2009; Grant et al., 2009; Grant et al., 2010) with large variability in sample sizes, infant's age, measures of maternal stress (i.e. psychiatric diagnosis, self-reported stressful life events, subjective and objective stress following exposure to a natural disaster) and caregiving (i.e. maternal sensitivity, sensitivity to distress, structuring and mother-infant attachment) making difficult to draw any comparisons. Furthermore, while Bergman and colleagues (2008) have investigated the moderating role of postnatal care in the link between maternal antenatal cortisol and 17-month-olds' cognitive development (Bergman et al., 2008), no studies to date have focused on early infancy and have included alternative biological measures of maternal antenatal stress, such as salivary alpha amylase (sAA), C-Reactive Protein (CRP) and Interleukine-6 (IL-6).

In chapter 5, we investigated the influences of variations in maternal antenatal depressive symptoms, stress and inflammatory markers on 3-month-olds neurodevelopmental outcomes, while controlling for postnatal symptomatology. In the current study, we aimed to extend research into the effects of antenatal stress by exploring the role of the quality of maternal early caregiving, as assessed through the Emotional Availability (EA) scales (Biringen, 2008). The EA scales is a well-validated instrument that allows to obtain reliable indicators of the quality of mother-child relationship and that, compared to other rating systems, emphasizes the emotional

feature of the interaction (Biringen et al., 2012), which might be an important aspect to consider in mothers experiencing depressive symptoms. Despite being widely used in developmental studies, the EA scales are still rarely employed in research within the DOHaD field.

Building upon existing knowledge, we aimed to provide additional empirical evidence for a role of maternal EA on 3-month-olds' developmental outcomes independent from prenatal and postnatal depressive symptoms and to investigate the interplay among prenatal and postnatal maternal influences in shaping infants' bio-behavioral development. For this reason, we examined the independent contribution of postnatal maternal EA and the interactive influence with prenatal maternal factors (i.e. maternal depressive symptoms, cortisol, sAA, CRP and IL-6) on a broad range of infants' outcomes including stress reactivity, temperament and psychomotor development, while controlling for several potential confounders. We evaluate several different outcomes tapping into distinct aspects of development in order to test the robustness of the effects and evaluate whether prenatal and postnatal factors interact in a similar way to influence different developmental outcomes. Additionally, considering the strong association between maternal depressive and anxiety symptoms, we investigated whether adjusting for maternal anxiety, rather than depression, changes the direction and significance of the associations among maternal EA and child outcomes and whether maternal antenatal anxiety interact with postnatal EA to predict any outcomes in the offspring.

Based upon the available literature, we hypothesized that maternal EA would be associated with several developmental outcomes in offspring and in, particular, we expected infants of less emotionally available mothers to show poorer cognitive and motor performances, more difficult temperament and greater stress reactivity. Furthermore, with respect to the role of maternal caregiving in the DOHaD hypothesis, we tested two main hypotheses. First, we hypothesized that women with higher prenatal depressive symptoms would show lower levels of maternal EA, as compared

to women with lower depressive symptoms, and that this subsequently could mediate the effects of antenatal depression on infants' neurodevelopmental outcomes. Secondly, we explored for the first time whether maternal EA would moderate the relationship between several indices of prenatal maternal stress (i.e. depressive symptoms, cortisol, sAA, CRP and IL-6) and infant outcomes. Due to the limited available literature in humans, we made only a very broad a priori hypothesis. In particular, we hypothesized that higher maternal EA at 3 months of age would protect infants from the negative impact of prenatal stress, while we expected the worst outcomes in infants of mothers who showed higher levels of antenatal stress and were also less emotionally available at 3 months of age.

6.3 Material and methods

6.3.1 Participants

One hundred and ten predominantly Italian (97.1%) middle-high class (87.5%) primiparous (91.3%) women were recruited between 30-33 gestational weeks at 3 hospitals in Italy as part of the EDI (Effects of Depression on Infants) study. Women with singleton uncomplicated pregnancy, no smoking or drug using and not afflicted by any chronic disease or taking any chronic medications were eligible to participate. From the initial sample of 110 women, 3 were excluded for reasons related to intrauterine death and newborn's health problems, while 3 women did not attend the postnatal session due to logistic reasons (i.e. living too far from the Medea Institute), resulting in 104 mother-infant dyads included in the current study. Infants (51% males) were mostly born by vaginal delivery (81.7%) and were full term, except for two babies born, respectively, at 35 and 36 gestational weeks in good health. Women who were excluded from the 12-weeks postnatal phase did not differ from participants on any demographic variables, depression or anxiety scores.

6.3.2 Procedure

Maternal depressive and anxiety symptoms, biological functioning and cognitive function were assessed in late pregnancy (mean gestational age= 34.72; SD= 1.14) as described below. Health and pregnancy-related data were also collected at that time. The 12-week postnatal assessment included one session for the evaluation of infants' cortisol and behavioral reactivity to the inoculation and a subsequent session at the Medea Institute for the assessment of infants' cognitive and motor development and quality of mother-infant relationship. On the same occasion, mothers were asked to report on infants' health, temperament and, once again, on their depressive and anxiety symptoms.

The Ethics Committee of University College London, of Scientific Institute Eugenio Medea and of the hospitals and pediatric health centers involved approved the study protocol.

6.3.3 Maternal assessment

Maternal pre- and postnatal depressive and anxiety symptoms. Maternal depressive symptoms were evaluated through the Italian version (Benvenuti et al., 1999) of the Edinburgh Postnatal Depression Scale (EPDS; Cox, Holden, & Sagovsky, 1987), a 10-items scale widely used to screen for perinatal depression. Items are scored on a 4-points Likert scale ranging from 0 to 3, with a maximum score of 30. Maternal anxiety symptoms were evaluated through the Italian version (Pedrabissi & Santinello, 1989) of the trait anxiety subscale of the State-Trait Anxiety Inventory (STAI) (Spielberger, Gorsuch & Lushene, 1970) a 20-item scale generally employed to identify symptoms of anxiety as a general trait. Responses are rated on a 4-point Likert scale ranging from 1 (not at all) to 4 (very much), with a maximum score of 40.

Maternal antenatal cognitive function was assessed through the Raven Standard Progressive Matrices (RSPM, Raven et al., 1998), a widely employed test that evaluate non-verbal general cognitive ability. It consists of 60 multiple choice

items that require participants to identify the missing element that completes a pattern within 30 minutes. The RSPM raw scores, as a measure of individual general cognitive ability, were employed in the analyses.

Maternal antenatal biological assessment. Blood samples, available from 94 women out of 104, were drawn in the morning by venipuncture and kept refrigerated at +4° until they reached the Biological Lab of Medea Institute were serum was centrifuged, aliquoted, and stored at −80 °C. Biological assay for CRP and IL-6 levels were run in duplicate by using Quantikine High Sensitivity ELISA kits (R&D Systems Europe, LTD) at LaboSpace in Milan. Intra-assay coefficient of variation (CV) was <6% for IL-6 and <3% for CRP, inter-assay CV was <10% for both markers.

Saliva samples were collected at home on two consecutive days immediately upon awakening, 30 minutes post-awakening and before going to bed by passive drool (Granger et al., 2007) to provide a general index of diurnal cortisol and sAA functioning (O'Donnell et al., 2013). Participants were instructed to record time of collection on a diary and asked to avoid eating, tooth-brushing and exercising in the 30 minutes before collection and eating a major meal in the hour before the evening sample. As 1 woman collected the 30 minutes post-waking sample on day 2 more than one hour from awakening this sample was excluded from analyses (e.g. O'Donnell, 2013; O'Connor, 2014). All saliva samples were stored frozen at -80° until assayed for salivary cortisol at the Biological Lab of Medea Institute, using a competitive high sensitivity enzyme immunoassay kit (Expanded Range High Sensitivity Cortisol EIA Kit, Salimetrics), and for sAA at the Salimetrics Centre of Excellence testing lab at Anglia Ruskin University, using a kinetic enzyme assay kit (Salimetrics α-Amylase Kinetic Enzyme Assay Kit). All samples from one woman were run in the same assay to minimize method variability. Salivary cortisol assays were run in duplicate and averaged, except for 2 samples with minimal volume. Average intra- and inter-assay coefficients were <6% and <8%, respectively. Results were computed in µg/dl. A

random 10% of the sAA assays were run in duplicate to confirm reliability. The intra-assay coefficient of variation was < 3%. Results were computed in U/mL.

Maternal postnatal caregiving. The quality of maternal caregiving was evaluated through the Emotional Availability (EA) Scales, Infancy/Early Childhood Version (4th edition, Biringen, 2008) during a 15 minutes-videotaped free-play session. Mothers were left with their 3-month-olds in a room equipped with a standard set of age-appropriate toys and were told to interact with their child as they normally would do at home. Maternal behaviours towards the child were rated on four dimensions, namely: 1) Sensitivity, which refers to the ability to be warm, emotionally connected and responsive to the child's signals; 2) Structuring, which refers to the ability to appropriate scaffold and structure the child's play; 3) Non-intrusiveness, which refers to the ability to be available to the child without interfering with the child's age-appropriate autonomy and 4) Non-hostility, which refers to non-negative, abrasive, impatient or antagonistic style of interaction with the child. Each scale is rated with a direct score ranging from 1 (non-optimal) to 7 (optimal), as well as along 7 sub-scales generating a total score ranging from 7 to 29. In order to maximize variability in the EA scores, the total scores were employed in data analyses, as previously done (Austin et al., 2017). Additionally, as the four maternal EA scales were moderately inter-correlated (rs= 0.73–0.50), they were standardized and summed to create an overall index of maternal EA (Cronbach's α= .85) which also reduces the number of variables for analyses, as done in prior work (Taylor-Colls & Fearon, 2015). Two clinical psychologists, both trained by Zeynep Biringen and reliable in the use of the EA scale, 4th Edition, both with the EA's author, and with each other, coded independently the videotaped interactions after the end of data collection. Both raters were blind to all prenatal and postnatal data. A subsample of 20 randomly chosen dyads (around 19.5% of the sample) were coded by both raters. Intra-class correlation (ICC) coefficients ranged from .75 to .91 with a mean ICC of .84 ($p<.001$).

6.3.4 Infant assessment

Infant temperament was assessed through the 37-items very short form of the Infant Behavior Questionnaire (IBQ-R-VSF), a widely employed standardized measure designed to evaluate 3 to 12 months infants' temperament (Putnam et al., 2014). Items describing specific infants' behaviors are rated by the caregiver on a 7-point scale and evaluate three broad, empirically derived, temperamental dimensions, namely negative affectivity, positive affectivity/surgency, and orienting/regulatory capacity.

Infant mental and motor development was assessed through a well-standardized and widely employed developmental assessment, namely the Bayley Scales of Infant and Toddler Development – Third Edition (Bayley III, Bayley 2006), that was performed by a trained clinical psychologist blind to prenatal data, according to the standardized protocol. Age-standardized composite scores for the Cognitive and Motor scales were calculated by using test norms (mean= 100; SD= 15).

Infant behavioral stress reactivity. Infants' cortisol and behavioural response to the inoculation were evaluated during the routine first inoculation visit at 12 weeks of age (SD=1.84). Infants were administered two injections (mean duration=1.06 minutes, SD=00.30) in the thighs and infants' behaviour was videotaped from the undressing before the inoculation until 2 minutes after the last needle was retracted. Coding of infant behavioural distress began once the last needle was retracted for a 2 minutes period at 5-seconds intervals on a 4-point scale (Jahromi et al., 2004) ranging from 0 (no vocalization) to 3 (very intense loud crying with a out-of-control quality). The predominant (>2.5 seconds) level of intensity during each 5-seconds interval was scored. The scores were averaged across intervals to obtain an overall measure of intensity of distress, while a measure of overall distress duration was calculated by adding the number of intervals during which the infant was distressed (i.e. received a rating >0) and multiplying by 5. All videos were coded by a trained graduate student, blinded to all prenatal and postnatal study data. Twenty-one percent of the observations were coded by an independent trained coder. Intra-class correlation was

0.998 (p<.001). Data concerning the inoculation were available for 91 infants out of 104, as 2 infants' parents did not consent to the inoculation, while 11 infants were vaccinated in a different district from those involved in the project.

Cortisol collection and assay. Three infants' saliva samples were collected in the waiting room right before entering in the doctor room for the inoculation (baseline) and 20 and 40 minutes after the inoculation, through specifically designed swab (SalivaBio Infant's Swab, Salimetrics), and were stored at -80º until assayed for cortisol according to the same procedure described for maternal cortisol. With the exception of 1 infant who was fed 11 minutes before the first saliva collection, babies were not fed in the 30 minutes before the saliva collection (mean time from last feeding=102.99 minutes, SD=54.60). Complete cortisol data were available for 78 infants, while one or two samples were missing for 16 infants. Infants with complete or partial data did not differ on any sociodemographic/maternal or infants' variables or situational factors (i.e. time of the day, length of the inoculation, behavioral score). All samples from one infant were run in duplicates on the same assay, with the exception of 8 samples that were run in singlet due to minimal saliva volume. The average intra- and inter-assay coefficients of variance were below 7% and 10%, respectively.

6.3.5 Statistical analyses

Variables were preliminarily examined for outliers and skewness. Samples greater than 3 SD from the mean were removed (n=7 for prenatal cortisol, n=4 for sAA, n=3 for prenatal IL-6, n=4 for infants' cortisol) and variables were natural log (ln) transformed to approximate normal distributions. As described in the previous chapters, cortisol and sAA values at each time point were averaged across the 2 days and mean values were used to calculated four summaries measures of maternal cortisol and sAA diurnal functioning, specifically: awakening values, response to awakening (calculated by subtracting waking values from the 30min post-waking levels), diurnal slope (calculated by subtracting the bedtime values from the waking

values) and total diurnal output (calculated as the area under the curve (AUCg) using the trapezoid method with respect to the ground, Pruessner et al., 2003). The AUCg was calculated for each day separately and as the two values were highly correlated across the 2 days ($r=.57$, $p<.001$, for cortisol, while $r=.75$, $p<.001$ for sAA) the mean of the 2 days was used.

Preliminary Pearson bivariate correlations and univariate analysis of variance (ANOVA) were conducted to investigate the effect of maternal, infant or situational variables on infants' outcomes. Subsequent analyses were adjusted for those variables that emerged as significantly related to the dependent variables in these preliminary analyses.

Separate hierarchical regression analyses were performed to evaluate the impact of maternal EA on infant's outcomes (i.e. IBQ-R-VSF scales scores, Bayley III scales scores and behavioral intensity and duration of distress), while adjusting for covariates. Regarding the role of maternal EA in the association between antenatal maternal depression and infants' development, we first employed Pearson bivariate correlations to investigate the association between maternal prenatal (and postnatal) depressive symptoms and EA score. A full mediation model was tested only when significant associations were found between maternal depressive symptoms and EA score (the a-path of the mediation model; Baron & Kenny, 1986) and maternal depressive symptoms and infants' outcomes (the c-path of the mediation model; Baron & Kenny, 1986). Additional hierarchical regressions models were conducted to determine the interactive effects among maternal postnatal EA and prenatal variables on infant's outcomes. Covariates were entered in the first step. Prenatal maternal variables were entered in a second step jointly with maternal EA scores, while the interacting effect of maternal prenatal and postnatal variables was entered in the last step. Similarly, Hierarchical Linear Models (HLMs) were estimated to explore the independent influence of maternal EA as well as the interactive influence of antenatal and postnatal maternal variables on infants' cortisol response to the inoculation, while

accounting for the hierarchical structure of the data. HLMs were specified at two levels where individuals were level 2 and time was level 1. Time was centered at baseline so that the model intercept represents the mean cortisol level at baseline. Before fitting explanatory models including level-2 predictors, a baseline model of cortisol response was fitted, including linear and quadratic slopes for time. A random intercept and a random linear slope were included to allow between-person variability. The explanatory variables were centered around the grand mean and entered in the model one-by-one. Gender was centered at males. Model fit was tested with likelihood deviance difference test for nested models. Specifically, variables were kept in the model when their presence resulted in a significant ($p<.05$) reduction of the likelihood ratio statistic.

Maternal depression and anxiety both during pregnancy ($r=.66$, $p<.001$) and postnatally ($r=.58$, $p<.001$) were quite strongly inter-correlated, thus a supplementary set of analyses was conducted to evaluate the independent effects of maternal EA while controlling for maternal anxiety, rather than depression. Additionally, the interactive effect between maternal prenatal anxiety and postnatal EA was tested.

Statistical analyses were performed using SPSS 24 and MLWiN.

6.3.6 Covariates.

Several factors known to affect infants' psychomotor development, temperament or stress physiology, such as sociodemographic factors, health-related and factors related to the day of inoculation were examined as potential confounders of the association between maternal variables and infant's outcomes.

Maternal age was significantly associated with infants' 20-min post-stressor cortisol levels ($r=.22$, $p=.04$) and females showed marginally higher levels of cortisol at the 40-min post stressor collection as compared to males ($F(1, 81)=2.98$, $p=.08$). Thus, maternal age and infant gender were included as covariates in subsequent analyses on stress reactivity.

Both gestational age at birth and maternal IQ were significantly associated with infants' cognitive development (respectively, r=.23, p=.02 and r=.24, p=.01) and gestational age at birth was significantly related to infants' PAS scores (r=.28, p=.01). In addition, females tended to perform higher on the Motor scale, as compared to males (F(1, 101)=3.53, p=.06). Thus, gestational age, gender and maternal IQ (only for analyses on psychomotor development) were retained as covariate in the subsequent hierarchical linear regression models.

Lastly, maternal EPDS scores at 3 months were significantly related to infant's NA (r=.31, p=.003) and, marginally, with infants' 20-min post-stressor cortisol levels (r=-.20, p=.06). Thus, maternal postnatal depression was included as a possible confounder in all regression and multilevels models. All analyses were also performed without including postnatal depression and are reported in the supplementary section.

6.4 Results

6.4.1 Descriptive analyses

Descriptive statistics for study variables are presented in Table 6.1. Bivariate correlations showed no significant associations between maternal EA scores and prenatal or postnatal EPDS or STAI scores (range in Pearson rs=.07-13, all ps>.15), thus indicating that both antenatal and current depressive/anxiety symptoms were unrelated to maternal quality of caregiving, thus precluding further testing of mediation hypothesis (Baron & Kenny, 1986). However, there was a significant association between maternal EA and prenatal IL-6 levels (r=-.22, p=.04). Additionally, there was a significant association between higher maternal EA and both higher infants' cognitive and motor composite scores (respectively, r=.20, p=.04 and r=.28, p=.005), as well as between higher EA and lower intensity of infant's behavioural distress in response to the inoculation (r=-.22, p=.04). In contrast, infants' cortisol levels and temperamental traits were not significantly associated with maternal EA scales scores (all ps>.05).

Table 6.1 – Descriptive statistics for study variables at the prenatal and postnatal assessment

Study Variable	Mean	SD	Range
Prenatal			
Maternal cortisol (µg/dl)			
Waking	0.38	0.13	0.13-0.83
Waking +30’	0.50	0.15	0.10-0.91
Bedtime	0.18	0.06	0.01-0.41
Maternal sAA (U/ml)			
Waking	69.89	64.68	3.00-463.84
Waking +30’	48.33	38.65	2.80-190.10
Bedtime	97.90	80.39	3.28-562.71
Maternal CRP (ng/ml)	3749.60	2714.57	480.04-11179.80
Maternal IL-6 (pg/ml)	1.69	1.03	0.48-6.47
Maternal depression (EPDS)	5.34	4.33	0-19
Maternal anxiety (STAI-T)	36.32	9.07	21-63
Postnatal			
Cognitive composite score	102.36	10.81	75-125
Motor composite score	99.73	8.14	85-121
NA	3.46	0.88	1.58-5.42
PAS	3.87	0.76	2.30-5.54
ORC	5.60	0.57	4.30-6.75
Behavioral response to stress	1.39	0.78	0.25-3.00
Infants’ cortisol (µg/dl)			
Baseline	0.31	0.23	0.03-1.10
20-min post-stressor	0.78	0.35	0.01-1.86
40-min post-stressor	0.47	0.25	0.11-1.37
Maternal sensitivity	22.23	3.32	15-29
Maternal structuring	23.06	2.81	14-29
Maternal non-intrusiveness	22.09	4.07	13-29
Maternal non-hostility	26.28	1.92	21-29

6.4.2 Independent effects of maternal EA scores on infant's outcomes

As shown in Table 6.2, multiple hierarchical regression analyses revealed significant associations between maternal EA scores and infant motor development, after controlling for infant's gender, gestational age, maternal IQ, prenatal and postnatal depression. Specifically, higher maternal EA was significantly associated with higher infants' motor scores at 3 months (β=.27, t=2.77, p=.007), while the association between maternal EA scores and infant cognitive development was not significant (p=.085).

Table 6.2 – Hierarchical linear regression analyses for the independent effects of maternal EA on infants' cognitive and motor outcomes, adjusted for covariates

	Cognitive scores		**Motor scores**	
	β	p	β	p
Step 1				
Gestational Age	**.22**	**.03**	**.26**	**.01**
Maternal IQ	**.22**	**.03**	.01	.94
Gender	.13	.18	**.20**	**.05**
Prenatal EPDS	-.13	.29	.04	.75
Postnatal EPDS	.12	.31	.13	.19
ΔR^2 for step 1	.12	.02	.13	.03
F_{model}	2.68	.03	2.67	.03
Step 2				
Maternal EA	.17	.09	**.27**	**.01**
ΔR^2 for step2	.03	.09	.07	.01
F_{model}	2.78	.01	3.67	.003

Furthermore, as presented in Table 6.3, HLMs revealed a significant effect of maternal EA on both the linear and quadratic slopes of infants' cortisol response (both ps=.04), while controlling for relevant covariates. Specifically, as shown in Figure 6.1, lower maternal EA was associated with 3-month-olds' greater cortisol reactivity, as

indicated by a steeper linear and quadratic slope of cortisol response. However, maternal EA was not significantly associated with cortisol baseline levels (p>.05). The inclusion of maternal EA significantly improved the model fit over the baseline model (deviance difference (3)=8.1 p=.04).

Lastly, there were no significant associations between maternal EA scales scores and any temperamental dimension as well as infant intensity and duration of behavioral distress in response to the inoculation, after adjusting for covariates (all ps>.05).

Table 6.3 – HLM for the independent effects of maternal EA on infants' cortisol response, adjusted for covariates.

	Cortisol response	
	Estimate (SE)	p
Fixed effects		
Intercept	0.262 (0.022)	<.001
Maternal Age	0.005 (0.003)	.13
Prenatal EPDS	0.034 (0.021)	.11
Postnatal EPDS	-0.029 (0.020)	.15
Maternal EA	0.002 (0.005)	.79
Linear	0.021 (0.002)	<.001
Maternal EA	**-0.001 (0.000)**	**.04**
Quadratic	-0.000 (0.000)	<.001
Maternal EA	**0.000 (0.000)**	**.04**
Random effects		
Level 2 (individual)		
Intercept variance	0.009(0.004)	.05
Linear slope variance	0.000 (0.000)	.02
Intercept/Linear slope covariance	-0.000(0.000)	.19
Level 1 (occasions)		
Intercept variance	0.017(0.003)	<.001

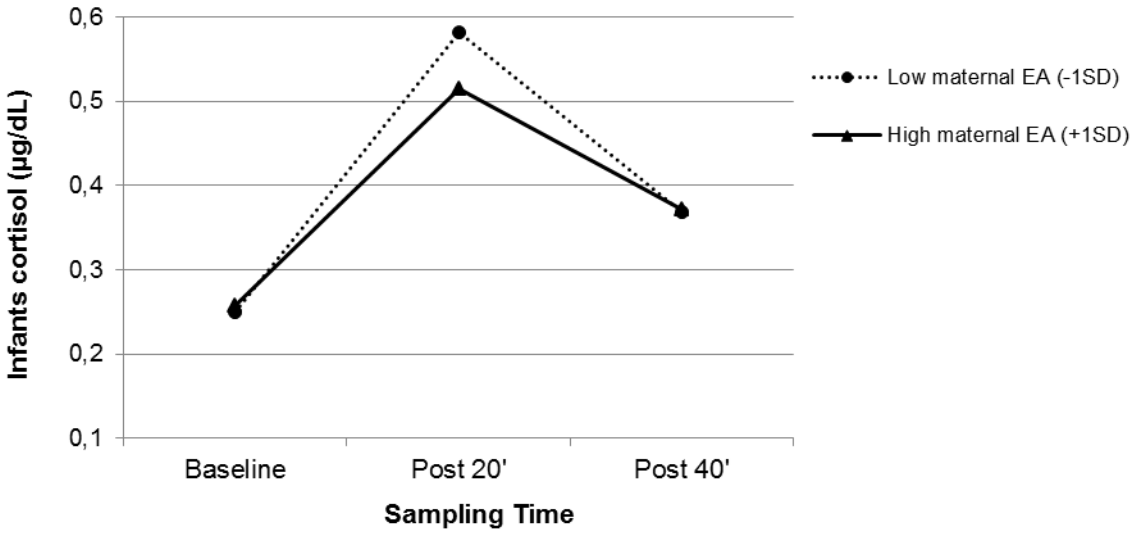

Figure 6.1 – Averaged cortisol values before and after the inoculation for infants of higher (+1 SD) and lower (-1SD) emotionally available mothers, after adjusting for covariates

6.4.3 Interactive effects between prenatal maternal variables and postnatal EA scores on infant's outcomes

In Chapter 5, the independent effects of antenatal maternal influences (i.e. depressive symptoms and biological markers) on infant psychomotor development, temperament and stress reactivity was evaluated in separate hierarchical regression or hierarchical linear models, as appropriate. We now extend those models to include both the independent effects of maternal EA and the interactive effects with the maternal prenatal variables above mentioned, in order to evaluate the moderating role of maternal postnatal EA in the association between maternal prenatal influences and infant outcomes, while controlling for possible confounders. Our interest was primarily in the interaction terms.

6.4.3.1 Motor and cognitive development

Hierarchical regression analyses showed no significant interaction between maternal prenatal variables (i.e. depressive symptoms, cortisol, sAA, CRP and IL-6)

and postnatal EA scores on infant cognitive and motor development, after adjusting for covariates. In contrast, as shown in Table 6.4, the main effects of prenatal maternal cortisol AUCg and CRP levels on cognitive development, already outlined in Chapter 5, remain significant even when maternal EA was included in the model.

Table 6.4 – Hierarchical linear regression analyses for the interactive effects between maternal postnatal EA and, respectively, prenatal cortisol and CRP levels on infants' cognitive scores.

	Cort AUCg		**CRP**	
	β	P	β	p
Step 1				
Gender	.15	.13	.10	.37
Gestational Age	**.21**	**.04**	**.20**	**.07**
Maternal IQ	**.22**	**.03**	**.17**	**.13**
Postnatal EPDS	.13	.27	.14	.29
Prenatal EPDS	-.14	.26	-.14	.31
ΔR^2 for step 1	**.13**	**.03**	.09	.15
F_{model}	**2.68**	**.03**	1.65	.15
Step 2				
Prenatal biological predictor	**-.37**	**<.001**	**-.21**	**.04**
Maternal EA	.16	.09	.21	.05
ΔR^2 for step 2	**.15**	**<.001**	**.11**	**.01**
F_{model}	**4.90**	**<.001**	**2.56**	**.02**
Step 3				
Maternal EA X Biological predictor	.09	.34	-.19	.10
ΔR^2 for step 3	.01	.34	.03	.08
F_{model}	**4.40**	**<.001**	**2.42**	**.02**

6.4.3.2 Temperament and behavioral distress in response to stress

None of the interaction coefficients between maternal prenatal variables and postnatal EA predicting temperamental scores was statistically significant (all ps>.05), after adjusting for infant's gender, gestational age and postnatal depression. In contrast, the main effects of maternal prenatal depressive symptoms and maternal sAA response to awakening on infants' NA scores, already reported in Chapter 5, remain significant after adjusting for postnatal EA. Specifically, higher depressive symptoms and greater morning sAA decline after awakening were associated with infants' higher scores on NA (respectively, β=.37, t= 2.97, p<.01 and β=-.31, t= -3.14, p<.01).

Similarly, there were no significant interaction between maternal prenatal variables and postnatal EA scores in predicting infant's intensity and duration of behavioral distress in response to the inoculation, after controlling for infant's gender, maternal age and postnatal depression.

6.4.3.3 Cortisol response to stress

As shown in Table 6.5, there was a significant three-way interaction among maternal prenatal cortisol AUCg, maternal EA at 3 months and both the linear and quadratic slopes of time (p<.05) on infants' cortisol levels, after controlling for maternal age, infant's gender, postnatal and prenatal depression. Specifically, as illustrated in Figure 6.2, at higher levels of maternal prenatal cortisol (+1SD), there was a significant association between maternal EA and infant's cortisol reactivity (for both linear and quadratic slopes p<.01), with lower levels of maternal EA being associated with greater cortisol reactivity. In addition, at higher level of maternal EA (+1SD), there were no differences in infants' cortisol response to the inoculation depending on levels of prenatal maternal cortisol (p=.26), while at lower levels of maternal EA (-1SD), infants prenatally exposed to higher maternal cortisol levels showed a significantly steeper linear and quadratic slopes (both ps<.05). The overall improvement of the

model fit over the baseline model was significant (deviance difference (9)=17.24, p=.05).

Table 6.5– Full prediction model for the independent and interactive effects of maternal postnatal EA and prenatal cortisol AUCg on infants' cortisol response

	Cortisol Response	
	Estimate (SE)	p
Fixed effects		
Intercept	0.268 (0.023)	<.001
Maternal Age	0.005 (0.003)	0.18
Postnatal EPDS	-0.040 (0.021)	0.06
Prenatal EPDS	**0.046 (0.023)**	**0.04**
Maternal EA	0.000 (0.005)	0.98
Prenatal Cortisol AUCg	-0.000 (0.000)	0.15
Prenatal Cortisol AUCg X Maternal EA	0.000 (0.000)	0.80
Linear	0.027 (0.023)	0.07
Maternal EA	0.003 (0.002)	0.15
Prenatal Cortisol AUCg	0.000 (0.000)	0.19
Prenatal Cortisol AUCg X Maternal EA	**-0.000 (0.000)**	**0.04**
Quadratic	-0.000 (0.000)	0.07
Maternal EA	-0.000 (0.000)	0.15
Prenatal Cortisol AUCg	-0.000 (0.000)	0.27
Prenatal Cortisol AUCg X Maternal EA	**0.000 (0.000)**	**0.05**
Random effects		
Level 2 (individual)		
Intercept variance	0.009(0.004)	0.04
Linear slope variance	0.000(0.000)	0.01
Intercept/Linear slope covariance	-0.000(0.000)	0.15
Level 1 (occasions)		
Intercept variance	0.016(0.003)	<.001

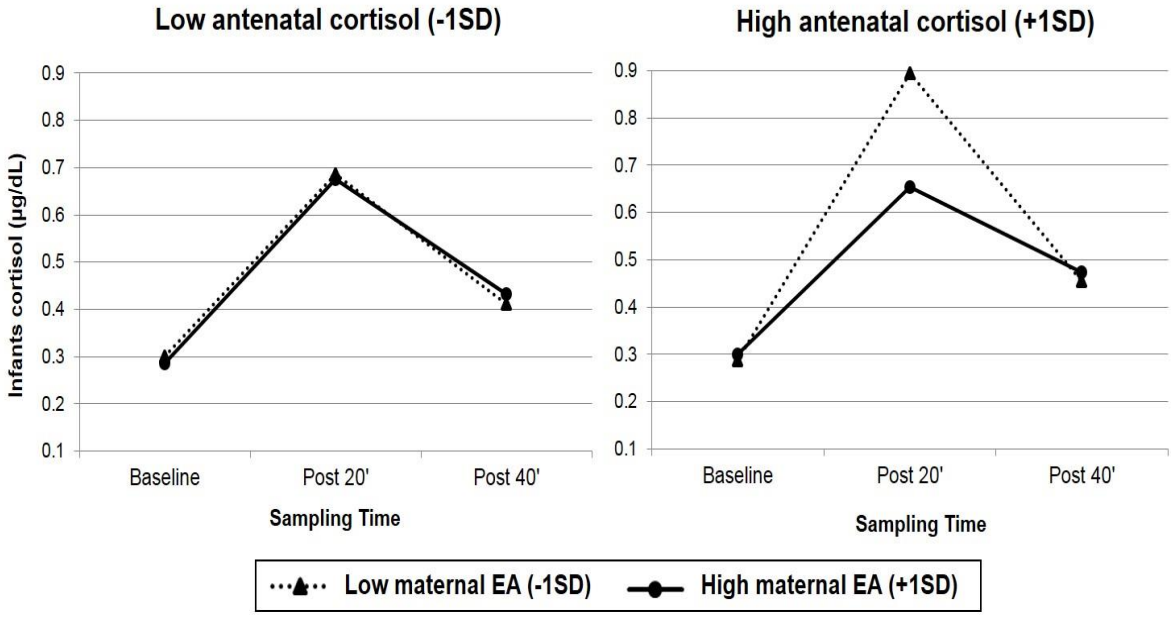

Figure 6.2 – Averaged cortisol values before and after the inoculation for infants of higher (+1 SD) and lower (-1SD) emotionally available mothers jointly with higher (+1 SD) and lower (-1SD) maternal antenatal cortisol, after adjusting for covariates.

No significant interaction between postnatal EA scores and all other prenatal variables (i.e. maternal depressive symptoms, sAA, IL-6 and CRP levels) on infant cortisol response was found. Additionally, the effects of maternal prenatal variables, including maternal depressive symptoms, on infant cortisol reactivity, were no longer significant once maternal EA scores were included in the model.

6.4.4 Supplementary analyses

A supplementary set of analyses was run to evaluate the effects of maternal EA on infants' outcomes while controlling for maternal anxiety, rather than depression. The analyses yielded comparable results with maternal EA remaining a significant predictor of infants' motor outcomes (β=.26, t=2.74, p=.007) and cortisol response to the inoculation (for both linear and quadratic slopes, ps=.04), while no significant associations between maternal EA and infants' temperamental or behavioral stress

scores were found (all ps>.05). Similarly, the three-way interaction among maternal prenatal cortisol AUCg, maternal EA at 3 months and both the linear and quadratic slopes of time on infants' cortisol levels remain significant (both p=.05), after controlling for maternal anxiety rather than depression. Lastly, no significant interaction between maternal prenatal anxiety and postnatal EA on any outcomes examined (all ps>.05) was found.

Furthermore, as multicollinearity could be an issue, all models were re-run without including postnatal depression as a covariate. Results showed that adjusting for maternal depressive symptoms at 3 months did not affect the statistical significance of the associations between maternal EA and infants' outcomes previously described. The only exception concerns the association between maternal EA and infants' cognitive scores that reached the statistical significance (β=.20, t=2.07, p=.04) once maternal postnatal depression was excluded from the model.

6.5 Discussion

The current study was aimed at investigating the effects of maternal postnatal caregiving on infants' early bio-behavioral development, as well as the possible role of variations in early maternal care in the association among maternal prenatal influences (i.e. depressive symptoms and stress-related biology) and 3-month-olds outcomes. As expected, maternal Emotional Availability (EA) emerged as a significant predictor of infant's motor development and stress reactivity at 3 months of age. However, contrary to our predictions, maternal EA was not associated with infants' early cognitive development and temperament. Concerning the role of maternal EA in the associations between prenatal maternal influences and infants' neurodevelopment, no support for a mediating role of maternal caregiving in the observed associations was found. In contrast, the current study provided novel evidence for a role of maternal EA in moderating the association between prenatal maternal cortisol and cortisol stress

reactivity in a low risk sample of healthy women and infants. These results were independent of prenatal and postnatal depressive or anxiety symptoms.

6.5.1 Psychomotor development

The current findings provide partial support for the hypothesized association between maternal postnatal EA and infant's psychomotor development in healthy full-term 3-month-old babies. As predicted, higher maternal EA was associated with enhanced motor development and the association remained significant when controlling for infant's sex, gestational age, maternal IQ, prenatal and postnatal maternal depression. In contrast, the association between maternal sensitivity and 3-month-olds' cognitive performance, although in the expected direction, did not reach the statistical significance when the model was adjusted for postnatal maternal depression.

The fundamental role of the caregiver in supporting infant development is one of the central tenets of attachment theory and empirical evidence suggests that maternal sensitivity, defined as the ability to respond in a timely and appropriate manner to the child's signals, is a robust predictor of several developmental outcomes (e.g. Kok et al., 2013; Leigh, Nievar, & Nathans, 2011; Verhage et al., 2016). However, the role of parenting in fostering motor development has been less investigated. In particular, while a number of studies examined the role of parenting on motor development in samples at risk for poor developmental outcomes, such as preterm or very-low-birth-weight infants (Forcada-Guex et al., 2006; Grunau, 2009; Sansavini et al., 2015; Treyvaud et al., 2009), studies on normative samples of typically developing children are lacking. To our knowledge, our study is among the first to report an association between maternal postnatal EA and early infant's motor development in a sample of healthy full-term babies. This finding is in line with accumulating evidence showing that even normal variation in the quality of mother-infant relationship might influence infant brain development (e.g. Rayson et al., 2017; Taylor-Colls & Pasco

Fearon, 2015) and it is particularly relevant because, across the first year of life, motor development play a crucial role in fostering babies' daily interactions and directly influence all other domains of development, with important implications for the child's overall functioning (Karasik, Tamis-LeMonda, & Adolph, 2011; Leonard & Hill, 2014). However, it is important to note that the correlational nature of these findings, does not allow to establish causality and direction of this relationship. In particular, it has been suggested that children's characteristics may affect maternal styles of interaction (Sameroff & Fiese, 2000). For example, a child who has developmental delays, including in the motor domain which play a fundamental role in sustaining mother-child interactions at the very early stages of life, may discourage maternal attempts to initiate interaction or compromise maternal interactional style as a reaction to her child's abilities, resulting in less optimal mother-child interaction (Wheeler, Hatton, Reichardt, & Bailey, 2007). Nonetheless, it is noteworthy that the present result is in line with experimental studies showing an effect of interventions aimed at promoting sensitive and supportive parenting in improving several domains of children's development (Landry, Smith, & Swank, 2006; Morrison et al., 2014), including the motor domain (e.g. Natrasony & Teitelbaum, 2016), as well as with studies on adopted children showing that foster-reared children showed less mental and motor delay in the first year of life as compared to orphanage-reared children (Miller et al., 2005). Additionally, while it has been shown that the development of reflexes mainly depends on a neurophysiological maturation of the central nervous system, reach-to-grasp movements, shared attention for an object, as well as gross-motor milestones are strongly influenced by environmental differences in stimulations, such as those provided by the caregiver (Zoia et al., 2013). Thus, we might hypothesize that highly emotionally available mothers provide an optimal stimulating and positive interactive environment that facilitates infants' physical exploration of the surroundings and engagement in rewarding exchanges that could foster infant's motor development from the very beginning of life. As consistent inter-individual variability in the acquisition of

motor milestones has been reported (Camaioni & Di Blasio, 2002), it would be important to evaluate whether the observed association would persist later in development and whether maternal EA at 3 months of age might predict later developmental trajectories.

The lack of a significant association between maternal EA and 3-month-olds cognitive performance is in contrast with our expectations and with previous findings (e.g. Austin et al., 2017; Firk et al., 2018; Mills-Koonce et al., 2015; van Bakel & Riksen-Walraven, 2002). We might hypothesize that this reflects a lack of power and that a larger sample size would have the statistical power to detect smaller effects than the current study was able to (and indeed the association was significant in the non-adjusted model). However, it is also possible that the influence of maternal EA we reported on motor outcomes might be observed on cognitive performance as the children age. In line with this hypothesis, Mills-Koonce and colleagues (2015) reported modest correlations between sensitive parenting at 6 months and concurrent infants' cognitive ability, but larger correlations at 24 months. Similarly, Firk and colleagues (2018) found a significant association between maternal sensitivity and cognitive development at 21 months, but not at 5 or 14 months and Grunau and colleagues (2010) reported a significant effect of parenting on cognitive development at 18 months but not at 8 months in a sample of preterm infants. This is also in line with research on developmental risk factors which show that cognitive delays among high-risk children are not apparent until late toddlerhood (Black, Hess, & Berenson-Howard, 2000; Mackner, Black, & Starr, 2003). Thus, we might speculate that different sensitive windows of higher plasticity to environmental influences might underline the development in motor and cognitive development, with the quality of postnatal rearing experience appearing to play a greater role in the gradual acquisition of motor skills rather than in the cognitive domain at 3 months of age. A follow-up assessment at older ages when cognitive and motor skills are more developed and differentiated might revealed different findings.

Furthermore, differently from our initial hypothesis, we did not find any evidence for a moderating effect of maternal EA in the link between maternal prenatal factors and infants' psychomotor development. In contrast, the negative association between infant's cognitive outcomes and both maternal prenatal cortisol and CRP levels remain significant even after the inclusion of postnatal maternal EA. This result is in contrast with findings from Bergman and colleagues (2010) which reported a significant moderating effect of child-mother attachment in the association between prenatal cortisol exposure and 17-month-old infant's cognitive development, with higher prenatal maternal cortisol being significantly associated with poorer cognitive outcomes only in insecurely-attached children. Replication of these results in different and larger samples are needed before interpretation of these findings can be made. However, considerable methodological differences, particularly in the assessment of the quality of mother-child interaction and, importantly, in child's age might explain the lack of consistency. For example, it has been hypothesized that cognitive development in the first stages of life appears much more biologically determined and less permeable to postnatal environmental influences, while during the second year of life children appear to benefit more from enriching environments (Black et al., 2000). However, findings are only preliminary and further research is needed to understand how early caregiving experiences may modify the bio-behavioral outcomes associated with prenatal stress exposure.

Lastly, it is noteworthy that no associations between maternal EA scores and either prenatal or postnatal depression and anxiety symptoms were found in the current study. As mothers in the study were a community low-risk sample, with high levels of education and socio-economic status, the lack of important differences in maternal EA between mother with higher and lower prenatal depressive symptoms is not totally unexpected (e.g. De Weerth et al., 2003; Endendjik et al., 2005). Indeed, while the use of a low-risk sample has the potential to limit confounding influences of additional risk factors associated with psychosocial adversity (e.g. teenage

motherhood, unemployment, financial problems etc.), it is possible that this may have constrained the range in maternal EA. In line with this hypothesis, reduced maternal EA has been found in depressed mothers (e.g. Easterbrooks, Bureau & Lyons-Ruth, 2012; Trapolini et al., 2008; Easterbrook, Biesecker, & LyonsRuth, 2000) and in mothers with depression in remission (Kluczniok et al., 2016) from high-risk samples such as low-income mothers (e.g. Easterbrooks 2000; 2012) or mothers being referred to parent-craft centre for support after the delivery (e.g. Trapolini et al., 2008). In contrast, studies on low-risk samples, such as work from Endendijk and colleagues (2005), reported no impact of maternal depressive symptomatology on EA scores and failed to support a mediation role of maternal parenting in the link between antenatal depression/anxiety and child outcomes, consistently with the current findings. Interestingly, aspects of maternal prenatal physiology related to inflammation, as indexed by higher IL-6 levels, were weakly associated with reduced postnatal maternal EA. Despite these findings require further replication in larger samples, they are in line with several animal studies that showed less optimal mothering behaviors in prenatally stressed dams as compared to controls (Bosch et al., 2007; Meek et al., 2001; Pardon et al., 2000; Patin et al., 2002) leading to the hypothesis that alterations in maternal behaviors might explain, at least partially, the adverse outcomes in pups born from prenatally stressed dams. However, it is important to note that in the current study no significant associations between maternal IL-6 levels during pregnancy and infants' neurodevelopmental outcomes at 3 months of age were found, thus precluding further testing of a mediation model. Additionally, the association between maternal CRP levels and infants' cognitive development remains significant after controlling for postnatal EA. Thus, we found no support for a maternal EA-mediated pathway from maternal inflammation to adverse child outcomes.

6.5.2 Temperament

Contrary to our initial hypothesis, we found no evidence for an association between maternal EA and early infants' temperamental traits, as well as no significant interactive effects between prenatal and postnatal maternal variables in shaping 3-month-olds' temperament, after adjusting for covariates.

Traditionally, temperamental traits are believed to be relatively stable individual differences in the domains of affectivity, activity, attention levels and self-regulation (Shiner et al., 2012), that are biologically driven and become more open to environmental influences later in development. The lack of an association between maternal EA and infants' temperament, at least at 3 months of age, is consistent with this view and it is in line with few available studies reporting no associations between maternal sensitivity and infants' early temperamental traits (e.g. Kaplan et al., 2009; Thomas et al., 2017). Similarly, significant association between maternal sensitivity and maternal reports of infants' temperament at 9-12 month of age, but not at a younger age, has been reported (Kivijärvi et al., 2005; Seifer et al., 1996). We might speculate that, while early temperamental differences are rather independent of the quality of postnatal maternal care, they might be open to postnatal shaping later across the first year of age. Alternatively, methodological issues related to the sample size or the use of maternal reports of infant's temperament rather than observative measures might explain the null findings.

In addition, maternal EA was not found to moderate the link between prenatal maternal influences and infant's temperament. In contrast, both antenatal maternal depression and sAA response to awakening were observed to contribute in a unique and independent way to predict infants' negative affectivity scores, after adjusting for confounders and for maternal EA. This is in line with findings from Kaplan and colleagues (2009) that did not observe any significant effect of maternal sensitivity in moderating the link between prenatal psychiatric diagnosis and 4-month-olds' temperament. In contrast, Bergman and colleagues (2008) showed that Insecure-

Ambivalent mother-child attachment, accentuated the link between antenatal stress and 17-month-olds' fearfulness. However, methodological differences in the age range and in the assessment of temperament (i.e. self-reports versus observation) and quality of care (i.e. maternal EA versus attachment) makes difficult to compare results. Despite further replication is needed before the significance of the current results can be discussed, we might hypothesize that the interactive effects between prenatal and postnatal maternal factors on infant temperament might be delayed in its effects and be revealed later in development. Additionally, it is also possible that factors that were not examined nor controlled as part of the current investigation may play a more critical role in shaping infants' temperament, such as genetics. There is growing evidence for the influence of specific polymorphisms on individual differences in temperament which suggest that temperament should be considered, at least partially, under genetic control (reviewed in Papageorgiou & Ronald, 2013). We might hypothesize that pleiotropic genetic effects might account for the observed association between prenatal maternal factors and infant's negative affectivity. Future studies are needed to elucidate the complex interplay among genetic, prenatal and postnatal environmental influences that are likely to determine the final outcome.

6.5.3 Stress reactivity

Although all infants, as a whole, displayed the expected cortisol response to the inoculation procedure (Gunnar et al., 2009), maternal EA appeared as a significant independent predictor of infant's cortisol reactivity at 3 months of age. In line with our predictions, we found that infants of less emotionally available mothers showed a greater cortisol response to the immunization as compared to infants of more sensitive mothers. In contrast, no effects of maternal EA on infant's intensity and duration of behavioral distress was found. Furthermore, we provided novel evidence for an interactive effect between maternal prenatal cortisol and postnatal EA on infants' cortisol response to the inoculation, suggesting an interplay between prenatal and

postnatal environmental factors in shaping individual variability in stress reactivity with important theoretical and clinical implications.

The association between higher maternal EA and lower infant cortisol reactivity is in line with our hypothesis and consistent with previous work both in animals and humans showing that sensitive and responsive caregivers are able to buffer the physiological stress response (e.g. Grant et al., 2009; Gunnar et al., 1996; Levine, 1994; Nachmias et al.,1996; Suomi, 1991; Spangler & Grossman, 1999), reducing activity of stress-mediating biological systems, including the HPA axis. In particular, while during the first 3 months of life the HPA axis is highly reactive to a variety of stressors (Gunnar, Talge, & Herrera, 2009), it has been shown that it is also during this period that maternal caregiving behaviors appear to shape infants' developing stress regulation (e.g. Albers et al., 2008) and, by the end of the first year of life, a secure mother-child attachment relationship becomes a powerful stress-buffering agent (reviewed in Gunnar, 2017). The mechanisms underlying parental stress buffering during infancy are still to be fully elucidated and include the possibility that early in development the presence of a sensitive caregiver directly controls regions that regulate stress such as the hypothalamus to prevent the beginning of the HPA-axis hormonal cascade (Gunnar & Hostinar, 2015) or stimulates oxytocin release which has inhibitory effects on the HPA-axis (Heinrichs et al., 2003; Hostinar, Sullivan, & Gunnar, 2014). We might speculate that in infants of less emotionally available mothers, the parental stress buffering is less effective and, although the mother is present, these children display stronger elevations in cortisol in response to stress as a result. As impaired stress buffering is considered one possible mechanism through which early adverse experiences "get under the skin" and influence stress-related physiology (Hostinar, Sullivan, & Gunnar, 2014), the current findings could indicate one way in which very early experiences with the caregiver might shape the developing stress response systems with possible long-term implications.

However, because the current findings are correlational, it is difficult to establish the direction of the influence between infants' reactivity and maternal sensitivity. For example, we might hypothesize that more "reactive" infants or infants who get more "easily" stressed are also infants who might challenge the ability of a caregiver to remain emotionally available. However, it is important to note that maternal behaviors and infants' stress response were observed during different situations and, in particular, maternal EA was coded during a free-play session which should be a less challenging and more usual situation for a caregiver to handle as compared to the inoculation procedure. Additionally, as discussed in the previous paragraph, we found no evidence for an association between maternal reports of infants' temperament and EA scores, in line with previous reports (e.g. Kaplan, Evans, & Monk, 2008; Hane & Fox, 2006). Thus, the alternative hypothesis that infant's characteristics, such as a difficult temperament, constrains the quality of mothers' caregiving seems less likely in the current sample.

Alternatively, maternal EA might influence maternal soothing behaviors during the inoculation procedure which, in turn, might influence infants' stress reactivity. However, it is noteworthy that we did not report any significant association between maternal EA and infants' behavioral intensity and duration of distress to the inoculation, whereas maternal soothing behaviors are usually found to predict infants' behavioral response to the vaccination (e.g. Cohen, 2002; Jahromi, Putnam, & Stifter, 2004). On the one hand, it is possible that methodological reasons, including the lack of a baseline period of behavioral observation, might have limited our ability to capture the trajectory of infant's behavioral response to stress and possible associations with maternal EA. Nonetheless, the current results are in line with previous reports suggesting a dissociation between the physiological and behavioral levels of response to stress related to the sensitivity of a caregiver. In particular, it has been shown that while a sensitive caregiver is able to buffer the infant's HPA axis, this does not prevent the child from showing elevations in levels of behavioral distress in response to a

stressor (Gunnar et al., 1996; Nachmias et al., 1996). This dissociation is thought to have a functional role as it allows infants to experience and communicate distress to caregivers in a way that elicit help and care, without experiencing concurrent possible detrimental elevations in cortisol levels (Gunnar et al., 2015).

Lastly, we cannot rule out unmeasured confounding or third variables that might influence the observed association between maternal EA and infants' cortisol reactivity, including pleiotropic effects whereby shared genetic factors influences both maternal sensitivity and infants' stress reactivity.

Interestingly, in line with our exploratory hypothesis, the quality of maternal caregiving emerged as a significant moderator of the association between prenatal maternal cortisol and infant's cortisol reactivity. Specifically, while maternal prenatal cortisol diurnal levels alone were not associated with 3-month-olds stress reactivity, a different picture appeared when the interactive effects between maternal prenatal cortisol levels and postnatal EA were examined. In particular, the magnitude of the association between maternal parenting behaviors and cortisol reactivity depended upon maternal prenatal cortisol levels, so that maternal EA was significantly related to infants' cortisol response only at higher levels of maternal prenatal cortisol. In addition, high maternal EA buffered the negative association between prenatal maternal cortisol and infant's cortisol reactivity. Specifically, while we reported a significant association between maternal antenatal cortisol and greater cortisol response to the inoculation among infants of less emotionally available mothers, the association became non-significant among infants of highly sensitive mothers.

Despite increasing evidence for a role of a sensitive caregiver as an early external regulator of the offspring's HPA axis and, in parallel, mounting data on the effects of prenatal maternal cortisol in shaping the development of fetal stress physiology, the extent to which maternal prenatal cortisol and postnatal care have interactive organizational effects on the offspring's HPA axis reactivity in humans remains unclear. The current study is among the first to suggest that the processes

leading to associations between prenatal maternal cortisol and offspring's stress reactivity might be over–ridden by postnatal environmental factors such as high levels of maternal EA in a low risk sample. Further replication in larger and different sample is needed. However, our findings are consistent with evidence from animal models (e.g. Francis et al., 2003; Maccari et al., 1995; Sanchez, 2006; Sapolsky, 1997; Wakshlak & Weinstock, 1990) and with initial evidence in humans suggesting an interplay between prenatal stress influences and postnatal maternal sensitivity in shaping offspring's stress-related physiology. In particular, Kaplan and colleagues (2008) reported that maternal sensitivity moderates the impact of prenatal psychiatric diagnosis on 4-month-olds' resting cortisol levels, with infants of women with an antenatal diagnosis showing higher cortisol levels only if they received less sensitive parenting. However, although infant cortisol was measured in a laboratory session, it was assessed only once and not in response to a stressor, thus can only partially inform about the interactive influence of prenatal and postnatal maternal factors on infants' stress reactivity. In contrast, in the study by Grant and colleagues (2009) maternal sensitivity was not found to moderate the association between prenatal maternal anxiety and infant's cortisol reactivity to the still-face procedure at 7 months of age. Instead, prenatal anxiety and maternal sensitivity were found to contribute independently and in an additive manner to infant cortisol response. However, none of these studies included measures of maternal cortisol during pregnancy which is thought to represent one of the primaries in utero mediating mechanisms of programming of fetal stress reactivity. Although we did not report a main effect for prenatal maternal cortisol on 3-month-olds cortisol reactivity, the significant interaction we observed among prenatal cortisol and postnatal EA is consistent with the growing body of evidence suggesting that the combination of multiple factors, rather than a single one, is more likely to be involved in determining psychobiological developmental trajectories (e.g., Appleyard et al., 2005). It could be speculated that poor parenting, as indicated by low maternal EA, might exacerbate the negative association between

maternal prenatal cortisol and infants' stress reactivity, leading to greater cortisol reactivity to the stress of inoculation in infants prenatally exposed to higher levels of cortisol. This is in line with a "multiple hit" or "cumulative stress" hypothesis suggesting that adverse experience during sensitive periods early in life might predispose individuals to be more vulnerable to aversive challenges later in life in a cumulative manner (Nederhof & Schmidt, 2012).

Interestingly, the magnitude of the link between maternal EA and infants' cortisol reactivity that we observed in the whole sample, was found to depend upon the levels of maternal diurnal cortisol during pregnancy with a significant association between variations in maternal EA and stress reactivity reported only among infants prenatally exposed to higher levels of maternal cortisol. These novel findings are in line with the notion that individuals might differ in their susceptibility to environmental influences that has been proposed by several theoretical models such as the diathesis–stress (Monroe & Simons, 1991; Zuckerman, 1999) or dual-risk models (Sameroff, 1983), Belsky's (2005; 1997), differential susceptibility hypothesis and Boyce & Ellis (2005) biological sensitivity to context. Further research is needed to elucidate the underlying mechanisms, however, it could be speculated that infants of prenatally "stressed" women might be particularly affected by variations in maternal care because of a genetic predisposition (e.g. Belsky & Pluess, 2009). Or, we might hypothesize that the exposure to an altered intrauterine environment characterized by higher levels of maternal cortisol, could increase the sensitivity of the developing individual to environmental influences and make those infants more vulnerable to the effects of variations in postnatal environment, in line with the hypothesis that prenatal experience could programme humans postnatal plasticity and adaptation to environmental factors such as rearing conditions (Pluess & Belsky, 2011).

6.5.4 Limitations

The current findings should be regarded in light of several limitations. First, the moderate sample size might have limited our statistical power to detect significant interactions. Second, besides maternal EA, additional postnatal environmental factors may moderate the association between maternal prenatal stress and differences in psychomotor development, temperament and stress regulation, which were not addressed in the current study. For example, factors related to paternal caregiving which have been shown to buffer negative effects of maternal interactive behaviors (Malmberg et al., 2016; Mills-Koonce et al., 2015). Similarly, alternative explanations, including genetics, need to be considered when interpreting the current findings and, equally important, the observational nature of the design does not allow to establish causality or directions of the observed associations. Third, as the current sample consists of middle-class, well-educated women, future studies should examine whether the current findings generalize to mothers from more disadvantaged socio-economic samples or high-risk samples. For example, the lack of variance in demographics as well as the low rate of women at risk for depression might have constrained the range in maternal EA scores and explain, at least in part, the lack of association between maternal symptoms of distress and EA scores which is often found (e.g. Easterbrooks, Bureau & Lyons Ruth, 2012). Fourth, it is possible that the early assessment of healthy full-term infants at 3 months of age might have limited the variability in outcomes such as cognitive scores or temperamental traits thus making difficult to detect significant effects that might emerged later in development. It is our aim to follow up the current cohort of infants to evaluate whether maternal EA, both alone and in interaction with prenatal variables, might predict infants' developmental trajectories.

6.6 Conclusions

The current findings extend animal research into the biological impact of early maternal care by suggesting that naturally occurring variations in maternal EA are significantly associated with individual differences in early motor development and stress regulation in a low risk sample. In particular, data seems to indicate that highly emotionally available mothers, characterized by a sensitive, structuring, non-intrusive, and non-hostile style of caregiving, provide an optimal context for early brain maturation, fostering motor development and contributing to the shaping of physiologic regulation to future stressors.

Furthermore, the current findings raise an important theoretical and methodological issue, indicating that research into biological mechanisms involved in programming of bio-behavioral development needs to account not only for prenatal effects but also for the influence of early caregiving experiences, as variations in postnatal care may alter the unfolding of prenatal programming. In particular, our results suggest that the vulnerability related to exposure to higher prenatal maternal cortisol, either mediated by genetic factors or by in utero mechanisms, might be opened to postnatal shaping by maternal EA, thus supporting the need of a comprehensive model of early-life programming.

Equally important, from a clinical perspective, the data highlights the importance of maternal EA, and especially in situations of high stress in pregnancy, for later development in young children. Findings suggest that infants exposed to higher levels of prenatal maternal cortisol might benefit more from high maternal EA to support the development of their stress regulation abilities. Thus, postnatal interventions aimed at enhancing maternal EA might be particularly beneficial for women experiencing higher stress levels during pregnancy in order to improve their children's developmental outcomes.

Future studies addressing the moderating and mediating influences of postnatal maternal caregiving in the link between antenatal adversity and child

outcomes are expected to provide insights into the mechanisms through which antenatal exposure to maternal depression might affect children outcomes, as well as contribute to identify potential targets for interventions through which the long-term consequences of fetal adversity can be attenuated.

Chapter 7: General Discussion

The developmental roots of mental illness can often be traced to early life (Grossman et al., 2003; Gunnar & Quevedo, 2007; Schlotz & Phillips, 2009), yet it is still unclear how early environmental exposures influence later health outcomes and susceptibility to disease. The prenatal period is a time of exceptionally rapid growth and brain development including neurogenesis, neuronal differentiation, synaptogenesis and myelination (Grossman et al., 2003), which make the fetus particularly susceptible to environmental influences with potentially long-lasting consequences (Gluckman et al., 2007). In the past decades, a tremendous amount has been learned about the organizational influence of maternal antenatal stress on the developing fetus, yet there is still a great deal that is not understood.

The current thesis builds on and extends research into the effects of antenatal stress in humans by adopting a multi-systems approach, that involves the assessment of different indices of maternal antenatal stress (i.e. depressive/anxiety symptoms, stress hormones and inflammatory markers), and by taking the role of postnatal maternal care into account. We used a prospective longitudinal design and demonstrated that antenatal depressive symptoms, but not anxiety, were associated with an altered stress-related physiology (i.e. diurnal cortisol and inflammation) in late pregnancy and independently predicted higher infant negative affectivity (NA) at three months of age. In addition, we provided evidence that variations in maternal stress-related biology during pregnancy were independently associated with physiological and behavioural outcomes in offspring. Lastly, we found the first evidence in humans that the impact of antenatal maternal cortisol on infant cortisol reactivity to stress may be moderated by the quality of the mother-infant relationship. Our results are in keeping with the hypothesis that maternal antenatal stress signals can exert a programming effect on offspring's bio-behavioral development and that the impact is

partially dependent on the quality of the early rearing environment. This chapter describes and discusses the main findings, reviews limitations of the studies included in the current dissertation and puts forward suggestions for future research.

7.1 Main findings

7.1.1 Maternal stress-related biology and depressive symptoms during the perinatal period

In line with our predictions, higher depressive symptoms during pregnancy were associated with higher Interleukine-6 (IL-6) levels, and an altered cortisol diurnal pattern, as indexed by lower morning cortisol levels (i.e. at waking and 30 minutes after) and a flatter diurnal decline, while adjusting for potential confounders. In contrast with our initial hypothesis, however, no significant associations were found in the early postpartum period. Furthermore, exploratory analyses indicate that anxiety, but not depressive, symptoms were associated with an altered sAA diurnal profile and that the interplay between inflammatory and stress markers during pregnancy might be different in women with higher depressive symptoms.

Evidence exists of an altered diurnal cortisol pattern in clinically depressed (O'Connor et al., 2014) or minority high-stressed (Suglia et al., 2010) pregnant women; likewise, an association between depressive symptoms and elevations in circulating IL-6 levels has been reported in samples of low-income high-psychosocial risk (Cassidy-Bushrow et al., 2012; Christian et al., 2009) and clinically depressed (Haeri et al., 2013; S. Osborne et al., 2018b) pregnant women. The current findings support and extend those studies by showing that antenatal depressive symptoms, even without meeting the full criteria for a clinical diagnosis, are associated with an altered stress-related physiology in a low-risk community sample of healthy pregnant women. These results converge with evidence of depression as a dimensional "trait" in the general population, rather than a discrete entity, with a continuous measure of risk

underlying the clinical phenotype (e.g. Wray et al., 2018). It is important to note that composite measures of diurnal cortisol, such as the overall diurnal cortisol output, as indexed by the Area Under the Curve (AUCg), were not significantly associated with depressive symptoms. One methodological implication is that a detailed characterization of the diurnal pattern, both through multiple sampling and sophisticated analytical techniques such as hierarchical linear models (HLMs), is needed in order to detect reliable associations between psychosocial stress measures and subtle disturbances in diurnal cortisol profile that would be undetectable otherwise, especially in late pregnancy and in non-clinical low risk sample. Indeed, as cortisol levels increase (e.g. Allolio et al., 1990) and maternal HPA-axis becomes gradually less responsive to stress as pregnancy advances (Kammerer et al., 2002), the association between maternal distress and cortisol might become more difficult to detect; this might be particularly true in low risk samples not exposed to excessive stressors (O' Connor et al., 2014).

The pathways underlying the observed associations are still to be understood. First of all, it has been suggested that depression and either inflammation or cortisol dysregulation share a common genetic background. In particular, genetic variants related to increased immune responses have been more frequently found in depressed patients (reviewed in Barnes, Mondelli, & Pariante, 2017). Similarly, glucocorticoid receptor polymorphisms were found to be associated both with an altered HPA axis function, with the occurrence of depression and response to antidepressants treatment (reviewed in Spijker & van Rossum, 2009).

Secondly, both the HPA axis and the Inflammatory Response System (IRS) have been shown to be extremely responsive to environmental adversities (Baumeister, Lightman, & Pariante, 2014) and it has been proposed that early adverse experiences, including intra-uterine ones, might affect their functioning and confer vulnerability for the development of mental health problems later in life (Pariante et al., 2017). For example, childhood trauma has been associated with increased

inflammation (see meta-analyses by Baumeister et al., 2016) as well as with a dysregulation of the HPA axis (Heim et al., 2008). Similarly, heightened inflammation has been found in young adults prenatally exposed to maternal depression (Plant et al., 2016), while a flatter cortisol diurnal slope was found in adolescents prenatally exposed to maternal anxiety (O'Connor et al., 2005), thus raising important questions concerning the intergenerational transmission of the risk for depression. In this view, it is important to note that depression is often conceptualized as a stress-related disorder, with adverse experiences and stressful life events influencing the onset and course of the symptomatology in humans (e.g. Pine et al., 2002; Kessler, 1997; Kendler et al., 1999). As both the HPA axis and the IRS are considered candidate mechanisms through which stress can "get under the skin" to affect mental and physical outcomes, current findings, despite observational in nature, could indicate possible biological pathways underlying the effects of stress on mental health outcomes. In particular, an alteration of the diurnal cortisol pattern (i.e. lower morning cortisol output and a flatter diurnal slope) could represent a promising candidate mechanism for explaining associations between psychosocial stress and mental health outcomes. Interestingly, while traditionally stress is thought to trigger an activation of the HPA axis, it is now clear that it could both increase and decrease HPA activity depending on several features of the stressor, including time (Miller et al., 2007). Meta-analytical evidence indicate that while the beginning of a stressor is associated with a greater activity of the HPA-axis, over time, there is a counter-regulatory response that leads to a rebound of cortisol output below normal, as indicated by lower morning levels, daily output etc. (Miller et al., 2007). Furthermore, there is now increasing evidence that a flatter diurnal cortisol decline can represent a biological marker of stress exposure (e.g. O'Connor et al., 2014, O' Donnell et al., 2013) and a recent meta-analysis provides evidence of an association between a flatter diurnal cortisol slope and a number of poorer mental and physical outcomes, including depression (Adam et al., 2017), thus suggesting that diurnal cortisol slopes might

represent a biological correlate of physical and emotional health. Likewise, it is now clear that psychosocial stress can activate the inflammatory response systems, leading to increased levels of pro-inflammatory cytokines, acute phase proteins, such as CRP, or other inflammatory markers (Segerstrom & Miller, 2007) and it has been proposed that, besides the HPA-axis, inflammatory pathways could represent a mechanism for the effect of stress on mental health outcomes that deserve further investigation (Miller & Raison, 2016). However, it is important to emphasize once again that the current findings were correlational and causative pathways cannot be established. Future research is needed to establish whether the changes we observed in the HPA axis and IRS functioning of women with higher depressive symptoms during pregnancy might represent a mechanism by which depressive states emerge or an effect of emerging depressive symptoms.

Importantly, the inflammatory response system and the HPA axis exhibit bidirectional links and function in a complex interdependent manner over time. Thus, abnormal inflammation or dysregulation of the HPA axis has the potential to disrupt this circuit, carrying potentially adverse consequences for mental and physical health. Our exploratory analyses provide evidence of a positive association between maternal IL-6 levels and both higher diurnal salivary alpha amylase (sAA) and, to a lesser extent, cortisol concentrations in women with higher depressive symptoms during pregnancy. While increased inflammation together with hyperactivity of the HPA axis activity have been often reported in depressed patients outside the perinatal period and is considered suggestive of glucocorticoid resistance (Pariante et al., 2017), the interplay among stress hormones and inflammation during pregnancy is still largely unexplored. Our initial findings are in line with our exploratory hypothesis and with the only two existing studies showing an impairment of the negative feedback relationship between cortisol and pro-inflammatory cytokines during pregnancy in high-risk samples (Corwin et al., 2013; Walsh et al., 2016), suggesting that bidirectional stress-immune interactions offer a promising avenue for future endeavor.

The current study did not find any substantial evidence of an association between diurnal sAA levels and depressive symptoms during pregnancy. In contrast, maternal antenatal anxiety symptoms were significantly associated with an altered sAA diurnal pattern, as indicated by lower sAA levels at awakening and a flatter decline in sAA levels 30 minutes after awakening, in line with few reports of an alteration of sympathetic function in prenatally anxious women (e.g. (Field et al., 2004; Field et al., 2006; Giesbrecht et al., 2013). These findings indicate that, despite being comorbid, maternal depression and anxiety during late pregnancy may differ in some respects in their underlying biology, raising important issues with regards to possible mechanisms involved in fetal programming and challenging the general notion that different forms of maternal antenatal stress are reliably associated with increased glucocorticoids levels. Replication of this finding, particularly with larger samples and those containing more clinical depressed or anxious individuals would be invaluable.

The lack of association between maternal depressive symptoms and diurnal cortisol or inflammatory markers levels soon after delivery is unexpected, although it is in line with some studies that failed to find any significant association between maternal depression and either cortisol (reviewed in Garcia-Leal et al., 2017) or circulating levels of inflammatory markers (e.g. Corwin et al., 2015; Skalkidou et al., 2009) in the immediate postpartum period. On the one hand, these null findings might be related to limited statistical power, as the sample size at the postnatal phase was decreased (N=89 women of the initial 110 included). Alternatively, it can be hypothesized that the considerable elevations in both stress hormones and inflammatory markers in response to labor and delivery might mask the association with postpartum depressive symptoms, so that a major event would be needed to raise additionally the already elevated levels.

7.1.2 Maternal depressive symptoms and infants' outcomes

As hypothesized, higher maternal antenatal depressive symptoms were significantly associated with greater infants' negative affectivity (NA) at 3 months of age. This association was specific for maternal depression, rather than anxiety, and remained significant after controlling for postnatal symptomatology and maternal care. Conversely, the hypothesized association among maternal depressive symptoms during pregnancy and 3-month-olds' cortisol stress reactivity, outlined in Chapter 5, became non-significant once accounting for the quality of maternal care (Chapter 6). Furthermore, in contrast with our initial predictions, no associations were found between maternal depressive symptoms during late pregnancy and either infant's birth outcomes or cognitive and motor development.

Findings of an association between prenatal exposure to maternal depression and infants' NA, defined as the individual propensity in experiencing negative emotions, are in line with several studies (Austin et al., 2005; Davis et al., 2007; Huot et al., 2004; Rouse & Goodman, 2014). As high NA levels represent a key temperamental risk factor for later internalizing and externalizing symptoms, including anxiety and depression (e.g. Gartstein, Putnam, & Rothbart, 2012; Sayal et al., 2014), the observed association might indicate a possible early pathway from antenatal exposure to maternal depression to children's later behavioral maladjustment. However, as the reliance on maternal reports for both the independent (i.e. depressive symptoms) and dependent (i.e. infant's NA) variables produces shared method variance and potentially leads to an overestimation of the effects of antenatal maternal depression on child temperament, the current findings are to be interpreted with caution.

The mechanisms underlying the association between antenatal maternal depression and infants' NA are still to be elucidated. On the one hand, bidirectional evocative effects might occur with either postnatal maternal depression negatively affecting infant temperament or infant difficult temperament having a negative impact

on maternal psychological state, or both. However, it is noteworthy that the effects of maternal antenatal depressive symptoms remained significant even after controlling for maternal postnatal mood and quality of caregiving, thus suggesting that these postnatal environmental factors cannot fully account for this result. Heritability might be one plausible explanation, with infants of mothers reporting greater depressive symptoms during pregnancy inheriting a predisposition to experience greater NA (L. Schumann et al., 2017), which is then expected to interact over time with environmental factors, such as postnatal maternal depression, through complex Gene x Environment interplays (Gartstein et al., 2012; Rothbart et al., 2011). Furthermore, intrauterine mechanisms of fetal programming might explain the observed associations with maternal antenatal depression influencing fetal development through intrauterine biological mechanisms, involving for example stress hormones (e.g. Braithwaithe et al., 2017; Davis et al., 2007) or inflammatory pathways (Gustaffson et al., 2018), and leading to greater infant NA. This latter possibility will be further discussed in the following section.

While maternal antenatal depressive symptoms predicted individual differences in 3-month-olds' cortisol stress reactivity, with infants prenatally exposed to higher maternal depression displaying a slower cortisol recovery after the inoculation, unexpectedly, the association was no longer significant when adjusting for maternal postnatal emotional availability (EA). This suggests that maternal antenatal depression does not account for significant unique variance in cortisol reactivity when EA was included in the model. The literature concerning an association between maternal antenatal depression and children cortisol reactivity is mixed, with either positive (e.g. Brennan et al., 2008; Swales et al., 2018) or negative (e.g. Vedhara et al., 2012; Waters et al., 2013) or null associations (e.g. Braithwaite, Murphy, & Ramchandani, 2016; Davis et al., 2011) being reported. The current findings might indicate that the quality of maternal postnatal caregiving represents a stronger predictor of infants' cortisol stress reactivity, as compared to antenatal maternal depression. It can be

hypothesized that antenatal depression represents a distal marker of adversity, as compared to infants' proximal interactive experiences with the caregiver, which are known to play a key role in the development of the stress-response systems (Hostinar & Gunnar, 2013). From a methodological perspective, the current findings highlight the importance of including measures of infants' postnatal early environment, along with prenatal ones, when investigating processes involved in early programming, as a failure to account for postnatal factors, particularly related to early caregiving, might confound the observed associations and lead to erroneous conclusions. Interestingly, maternal antenatal depressive symptoms were not found to predict cortisol stress reactivity among healthy full-term neonates at birth, thus largely independently from postnatal influences, in line with findings from Davis and colleagues (2011). More studies evaluating infants soon after birth are needed to replicate our and Davis and colleagues' (2011) findings. However, taken together the current results did not provide evidence for a prenatal effect of maternal depressive symptoms, independent from postnatal factors, in influencing infants' stress reactivity from birth to three months of age in line with the results of a recent systematic review (Bleker et al., 2018) and suggest that inadequate control of postnatal factors related to the quality of maternal care might, at least partially, account for inconsistencies in the literature.

The lack of effects of maternal depressive symptoms during late pregnancy on infants' birth outcomes and cognitive development at 3 months is somewhat unexpected. Meta-analytical findings indicate a negative association between clinically significant levels of maternal depression during pregnancy and infants' weight at birth (Grote et al., 2010; Jarde et al., 2016). However, it is important to note that the association was weaker in low-risk samples and becomes non-significant when using a continuous measure of depression, such as the EPDS (Grote et al., 2010; Alder et al., 2007), or adjusting for relevant covariates, such as gestational age (Accort et al., 2015). Similarly, a number of large studies reported a link between maternal depression during pregnancy and lower cognitive development in infants ranging from

18 months of age to 8 years-old (Evans et al., 2012; Koutra et al., 2013; Lin et al., 2017), although the opposite association (DiPietro et al., 2006; Sandman, Davis, & Glynn, 2012) or null association between antenatal depression and infants' cognitive and motor scores from 6 to 78 months in equally methodologically rigorous studies have also been reported (Bandoli et al., 2016; Osborne et al., 2018b; Santucci et al., 2014). Although these null findings might be reliable, alternative explanations are also possible, including the limited sample size, which might have reduced the chance to detect a true effect, or the possibility that mild levels of maternal depression might have little effect on the developing foetus, as compared to more extreme exposures (Grote et al., 2010; Vedhara et al., 2012; Waters et al., 2014). Additionally, we cannot rule out that the exclusion of pregnant women with chronic medical problems or obstetric complications, resulting in a highly selected sample, might have biased the observed associations, particularly with respect to birth outcomes. It is also possible that the effects of prenatal maternal depression on offspring's stress reactivity and cognitive development might emerge later in development. A follow-up of the current cohort will provide further insight around this issue.

7.1.3 Maternal stress-related biology and infants' outcomes

As discussed in the following sections, findings from the current thesis provide further support for an association between maternal prenatal diurnal cortisol and individual differences in infants' stress reactivity and cognitive development, in line with our predictions. Furthermore, the results add to the limited literature on alternative biological mechanisms involved in fetal programming, by highlighting significant associations between variations in markers of maternal SNS (i.e. sAA) and IRS (i.e. C-Reactive Protein (CRP) and IL-6) functioning and infants' outcomes in a low-risk sample of mother-infant dyads. Lastly, we provided the first evidence in humans that the association between antenatal maternal cortisol and infants' stress reactivity can be over-ridden by sensitive maternal caregiving.

7.1.3.1 Maternal cortisol and infants' outcomes

Cortisol has been the most studied candidate mediator of maternal antenatal stress on fetal development, yet there are still several lacunae as regards the impact of antenatal cortisol on child development (see Zijlmans et al., 2015 for a review). In line with our hypothesis, maternal cortisol diurnal levels during pregnancy were associated with infants' stress reactivity and cognitive development. These results were independent from prenatal and postnatal depressive symptoms and adjusted for a number of covariates. However, contrary to our predictions, we failed to detect any association between maternal antenatal cortisol and infants' birth outcomes and temperament.

The association between variations in maternal diurnal cortisol during pregnancy and offspring's stress reactivity is one of the major positive findings of the current dissertation. Specifically, at birth, a flatter infant cortisol response to the heel-stick was associated with a higher maternal cortisol awakening response (CAR) during pregnancy, while greater infant behavioural reactivity was related to a flatter maternal diurnal cortisol slope, supporting a role for variations in maternal diurnal cortisol rhythm during late pregnancy, largely independent from postnatal environmental influences, in influencing offspring's stress reactivity, in line with few initial evidence (de Weerth, Buitelaar, & Beijers, 2013; Giesbrecht et al., 2017; Osborne et al., 2018). Additionally, at three months of age, we reported a significant association between antenatal maternal diurnal cortisol and greater cortisol response to the inoculation among infants of less emotionally available mothers. These findings suggest an interplay among prenatal and postnatal maternal influences in shaping offspring stress reactivity with important conceptual and methodological implications.

First, the current findings suggest that variations in maternal diurnal cortisol during pregnancy are associated with altered patterns of stress reactivity in the offspring's at birth and that these effects are likely to persist until 3 months of age if the infant is postnatally exposed to a less sensitive rearing environment. These results are

consistent with the hypothesis, mainly based on animal models, that the effects of prenatal stress exposure might arise from an alteration of the foetal stress response systems (see Glover et al., 2010) that might carry possible long-lasting effects on how the brain will respond physiologically and behaviourally to stress and enhance the risk of developing later psychopathology, such as mood or anxiety disorders (Tarullo & Gunnar, 2006; Gunnar & Vazques 2001; Heim & Nemeroff, 2001). Furthermore, the current results indicate that postnatal environmental adversity, such as poor parenting, may exacerbate the negative association between maternal antenatal cortisol and infants' stress reactivity, in line with a "multiple hit" or "cumulative stress" hypothesis (Nederhof & Schmidt, 2012). Mechanisms for the effects of maternal postnatal care in the link between prenatal cortisol exposure and infants' stress reactivity will be fully discussed in section 7.1.3.4.

Secondly, it is noteworthy that variations in maternal diurnal cortisol pattern during pregnancy were associated with a dampened infant cortisol reactivity (though an amplified behavioral response to stress) at birth and with a greater cortisol reactivity at three months of age in infants of less sensitive mothers. Although replication in larger and different samples is needed, we might speculate that neurodevelopmental physiological changes in HPA axis responsivity occurring across the first year of age might play a role in the observed pattern of association. In particular, it has been shown that the effects of similar stressors, such as painful procedures, in inducing a cortisol response depend strongly upon the child's age, with a normative decrease in cortisol reactivity to painful stimuli with increasing months of age (Jansen et al., 2010). Thus, we might speculate that infants of mothers showing altered cortisol diurnal pattern during pregnancy might failed to demonstrate the expected substantial increase in cortisol concentrations after the heel-stick at birth. In contrast, they might exhibit a stronger response than expected to the inoculation at a later developmental age, when the HPA axis responsivity begins to decrease, if they do not benefit from a stress-buffering mother-child relationship.

Beyond the effects on offspring stress reactivity, in line with our hypothesis, higher levels of maternal diurnal cortisol in late pregnancy were associated with infants' lower cognitive scores at 3 months of age, after accounting for several confounders, including the quality of postnatal care. This finding is in line with a number of studies in humans (e.g. Bergman et al., 2010; Lewinn et al., 2009) and substantial data from animal models (reviewed in Owen, Andrews, & Matthews, 2005), linking prenatal exposure to either endogenous or synthethic glucocorticoids and poorer cognitive performance.

Taken together, the current findings indicate an association between maternal antenatal diurnal cortisol and physiological, behavioral and cognitive outcomes in the offspring, possibly suggesting that an alteration in maternal diurnal cortisol levels during pregnancy represent a broad risk factor for later infant development. However, it is essential to emphasize that the correlational nature of the current findings does not allow us to draw causal conclusions and that the pathways possibly underlying the observed associations are still to be clarified. On the one hand, genetic or epigenetic mechanisms might account for the observed associations (e.g. Van Hulle et al., 2012). On the other hand, in light of the widespread observed effects of maternal antenatal cortisol on offspring development, it is tempting to interpret the current findings as suggestive of a role of maternal diurnal cortisol in influencing fetal brain development, leading to later altered biological and behavioral stress reactivity and cognitive development. Future studies should examine whether this might be the case and possible mechanisms underlying the impact of maternal cortisol on fetal brain development. In particular, future investigation should determine whether alterations in maternal diurnal cortisol during pregnancy might result in fetal cortisol overexposure. As the expression of the 11β-HSD2 placental enzyme has been found to be reduced in the last stages of gestation (Murphy et al., 2006), it is possible that even mild elevations in maternal cortisol in late pregnancy might affect fetal brain development. Notably, both the hippocampus, crucially involved in cognition, and the HPA axis are

particularly sensitive to excessive levels of glucocorticoids (Noorlander et al., 2006; Owen et al., 2005), this fact might account for the widespread effects of cortisol on different domains of child development. Alternatively, maternal cortisol might influence fetal brain development indirectly, by affecting vessel tone and possibly leading to reduced utero-placental blood flow, with long-term consequences on neurodevelopmental outcomes (Rakers et al., 2017).

Lastly, contrary to what we expected, we did not find any significant association between maternal antenatal diurnal cortisol and both infants' temperament and birth outcomes. These null findings deserve further replication in order to elucidate whether they are veridical or might be related to methodological issues. However, they are in line with the results of a recent literature review (Zijlmans et al., 2015) showing that no evidence exists for an association between maternal cortisol levels during gestation and maternal report of children's temperament and with a number of large studies in humans finding no significant associations between maternal cortisol and birth outcomes after adjusting for covariates, such as gestational age (e.g. Goedhart et al., 2010; Li et al., 2012). It is noteworthy that despite models were adjusted for infants' gender, we did not examine how infants' gender interact with antenatal cortisol exposure to determine the observed outcomes. As initial evidence exists for gender differences in the link between maternal antenatal cortisol and both infants' temperament (Braithwaite et al., 2017a) and birth outcomes (Sandman et al., 2013; Clifton et al., 2010), this remains an important area for future research.

7.1.3.2 Maternal sAA and infants' outcomes

The role of antenatal maternal sAA, a non-invasive biomarker of SNS, in fetal programming is just beginning to be investigated. Exploratory analyses in the current dissertation provide preliminary evidence of an association between diurnal patterns of maternal sAA during late pregnancy and both infants weight at birth and NA at 3 months of age, independent from prenatal and postnatal depressive symptoms and

from several confounders. In contrast, no significant associations were detected among antenatal sAA levels and stress reactivity or cognitive outcomes.

Findings of a positive association between antenatal maternal sAA levels at awakening and throughout the day and infants' birth weight are partially in line with results from the only available study on the link among maternal antenatal sAA and birth outcomes (Giesbrecht et al., 2015). We speculated that heightened maternal sAA levels might reflect an upregulation of the SNS with advancing gestation to sustain maternal and fetal energetic demands, however this hypothesis needs to be explicitly tested in future research. Furthermore, a greater maternal sAA decline after awakening was related to higher infants NA scores at 3 months of age, after adjusting for covariates and accounting for the quality of postnatal maternal care. The influence of maternal antenatal sAA levels during pregnancy on infants' temperament has been evaluated in only one study reporting significant sex-dimorphic associations between maternal sAA diurnal levels and 2-month-olds negative emotionality (i.e. high levels of maternal sAA being marginally associated with reduced negative emotionality in males; Braithwaithe et al., 2017).

Literature on a possible role of sAA in the DOHaD hypothesis is very limited and our understanding of the role of alternatives to HPA-axis mediated mechanisms in fetal programming is still patchy, thus limiting possible interpretation of the current findings. While sAA is thought to provide a reliable index of the SNS functioning (U. M. Nater & Rohleder, 2009), studies on sAA during pregnancy are limited (Giesbrecht et al., 2013; Giesbrecht et al., 2015; Nierop et al., 2006). Thus, it is currently unclear what sAA levels assessed during pregnancy could actually reflect. For example, an increased SNS activity during pregnancy causes an increase in vasoconstriction and a reduction of placental perfusion, however, the association between maternal prenatal sAA levels and foetal blood flow has never been examined. The preliminary and correlational nature of the current findings require caution in interpretation. However, taken together these initial results point to maternal antenatal sAA as a promising

marker for research into early programming of later health and behavioral outcomes. In addition, from a methodological perspective, they converge with the current findings on the effects of cortisol on child outcomes to underscore the importance of diurnal assessment of stress biomarkers, as a failure to do so might lead to miss possible significant associations between maternal physiology and child outcomes.

7.1.3.3 Maternal inflammation and infants' outcomes

Literature on the effects of maternal inflammation during pregnancy on offspring outcomes in humans is very limited. Although observational epidemiological studies report that maternal inflammatory response to infections during pregnancy is associated with increased risk of neuropsychiatric disorders, such as schizophrenia, in the resulting offspring (Estes & McAllister 2016), the extent to which there is a programming effect of maternal antenatal cytokines is unknown.

The current study is among a small number of other very recent reports prospectively examining the association between maternal inflammatory markers during pregnancy and early neurodevelopmental outcomes (Osborne et al., 2018; Gustaffson et al., 2018; Rasmussen et al., 2018; Graham et al., 2017). Our exploratory analyses indicated that maternal antenatal IL-6 levels were negatively associated with head circumference at birth, while maternal prenatal CRP concentrations were negatively associated with infants' cognitive scores at 3 months of age in a low risk sample of healthy women and infants. These associations were not confounded by maternal BMI, infant's gender and gestational age and were independent from maternal depressive symptoms and from the quality of postnatal maternal care. In contrast, we did not observe any significant association among maternal inflammation and infants' stress reactivity and temperament.

The association between higher prenatal maternal IL-6 levels and smaller head circumference at birth, which is considered a marker for intrauterine brain development (Broekman et al., 2009b), is a novel finding. Instead, findings of an association among

higher maternal CRP levels during pregnancy and lower 3-month-olds' cognitive scores converge with the modest available evidence in humans indicating an association between maternal inflammation during pregnancy and offspring neurodevelopment across the first year of age (Osborne et al., 2018; Rasmussen et al., 2018). Despite the fact that the observational nature of the current study does not allow us to draw causal inferences, taken together our findings are consistent with results from animal studies (e.g. Meyer et al., 2006) and with very recent evidence from brain imaging studies in human infants (Grahman et al., 2017; Rasmussen et al., 2018) suggesting a role of maternal inflammation in affecting fetal growth and neural development, although the in utero pathways are still to be clarified.

IL-6 and CRP are both considered biomarkers of chronic or systemic low-grade inflammation (Rohleder, Aringer, & Boentert, 2012) and, as expected, in the current sample were moderately correlated. However, it is unclear why different associations with child outcomes were detected for IL-6 or CRP levels. Differences might be related to methodological or limited statistical power issues or, might also be suggestive of distinct inflammatory pathways involved in fetal programming. IL-6 has been proposed as a key biological mediator of environmental conditions on the developing fetal brain (Entringer, Buss, & Wadhwa, 2015), being able to cross both the placental and blood–brain barriers (Banks, Kastin, & Gutierrez, 1994; Zaretsky et al., 2004) and possibly directly influencing the fetal brain development (Boulanger, 2009; Deverman & Patterson, 2009; Smith et al., 2007). Alternatively, elevated levels of maternal IL-6 might indicate an imbalance of pro-inflammatory and anti-inflammatory influences (Kamimura, Ishihara, & Hirano, 2003) which has been proposed as one possible mechanism related to the effects of maternal antenatal inflammation on fetal brain development (Meyer et al., 2008). While CRP is often considered as a proxy for IL-6 (S. Black, Kushner, & Samols, 2004), evidence suggests that elevations in CRP itself can affect fetal development through several pathways. For example, heightened maternal CRP levels could interfere with fetal growth and brain development by

contributing to vascular and placental dysfunction (Ernst et al., 2011) or by directly altering offspring's synaptic connectivity (Canetta et al., 2014; Stephan, Barres, & Stevens, 2012). Lastly, higher CRP levels may indicate poor maternal health (Wium-Andersen & Nielsen, 2013), which can themselves influences offspring's brain development and behavioral outcomes (Chittleborough, Lawlor, & Lynch, 2012; O'Neil et al., 2014) and, therefore, account for the current findings. Alternatively, hereditary transmission might explain the observed associations, with mother-infants shared genes accounting for variation in both maternal inflammation and offspring's outcomes though pleiotropic effects. Additionally, epigenetic mechanisms could mediate the impact of prenatal maternal inflammation on fetal brain development with potentially long-lasting effects, as suggested by results of animal studies (Kundakovic & Jaric, 2017).

We did not find any evidence of a significant association between maternal antenatal inflammation and both infants' stress reactivity and temperament after adjusting for covariates. While animal findings suggest a possible role of maternal pro-inflammatory cytokines during pregnancy in the development of fetal stress response systems (e.g. Dahlgren et al., 2006; Samuelsson et al., 2006), existing evidence in humans is only preliminary. Osborne and colleagues (2018) very recently reported a positive unadjusted correlation between specific maternal inflammatory markers, namely IL-10, TNFα and VEGF, but not IL-6, and 12-month-olds' cortisol stress reactivity in a sample of clinically depressed pregnant women and healthy controls. Furthermore, Gustaffson and colleagues (2018) found that maternal antenatal pro-inflammatory cytokines (indexed though a latent variable including IL-6, TNF-α, and monocyte chemoattractant protein-1) mediated the association between prenatal depressive symptoms and maternal, but not observed, ratings of negative affect in 6-month-olds in a sample of pregnant women at risk for attention-deficit/hyperactivity disorder. Despite the possibility that the current null findings concerning temperament and stress regulation might be an issue of power, it is noteworthy that the current

sample size is slightly higher than the ones in the above mentioned studies (N=87 in Osborne et al., 2018; N=62 for Gustaffson et al., 2018). Alternatively, methodological heterogeneity in the composition of the samples and in infants' ages might account for different results. Lastly, maternal stress, rather than inflammatory, mediators might be more closely involved in influencing fetal development of stress regulation. Nonetheless, it is important to emphasize that we are at the very beginning of our understanding of the role of maternal inflammation in fetal programming of later health and mental outcomes. Future studies are needed in humans to investigate the association among direct measures of inflammatory markers and outcomes in the offspring and unravel the specific in utero inflammatory pathways involved in fetal brain development.

7.1.3.4 The moderating role of maternal postnatal care

Animal studies have consistently shown that the processes leading to the negative link between prenatal stress exposure and bio-behavioral outcomes in offspring can be over–ridden by postnatal environmental factors (e.g. Maccari et al., 1995; Wakshlak & Weinstock, 1990). Results from the current thesis provide the first evidence in humans indicating that the effects of maternal antenatal cortisol on 3-month-olds cortisol stress reactivity is eliminated by sensitive maternal caregiving, as measured through the Emotional Availability (EA) Scales (Biringen et al., 2008). Specifically, in line with our broad hypothesis, higher levels of maternal cortisol during pregnancy were associated with infants' greater cortisol response to the inoculation only among infants of less emotionally available mothers, while the association became non-significant among infants of highly sensitive mothers. Furthermore, the magnitude of the association between maternal EA and infants' cortisol reactivity depended upon maternal prenatal cortisol levels, in line with the emerging notion that prenatal experiences could modify individual susceptibility to postnatal environmental

influences (Pluess & Belsky, 2011). Contrary to our predictions, however, maternal EA did not moderate the link between prenatal maternal influences and infants' cognitive development or temperament. Furthermore, we did not find evidence for a role of maternal EA in mediating the effects of antenatal maternal depression on infants' outcomes.

From a methodological point of view, results from the current dissertation emphasize the strong need for models of fetal programming to account for the influence of postnatal care. While a considerable number of studies within the DOHaD field control for the influence of postnatal maternal symptomatology, which is thought to represent a proxy of postnatal environment, current findings indicate that postnatal symptoms of distress are not necessarily associated with the quality of maternal caregiving in a low risk sample. Furthermore, a failure to include measures of maternal care might lead to misleading findings. Indeed, in the current sample, measures of antenatal cortisol alone were no longer associated with infants' pattern of stress reactivity at three months of age, although the picture was somewhat different once the interactive effects between maternal prenatal cortisol and postnatal EA were taken into account.

The lack of association between maternal EA and either prenatal or postnatal depressive or anxiety symptoms we reported in the current study is potentially surprising. However, it should be noted that mothers in the study consisted of a community low-risk sample, mostly middle-high class, married and well educated. Thus, it is possible that the characteristics of the sample might have constrained the range in maternal EA. In line with this hypothesis, no differences in maternal EA between mother with higher and lower prenatal depressive symptoms have been reported in low risk samples (e.g. De Weerth et al., 2003; Endendjik et al., 2005), while reduced maternal EA in depressed mothers from high risk samples has often been reported (e.g. Easterbrooks, Bureau & Lyons-Ruth, 2012; Trapolini et al., 2008; Easterbrook, Biesecker, & LyonsRuth, 2000; Kluczniok et al., 2016).

The mechanisms underlying the moderating effects of maternal postnatal care in the link between prenatal cortisol exposure and infants' stress reactivity are still to be uncovered. Strong evidence across species indicates that caregiving experiences can affect emotional behaviour and physiology by influencing the neural circuits underlying emotion regulation and stress reactivity (Callaghan & Tottenham, 2015; Tottenham, 2015). In particular, the presence of a sensitive caregiver is hypothesized to act as an external regulator that buffer infants' neural, endocrine and behavioral responses to stress (Gunnar & Donzella, 2002; Gunnar et al., 2015) by suppressing the hormonal cascade leading to elevations in glucocorticoids and inducing greater prefrontal cortex regulation of the amygdala (Moriceau & Sullivan, 2006; Gee et al., 2016). Thus, early in development, caregivers are believed to operate fairly directly on the neural circuits underlying emotional and stress reactivity and influence their development (Tottenham et al., 2017; Gee et al., 2014), although the quality of caregiving matter. For example, pioneer studies have shown that the maternal presence can buffer infant's HPA axis reactivity for secure but not insecure attachment (Nachmias et al., 1996). We might speculate that the maternal "buffering power" is less effective in infants of less emotionally available mothers thus exacerbating the association between prenatal cortisol exposure and stress reactivity and leading to enhanced cortisol response to the inoculation. Additional mechanisms for the buffering effects of positive caregiving on infants' stress reactivity might involve oxytocin, which is known to have behavioral and physiological stress-attenuating effects, including inhibitory effects on the HPA-axis stress reactivity (Heinrichs et al., 2003). While oxytocin is known to play a role in social interactions, recent work indicates that this hormone responds to the quality of these interactions. For example, increases in infants' salivary oxytocin following parent-infant interaction have been found to be positively related to levels of tactile contact with the parent, parent-child affect synchrony and infants' social engagement (Feldman et al., 2010). We might speculate that adverse early caregiving, as indicated by low levels of maternal EA, might

negatively affect infants' HPA axis responsiveness, thus aggravating the impact of antenatal cortisol exposure, by impairing the ability of the oxytocin system to regulate cortisol stress reactivity, in line with animal evidence (Hostinar et al., 2014) and preliminary evidence in human adults (Meinlschmidt and Heim, 2007). Lastly, epigenetic mechanisms are an additional pathway through which early caregiving experiences can shape stress regulation abilities and interact with prenatal exposures. In particular, the work of the group led by Michael Meaney on rodent models has shown that maternal adequate caregiving stimulation (i.e. licking and grooming) over the first days of life reduce HPA-axis stress reactivity and anxiety-like behaviors in the pups by increasing the glucocorticoid receptor (GR) gene expression, mainly through the demethylation of the GR gene in the hippocampus of the offspring (Weaver et al., 2004). In humans early maternal postnatal stroking was found to buffer the association between prenatal maternal depression/anxiety and negative outcomes in the offspring (Sharp et al., 2012; 2014; Pickles et al., 2017), with initial evidence suggesting that these effects might be mediated by epigenetic mechanisms involving the reduction of the GR gene methylation (Murgatroyd et al., 2015). It is noteworthy that these studies focused on the ability of low-grade components of caregiving, such as tactile stimulation, to reverse the effects of antenatal stress and did not include measure of maternal caregiving. Future studies should investigate whether maternal EA or sensitive caregiving can exert similar epigenetic effects on infants' developing neural and endocrine system. Lastly, it is important to emphasize that, as the current findings are correlational, it is difficult to establish the direction of the association between infant's stress reactivity and maternal EA. Thus, while maternal caregiving is known to play a role in shaping the infants' stress response systems, it is also true that infants' characteristics, such as being more "reactive", "easily stressed" or "difficult to soothe" are likely to negatively affect maternal EA, in a process that is, by nature, dyadic and bidirectional.

While mechanisms underlying the effects of maternal postnatal EA in the link between antenatal cortisol exposure and infant's stress reactivity are still to be elucidated, these results contradict deterministic views of fetal programming, supporting the notion that the sensitive window for early life programming of later health and mental outcomes extends well beyond the prenatal period with important clinical implications. In particular, as attachment-based intervention has been proved to be successful in improving maternal EA (e.g. Ziv, Kaplan & Vezan, 2016), this can be thought of as a target of clinical intervention with women experiencing emotional distress during pregnancy.

Lastly, the lack of a moderating effect of maternal EA in the link between maternal prenatal factors and infants' psychomotor development or temperament is in contrast with our exploratory hypothesis. One previous study reported a significant moderating effect of child-mother attachment in the association between antenatal maternal stress and 17-month-old infant's cognitive development (Bergman et al., 2010) and fearfulness (Bergman et al., 2008). It can be speculated that both cognitive development and temperament early in life might be more biologically determined and less permeable to postnatal environmental influences (Black et al., 2000) and that the moderating effects of maternal postnatal care in the link between prenatal stress and child's outcomes might be revealed later in development. However, findings are only preliminary and further research is needed to understand the complex interplay between prenatal and postnatal experiences.

7.2 Limitations

The interpretation of findings from the current thesis should be cautious due to several limitations that have been already discussed within each chapter but are now briefly summarized.

First, data are based on a healthy and relatively high SES well-educated community sample and levels of self-reported depressive or anxiety symptoms were relatively low, as would be expected in a community sample. It is worth noting, however, that the prevalence of women at risk for depression according to the EPDS cut-off, was similar to that noticed in earlier studies employing the same instrument and cut-off (e.g. Johnson et al., 2012) and in line with the general prenatal prevalence of these symptoms (e.g. Bennett et al., 2004). In addition, the use of a structured diagnostic interview to exclude other psychiatric disorders, jointly with a perinatal-specific depression questionnaire, strengthens our results. The rationale for focusing on a low risk sample is based on the evidence that maternal prenatal stress effects are not generally limited to extreme stress levels or clinically diagnosed mental illness. Rather, they have often been found in unselected community samples and appear to occur across the whole normative range of distress that women might experience at some time during their pregnancies (reviewed in Madigan et al., 2018; Talge, Neal, & Glover, 2007; Van den Bergh et al., 2017). In addition, very little is known about the role of naturally occurring variations in maternal stress and inflammatory signals on offspring's normative neurodevelopment. Thus, a better understanding of the complex mechanisms underlying early programming of typical development in a healthy low risk sample might represent the first step before applying this knowledge to investigate prenatal programming of abnormal development in high risk samples. Furthermore, the use of a low risk sample has the potential to limit confounding influences of additional risk factors associated with psychosocial adversity (e.g. teenage motherhood, unemployment, financial problems etc.), thus possibly leading to fairly conservative associations. Findings obtained from a low-risk sample could provide "control" data for a future comparison with similar work on high risk samples. Nevertheless, the generalizability of the current results to high psychosocial risk or clinical populations is limited. Although it is tempting to imagine that findings would be

more profound among a sample of clinically depressed women, this remains an open question for future investigation.

Second, although the sample size was of necessity limited because, as described in Chapter 2, only healthy pregnant women not taking any chronic medication and with no pregnancy complications or medical conditions were included, a larger sample size would have allowed more power to detect small effects. Additionally, while a highly selected sample is appropriate for a psychobiological study in order to limit possible conditions that might themselves be associated with an altered functioning of the biological systems examined, it can also limit the generalizability of findings. Furthermore, as previously reported (Egliston et al., 2007), obtaining sufficient saliva volumes from newborns was a challenge, thus leading to a more limited sample size for cortisol analyses. HLMs were employed in order to maximize the number of infants included in the final analyses and obtain reliable estimates of effects despite missing values for one or more time-points. The results of a post-hoc power analysis calculated using G*power 3.0.10 suggest that for detecting medium sized effects, hierarchical linear regressions for the effects of prenatal maternal factors (i.e. depressive/anxiety symptoms, diurnal cortisol and sAA measures, inflammatory markers) on infants' outcomes had a power estimate ranging from .94 to .97. Therefore, the current sample size can be considered adequate for testing our hypotheses. This is also in line with results of a recent meta-analysis indicating a minimum sample size of N=60, based on the observed associations, in order to detect significant associations between maternal cortisol in the third trimester and infants' birth outcomes, while testing for 8 potential covariates (Cherak et al., 2018). For what concerns HLMs for the effects of prenatal maternal factors on infants' cortisol reactivity, we estimated that with the current sample size and 80% power, we were able to detect effects ranging from r=.18 to r=.22, while we would have miss effects smaller than this. Thus, it is important to emphasize once again that conclusions that can be drawn from the current study are to be regarded as

preliminary and require replication in different and larger cohorts.

Third, while the current study's impact comes from the simultaneous measures of several self-reported, observational and biological measures from both mothers and infants over repeated occasions, this brings the potential confounder of multiple comparisons (i.e. increase in the probability to observe associations that result only from chance - false positives - as the number of statistical comparison increases). While a standard practice is to adjust the threshold for statistical significance according to the number of pairwise tests performed, for example using the well-known Bonferroni correction (Dunn, 1961), these methods may result in overly conservative conclusions and are not free of criticisms (e.g. Nakagawa et al., 2004; Rothman, 1990; Perneger, 1998; Feise, 2002; Gelman et al., 2012; Altenhouse et al., 2016). For example, while adjusting the α levels for each test performed is effective in limiting Type I errors (i.e. rejection of the null hypothesis when the null hypothesis is true), it inevitably leads to an increase in the number of Type II errors (i.e. true effects that are discarded because they do not pass this threshold) and thus to too many false-negatives. This might be problematic in exploratory studies that, by nature, are more concerned of not missing on something potentially interesting and hence limiting Type II error. Additionally, more detailed researches (i.e. including and measuring more variables) have less probability of finding significant results and, thus, are more penalized by multiple comparisons corrections (Moran et al., 2003); not to mention the growing concern that these methods might lead to selective reporting and lack of scientific transparency (Fortsmeier et al., 2017). Furthermore, there is currently limited agreement concerning the choice of the number of tests that must be adjusted for and this is often a spurious decision (Altenhouse et al., 2016). In particular, it is not clear whether preliminary analyses should be included or not. As several preliminary analyses were run in each chapters to examine potential confounders of the observed associations, determine the number of actual comparisons made was problematic. Thus, given the exploratory nature of this study and the fact that in several cases we

do not have a priori specific hypothesis concerning the effects, we made no statistical adjustments for multiple comparisons (e.g. Ravicz et al., 2015; Okun et al., 2013). Instead, as recommended by some authors (e.g. Nakagawa et al., 2004), we reported all exact p values and stated all the comparisons made in order to allow the reader to interpret the results accordingly and evaluate the relative weight of the conclusions. A subsequent study with pre-planned hypotheses should be conducted to confirm the observed associations.

Fourth, the focus of the current dissertation was on the effects of late pregnancy exposure, thus the current findings might not generalize to different gestational windows. Furthermore, we cannot determine whether the observed associations are specific to the third trimester or might be the result of a continuous prenatal exposure. However, our results, particularly concerning the association between maternal antenatal influences and offspring's stress reactivity, are consistent with studies suggesting a stronger and specific effect of prenatal stress exposure in late pregnancy as compared to earlier exposure (e.g. Davis et al., 2011; Vedhara et al., 2012).

Fifth, maternal salivary samples were collected at home and despite considerable efforts being made to assure compliance with the protocol, this was not objectively measured. However, it is noteworthy that Okun and colleagues (2010) reported excellent concordance rates between objective and self-report measures of sample collection times during pregnancy. In addition, the expected typical diurnal patterns in salivary cortisol and sAA was found, thus suggesting that, as a whole, women were likely to have complied with the saliva sampling protocol.

Sixth, due to the observational nature of the design, it is difficult to disentangle the role of prenatal factors from the effects of other related factors, including maternal diet or lifestyle or shared genetic effect that are likely to play a role in the "risk" transmission from mother to infant. A wide range of possible confounders such as sociodemographic factors, health and pregnancy-related factors were controlled in

statistical analyses. Additionally, only healthy pregnant women free from any medication, alcohol or smoking use were included. However, genetic factors or factors related to changes in diet or lifestyle remain areas for future investigations.

7.3 Contribution to the DOHaD hypothesis

The current dissertation sought to extend existing literature on the DOHaD hypothesis by adopting a novel multi-systems approach, involving a comprehensive assessment of maternal antenatal psychological and biological influences, and taking into account the role of postnatal maternal care, in the effort to shed light on the mechanisms underlying the link between maternal antenatal depressive symptoms and infants' bio-behavioral development. Based on the hypotheses stated in Chapter 1, three major positive findings were obtained. First, results show new evidence in a low-risk sample that women with higher depressive symptoms, but not anxiety, during pregnancy have an altered cortisol diurnal pattern and increased levels of inflammation. Second, findings indicate that variations in maternal diurnal cortisol during pregnancy are associated with early infants' stress reactivity and cognitive development and, third, that maternal sensitive caregiving can reverse some of these effects. Furthermore, our exploratory analyses add to the limited literature on alternative biological mechanisms involved in fetal programming by showing that markers of maternal IRS and SNS functioning during pregnancy are associated with infants' outcomes. Contrary to our predictions, however, maternal depressive symptoms was significanty associated only with 3-month-olds NA, while no associations were found with infants' birth outcomes, stress reactivity and cognitive development once the models were adjusted for postnatal maternal symptoms and caregiving. Likewise, unexpectedly, maternal diurnal cortisol was not related to infants' birth outcomes and temperament. Furthermore, maternal sensitive caregiving, as assessed through the EA scales, was not related to maternal antenatal or postnatal

depressive symptoms. Reasons for these null findings have been already discussed previously and included, among others, the low-risk and highly selected nature of the sample, the limited statistical power to detect small effects, the gestational window under examination and a failure to account for sex differences or other relevant factors such as genetics.

Findings from the current dissertation are relevant to the field of child development, developmental psychopathology and psychoneuroendocrinology and make a contribution to the literature in the DOHaD hypothesis in several ways.

First, findings indicate that antenatal maternal depressive symptoms, even without meeting the full criteria for a clinical diagnosis, are associated with an altered stress-related physiology. With respect to the DOHaD hypothesis, these results further corroborate the notion that maternal antenatal depression might have a detectable impact on fetal development even at subclinical levels (O'Donnell & Meaney, 2017), and encourage further investigation into the effects of maternal depression across the whole spectrum of symptom severity. In addition, as meta-analytical findings from Valkanova and colleagues (2013) indicates that in non-pregnant adults higher CRP and IL-6 levels precede the development of major depression, while a cortisol flatter diurnal decline is increasingly regarded as a general marker of stress exposure (e.g. O' Donnell et al., 2013), the current findings lead the way for future studies into the identification of "early risk" biomarkers that might aid in the development of prevention strategies and, once an etiological pathway to depressive symptoms has been established, be feasible intervention target.

Second, the observed associations among maternal depressive symptoms and both biological alterations during pregnancy and infants' outcomes (i.e. heightened infant NA) were specific for depression. In contrast, maternal anxiety symptoms were related to an altered sAA diurnal rhythm during pregnancy and predicted poorer infants' cognitive performances at three months of age. Considering the high rate of comorbidity (Falah-Hassani et al., 2017) and proportion of shared genetic factors that

influence these two conditions (Middledorp et al., 2005), marked differences in the association between anxiety or depression and maternal physiology or child outcomes are quite unexpected. Methodological issues might account for these conflicting findings. For example, while the EPDS inquiries about the past week, the STAI-T investigates a "trait" measure of anxiety that refers to general functioning. However, it is important to mention that comparable results were found when using the "state" measure of the questionnaire that assess transient anxiety. There are also theoretical explanations for the differential association between prenatal depression versus anxiety and child outcomes. Indeed, while within the literature concerning the DOHaD hypothesis, maternal antenatal depression and anxiety are often considered psychological markers of the same underlying construct of "antenatal stress", current findings suggest that their biological underpinnings are to some extent different and, thereby, they possibly affect fetal development in different ways. A limited number of studies has examined both antenatal anxiety and depression in the same sample and conflicting findings have been reported. Few studies indicate that maternal depression, rather than anxiety, was more strongly associated with infants' outcomes (Madigan et al., 2018; Lin et al., 2017; Barker et al., 2011), while a handful of others found that prenatal anxiety symptoms, rather than depression, have a stronger effect on infants' development (Grant et al., 2009; O'Connor et al., 2002; 2003; 2005; Ibanez et al., 2015). While more studies are needed to determine the prenatal risk phenotype that might be more implicated in child development, our findings demonstrate that an accurate psychological and biological characterization of the type of antenatal stress under investigation is pivotal in order to advance understanding of the biological mechanisms underlying fetal programming, in line with the recent general call within the field of the DOHaD hypothesis (Graignic-Philippe et al., 2014; O'Donnell & Meaney, 2017).

Third, in line with the DOHaD hypothesis, small variations in maternal mood, stress hormones and inflammatory markers in late pregnancy were all associated with

a number of neurodevelopmental outcomes in offspring. However, we did not find support for a role of maternal stress-related biological alterations in mediating the effects of antenatal depression/anxiety on offspring outcomes. Rather, variations in levels of maternal depression, anxiety, stress hormones and inflammatory markers during pregnancy appear to exert an effect on offspring outcomes through independent and different pathways. This underscores the importance of combining both subjective and objective measures of maternal antenatal stress experience. Additionally, our initial evidence is possibly suggestive of specific associations between distinct antenatal influences and specific outcomes. Specifically, while maternal depressive symptoms were associated with heightened infant NA, which is considered a marker of risk for later mental health problems (e.g. Sayla et al., 2014), inflammatory pathways appeared to be more implicated in outcomes related to brain and cognitive development, while glucocorticoids showed more widespread effects on stress reactivity and cognitive development. However, it is crucial to emphasize once again, that, although findings of a prospective association between maternal antenatal influences and offspring's outcomes are in the expected direction and it is tempting to interpret them as suggestive of causative biological pathways, they are only supported by correlational analyses. Thus, as for any observed association, they could be the result of random error, bias or unmeasured confounding, or they may be the result of a true causal effect of prenatal exposure. Further replication and validation in larger samples are required.

Fourth, consistently with the DOHaD hypothesis, the observed associations mostly support an effect of antenatal maternal influences independent from the postnatal environment. First of all, findings of an association among maternal prenatal cortisol or inflammation and altered outcomes soon at birth reinforce the idea that programming effects might occur in utero. Secondly, the opportunity to control for both postnatal maternal symptomatology and caregiving, allows to obtain statistically independent estimates of prenatal effects on 3-month-olds outcomes. Future studies

should investigate the extent to which the observed associations are independent from genetic influences, which are likely to confound the associations among prenatal factors and child outcomes, by adopting genetically informed designs (Rice et al., in press).

Fifth, there has been surprisingly little emphasis on the role of postnatal environment in research on the DOHaD hypothesis. This dissertation provides the first human evidence that postnatal positive caregiving, as indicated by high maternal EA, eliminates the effects of prenatal cortisol exposure on infants' stress reactivity. This result provides a major translation of animal findings showing that a sensitive caregiving environment can reverse the effects of neurobiological vulnerability (e.g. Francis, Diorio, Plotsky, & Meaney, 2002) and extends research on the DOHaD hypothesis and, more generally, on the biological impact of early caregiving experiences. From a methodological-conceptual point of view, our findings offer insight into the inconsistent pattern of results that have been observed across previous studies assessing the association between prenatal cortisol exposure and infant cortisol reactivity (reviewed in Zijlmans et al., 2015). Indeed, while we did not detect any main effect of antenatal maternal cortisol on 3-month-olds' stress reactivity in the whole sample, we did report a positive association among infants of less emotionally available mothers. The current results underscore the need for research into mechanisms involved in early life programming of later risk for physical and mental health to account for the role of postnatal caregiving experiences which are likely to alter the unfolding of prenatal processes. From a clinical perspective, if the current findings are replicated, they suggest that the quality of maternal caregiving should be a major target for intervention, especially among prenatally stressed mothers. The lack of moderating effects of maternal EA in the link between prenatal maternal stress signals and both infants' temperament and cognitive development deserve further investigation, although it might indicate that the effects of maternal care in buffering prenatal influences vary depending on the nature of the outcome investigated.

Sixth, our findings support the emerging view that various forms of prenatal adversity may increase the sensitivity of the developing organism to the influences of the postnatal environment (Pluess & Belsky, 2011), as a significant association between maternal EA and infants' stress reactivity was reported only among infants prenatally exposed to higher cortisol levels. This hypothesis has been only rarely addressed within the DOAhD field. Initial studies showed that the effects of environmental factors, such as socio-economic status, maternal care or breastfeeding, on a number of neurodevelopmental outcomes in the offspring are greater among low as compared to normal birth weight (Buss et al., 2007, Jefferis et al., 2022, Kelly et al., 2001, Bonhert et al., 2008, Tully et al., 2004, Anderson et al., 1999). Furthermore, it has been shown that the effects of socio-economic status on long-term memory are greater among infants prenatally treated with synthethic glucocorticoids (Grant et al., 2015). However, to our knowledge, this is the first study to indicate that prenatal exposure to endogenous maternal cortisol might alter infants' susceptibility to the quality of maternal care. The possibility that the quality of in utero environment might influence, at least in part, the wide diversity among children in the degree to which developmental outcomes are influenced by the environment deserves further investigation as it holds the potential to inform and improve early interventions strategies

Collectively these findings bring to the forefront a new approach for research into the DOHaD hypothesis and represent a step forward in the understanding of early programming of both behavioral and biological outcomes by maternal prenatal and postnatal influences.

7.4 Future directions

The studies included in the current thesis have sought to address some of the issues outlined in the initial chapter and provide novel contribution to the state-of-a-science on an important topic concerning the effects of antenatal maternal depression.

However, many questions remain unanswered and the current results suggest many promising future directions for the study of the DOHaD hypothesis.

First, while stress-related biological mechanisms possibly involved in fetal programming were reviewed and examined separately, they are not likely to be alternatives to each other. Future studies on larger samples should address the complex bi-directional interplay among stress and inflammatory pathways that is likely to underline the effects of antenatal maternal depression on fetal development. In addition, we provided very preliminary evidence to suggest that the interaction among stress and inflammatory response systems might be altered among women with higher depressive symptoms during pregnancy; however, this finding deserves further investigation.

Second, we reported associations among alterations in maternal cortisol diurnal rhythm during pregnancy and later patterns of offspring stress reactivity. However, as discussed above, the mechanisms underlying this association are still to be clarified. In particular, it is unclear whether variations in maternal diurnal cortisol profile during pregnancy might actually lead to more cortisol passing through the placental barrier to directly affect fetal development. Future studies including more direct indexes of fetal intrauterine cortisol exposure such as amniotic fluid cortisol (Baibazarova et al., 2013; O'Connor et al., 2013) or cord blood cortisol (Miller et al., 2005) could provide greater leverage for testing the effects of antenatal cortisol exposure.

Third, future studies exploring the link between antenatal maternal stress and inflammatory markers and offspring's functional and structural alterations of the brain represent an important avenue for future research in that they might help to explain the pathways underlying the reported associations (e.g. Rasmussen et al., 2018; Buss et al., 2012).

Fourth, while the current thesis outlined significant associations among several indices of maternal antenatal stress and infants' outcomes from birth to three months

of age, our findings are limited by the short postnatal follow-up. Thus, it remains unknown whether the effects of antenatal maternal stress on later biological, behavioral and cognitive outcomes might persist later in development (e.g. O'Donnell et al., 2013), change accordingly to the offspring's age (e.g. Osborne et al., 2018) or whether the antenatal stress-related altered outcomes might be predictive of subsequent developmental outcomes (e.g. Thomas et al., 2017; Grahman et al., 2017). Furthermore, the interplay among maternal prenatal and postnatal influences in determining offspring outcomes later in development is unknown. To begin to address some of these questions, children from the current cohort were re-assessed at 13 months of age and are being currently re-assessed at approximately 40 months of age. While this work goes beyond the current thesis timeframe, the results of these later follow-ups will help to shed light on long-lasting effects of antenatal maternal stress as well as on the buffering role of postnatal maternal care.

Fifth, future studies are warranted in order to elucidate the differential effects of exposure during different critical periods along gestation by including multiple measures of stress and inflammation throughout pregnancy, at least during early, mid- and late pregnancy. While a similar approach will be quite expensive and demanding for women, it is expected to provide novel data on the most susceptible gestational periods for the effects of distinct stress-related mechanisms. In addition, recent evidence suggests that changes in cortisol secretion as gestation advances (Davis & Sandman, 2010; Davis et al., 2011) are more predictive of later infant development, as compared to single cortisol assessment. Thus, repeated biological assessments across gestation might help to evaluate the trajectory of change in each biological marker levels and better understand possible gestational timing effects.

Sixth, while the observed associations have been adjusted for infant gender, the current sample size did not allow us to directly examine possible gender differences in the link between maternal antenatal stress and child outcomes. Nevertheless, the possibility that the effects of prenatal stress may be sex-dependent

remains an important issue for future research as several lines of evidence converge to indicate that that the effects of antenatal stress might differ depending on fetal gender. First of all, it has been shown that maternal cortisol secretion during pregnancy differs as a function of fetal sex (e.g. Bleker et al., 2017; DiPietro et al., 2011; Giesbrecht et al., 2015; Hocher et al., 2009; Glynn & Sandman, 2011; Mitchell et al., 2017). In line with these findings, in Chapter 3, we reported lower cortisol levels at awakening in women carrying female fetuses, relative to males, although fetal sex was not found to moderate the association between antenatal maternal depression and the diurnal cortisol pattern. Additionally, sex differences in placental function, including expression of 11-βHSD2 (Mericq et al., 2009) and glucocorticoid receptors (Saif et al., 2015), glycemic control and blood pressure have been reported (Hocher et al., 2009; Petry et al., 2007). Furthermore, mounting data from animal models and preliminary evidence in humans suggest that, while both males and females are susceptible to the effects of maternal antenatal stress, the pattern of effects might differ depending on fetal sex, resulting in sexually-dimorphic fetal growth strategies and outcomes (Sandman, Glynn & Davis, 2013; Glover & Hill, 2012; Van den Bergh et al., 2017). For example, preliminary findings in humans suggest that sex moderates the effects of prenatal distress on children's stress reactivity, with males displaying blunted response, whereas females showing increase reactivity to stressors (reviewed in Carpenter et al., 2017). Interestingly, Giesbrecht and colleagues (2017) reported that sex significantly moderated the association between a higher maternal CAR and 3-month-olds cortisol reactivity to a blood draw in a large sample (N= 236), with males showing a flatter response, in line with our findings on the whole sample at birth (Chapter 4), and females showing a greater cortisol response. Despite we did not examine sex differences in newborns' cortisol response, the sex ratio in our sample slighlty favoured males (51.9%) over females (48.1%), thus it is possible that the direction of the observed effect might be partially driven by the composition of our sample (Gisebrecht et al., 2017). However, adjusting for sex did not change the

direction of the association. Sex differences in the effects of antenatal maternal stress might also account for unexpected findings in the current study. For example, in contrast with our initial hypothesis, we did not report any significant association between antenatal maternal cortisol levels and infants' NA (Chapter 5). We might speculate that a failure to account for sex differences might explain this null finding. Indeed, initial evidence indicates that heightened maternal cortisol in pregnancy is associated with a more difficult temperament (Sandman et al., 2013; Braithwaithe et al., 2017) in females, while opposite effects have been reported in some cases in males (e.g. Braithwaithe et al., 2017). As ignoring sex difference essentially takes the average of the effects for males and females, in the case they are opposite, this might lead to a null overall effect (Gisbrecht et al., 2017). Future studies in larger samples should be designed to directly examine sex-dependent processes underpinning the effects of prenatal stress on infants' developmental trajectories, whereby females tend to become more reactive to challenge and more anxious, and males become less reactive and more aggressive (Glover and Hill, 2012; Sandman et al., 2013). These findings may also have relevance to our understanding of sex differences observed in the incidence and presentation of psychiatric and stress-related disorders.

Seventh, studies investigating the interplay among prenatal and postnatal maternal influences in determining offspring's developmental trajectories are scarce. We provided novel evidence for a buffering effect of the quality of maternal care in the association between antenatal maternal cortisol and infants stress reactivity. However, future studies should examine the extent to which other environmental influences, such as partner support or positive family functioning, might moderate the association between antenatal maternal stress and child outcomes.

Eighth, the current findings need to be replicated in future studies adopting a genetically informed design (Rice et al., 2018) before strong conclusions can be drawn. For example, Rice and colleagues (2010) reported significant associations between prenatal maternal stress and some indices of later child development (infant

birth weight and conduct problems; though not all e.g. anxiety, ADHD) when using an in vitro fertilization-based prenatal cross fostering design which controls for genes shared between mother and child. In addition, while a number of genetic variants have been found to interact with an adverse early environment to increase vulnerability for later depression (e.g. Caspi et al., 2003) or anti-social behaviour (e.g. Taylor & Kim-Cohen, 2007; Thapar et al., 2005), comparatively, a small number of studies (Buchmann et al., 2014; Hill et al., 2013; Pluess et al., 2011; Zohsel et al., 2014) have investigated whether these genetic polymorphism might moderate the link between prenatal stress exposures and later emotional and behavioural outcomes. This kind of studies are expected to provide a valuable contribution to the DOHaD field.

Lastly, observational studies have inherently limited leverage to test whether the effects of prenatal maternal influences on child outcomes is truly causal. Future studies should be aimed to provide more experimental evidence in humans. In particular, findings from randomized controlled trials on evidence-based intervention to reduce antenatal depressive symptoms, hold promise for providing an experimental test of the DOHaD hypothesis. More strictly speaking, evidence demonstrating that reducing prenatal maternal depressive symptoms has an impact on child biological and behavioral outcomes (while controlling for relevant postnatal influences), thereby decreasing later risk for psychopathology would provide strong evidence for a true antenatal effect of maternal depression with substantial theoretical and clinical implications.

7.5 Concluding remarks

The amount of evidence linking maternal antenatal depression with an increased risk of altered biological, behavioral, emotional and cognitive outcomes in offspring is now substantial (reviewed in Van den Bergh et al., 2017). Knowledge concerning the underlying mechanisms is strikingly much less advanced. The current dissertation has addressed an existing gap in the literature by prospectively

investigating three stress-related biological pathways possibly underlying fetal programming by maternal antenatal depression and by examining the interplay between prenatal and postnatal maternal influences. Findings from the current thesis represent a first step towards understanding the role of inflammation and stress hormones in developmental programming, as well as the buffering role of postnatal sensitive caregiving. These are promising and largely unexplored areas for future scientific inquiries. Work is needed to elucidate the nature of the in-utero pathways leading to the observed associations between maternal antenatal influences and infant bio-behavioral outcomes in a normative sample. In addition, we need to understand more about gestational ages of sensitivity, sex differences and role of genetics and gene-environment interactions.

From a clinical perspective, the current thesis provides evidence that even without meeting full criteria for a clinical diagnosis, maternal depressive symptoms during pregnancy are associated with an altered stress-related biology and infant outcomes, thus outlining the need for additional attention to maternal mental health during the perinatal period. As prevalence estimates suggest that up to 30% of women during pregnancy experience depressive symptoms (Bennet et al., 2004; Banti et al. 2011), the importance of considering maternal mental health as a population health issue of high significance cannot be overstated. Maternal depression and anxiety is mostly undetected by health practitioners providing perinatal care (Alder et al., 2011). In contrast, we proposed that maternal psychological health during pregnancy should be addressed as fully as any other medical aspects of antenatal care and that midwifes and obstetricians should be trained to detect signs of women distress. Pregnant women who present high levels of depressive symptoms should deserve careful evaluation and monitoring as they likely represent a vulnerable population who may need additional help and support during the transition to parenthood. Paralleling to this, more research is needed into psychological interventions effective in reducing depressive and anxiety symptoms during pregnancy, as to date preventive antenatal

intervention have shown little effect in reducing maternal distress and prenatal intervention leads only to a small reduction in symptomatology (see Fontein-Kuipers et al., 2014 meta-analytical findings). For pregnant women who are severely depressed, more specialist care, possibly included antidepressants, may be needed.

Clinical implications of the observed associations among stress and inflammatory markers and both antenatal depressive symptoms and child outcomes are yet less clear. Future research is needed to understand whether the assessment of inflammatory markers into routine clinical practice may aid the development of prevention and treatment strategies. For example, inflammation has been found to predict response to antidepressants (Cattaneo et al., 2013) and there are evidence to suggest that adjunctive anti-inflammatory treatment might be needed in some treatment-resistant depression cases (Raison et al., 2013). In light of the demanding nature of the assessment of diurnal cortisol, involving multiple samples within the day, cortisol diurnal assessment seems a less feasible option to be included in standard care and might be offered only to sub-groups of women. There is still a long way to go before the assessment of biomarkers of risk for depression can be incorporated in routine clinical assessment and be prevention and/or intervention targets. Future studies should establish whether the dysregulation of IRS and HPA axis play a pathophysiological role in antenatal depression and whether psychological interventions for depression lead to changes in cortisol or inflammatory markers alongside mood improvements, as there is limited acceptance of pharmacological interventions during pregnancy (Goodman, 2009).

Lastly, findings from the current thesis indicate that whether or not the observed association between maternal antenatal cortisol and infant stress reactivity is mediated by genetic transmission (e.g. Van Hulle et al., 2012) or by in utero cortisol-mediated mechanisms (e.g. O' Donnell et al., 2009), changes in infant stress-related systems might be over-ridden by a postnatal sensitive environment, thus extending the window of opportunities for preventive interventions beyond the first nine months of

life. In contrast, the effects of poor parenting might exacerbate the effects of prenatal stress influences contributing to influence later vulnerability for health and mental outcomes (Schlotz & Phillips, 2009). Parenting is a challenging task and might become even more demanding and overwhelming for women experiencing antenatal distress (Easterbrooks et al., 2012). A number of well-validated attachment-based interventions, such as the Video-Feedback Intervention to Promote Positive Parenting (VIPP; Juffer, Bakermans-Kranenburg, & Van Ijzendoorn, 2012), have been proved to be effective in promoting mother-infant relationship, enhancing maternal sensitivity and EA, in several context (reviewed in Juffer, Bakermans-Kranenburg, & van IJzendoorn, 2017) including with depressed mothers (Stein et al., 2018), thus constituing a valid option to be offered to antenatally depressed mothers.

If the current findings are replicated, they suggest that researchers and clinicians should join their efforts to find the best way to effectively identify women experiencing depressive symptoms during pregnancy and provide them with accessible and effective antenatal and postnatal treatment in order to help all mothers and babies have the best start in life.

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Appendices

APPENDIX A: UCL Research Ethics Committee Approval

UCL RESEARCH ETHICS COMMITTEE
GRADUATE SCHOOL OFFICE

Professor Pasco Fearon
Research Department of Clinical, Educational and Health Psychology
UCL

7 May 2014

Dear Professor Fearon

Notification of Ethical Approval
Project ID: 5617/001: The effects of maternal depression during pregnancy on infants' psychological and biological development from birth to 3 months of age

I am pleased to confirm that your study has been approved by the UCL Research Ethics Committee for the duration of the project i.e. until October 2016.

Approval is subject to the following conditions:

1. You must seek Chair's approval for proposed amendments to the research for which this approval has been given. Ethical approval is specific to this project and must not be treated as applicable to research of a similar nature. Each research project is reviewed separately and if there are significant changes to the research protocol you should seek confirmation of continued ethical approval by completing the 'Amendment Approval Request Form'.

The form identified above can be accessed by logging on to the ethics website homepage: http://www.grad.ucl.ac.uk/ethics/ and clicking on the button marked 'Key Responsibilities of the Researcher Following Approval'.

2. It is your responsibility to report to the Committee any unanticipated problems or adverse events involving risks to participants or others. Both non-serious and serious adverse events must be reported.

   Reporting Non-Serious Adverse Events
   For non-serious adverse events you will need to inform Helen Dougal, Ethics Committee Administrator (ethics@ucl.ac.uk), within ten days of an adverse incident occurring and provide a full written report that should include any amendments to the participant information sheet and study protocol. The Chair or Vice-Chair of the Ethics Committee will confirm that the incident is non-serious and report to the Committee at the next meeting. The final view of the Committee will be communicated to you.

   Reporting Serious Adverse Events
   The Ethics Committee should be notified of all serious adverse events via the Ethics Committee Administrator immediately the incident occurs. Where the adverse incident is unexpected and serious, the Chair or Vice-Chair will decide whether the study should be terminated pending the opinion of an independent expert. The adverse event will be considered at the next Committee meeting and a decision will be made on the need to change the information leaflet and/or study protocol.

On completion of the research you must submit a brief report (a maximum of two sides of A4) of your findings/concluding comments to the Committee, which includes in particular issues relating to the ethical implications of the research.
With best wishes for your research.

Yours sincerely

Professor John Foreman
Chair of the UCL Research Ethics Committee

Cc:
Sarah Nazzari, Applicant
Professor Peter Fonagy, Head of Department

UCL Research Ethics Committee, c/o The Graduate School, North Cloisters, Wilkins Building
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ethics@ucl.ac.uk
www.ucl.ac.uk/gradschool

APPENDIX B: Scientific Institute Eugenio Medea Ethics Committee Approval

ASSOCIAZIONE la Nostra Famiglia
EUGENIO MEDEA
ISTITUTO DI RICOVERO E CURA A CARATTERE SCIENTIFICO

Comitato Etico
23842 Bosisio Parini (LC), Via Don Luigi Monza 20 -

Gentile Dr.ssa
Alessandra Frigerio
Responsabile Scientifico del Progetto
Sede di Bosisio Parini

epc Al Direttore Scientifico
Prof. Nereo Bresolin
Al Responsabile Segreteria Scientifica

Prot. no. 039/14-CE Bosisio Parini, 28 maggio 2014

PROTOCOLLO STUDIO 039/14 (da indicare in qualsiasi comunicazione)

Il Comitato Etico dell'IRCCS E.MEDEA Sez.Scient. dell'Associazione La Nostra Famiglia, riunitosi il giorno 29 aprile 2014, presso la sede di Bosisio Parini.

PRESENTI: Dr.ssa Paolo Arosio, Presidente (Pediatra), Dr.ssa Graziella Uziel (Clinico), Dr. Giovanni Manenti (Rapp.te Volontariato/Associazionismo) ,Dr.ssa Simona Caravita (Clinico), Dr.ssa Luisa Minoli (esperto in materia assicurativa), Dr.ssa Cristina Sobacchi (Genetista), Dr. Roldano Fossati (Medico di medicina generale), Dr.ssa Eliana Rulli (Biostatistico), Dr. Marco Pozzi (Farmacologo), Dr. Angelo Primavera (farmacista), Dr.ssa Francesca Villanova (Rapp.te Prof.Sanitarie), Ing. Paola Grigioni (Esperto Dispositivi Medici), Prof. Nereo Bresolin (Direttore Scientifico).
ASSENTI GIUSTIFICATI: Dr. Don Stefano Cucchetti (Teologo Moralista), Dr.ssa Simona Guarisco (Clinico), Prof. Leonardo Lenzi (Esperto in Bioetica), Avv. Anna Paola Manfredi (Giurista), Dr. Ambrogio Bertoglio (Direttore Sanitario).

VERIFICATA La presenza del numero legale, essendo presenti 13 membri su 18.

ESAMINATA la documentazione presentata :

1) Progetto di Ricerca "GLI EFFETTI DELLA DEPRESSIONE MATERNA IN GRAVIDANZA SULLO SVILUPPO PSICO-BIOLOGICO DEL BAMBINO DALLA NASCITA AI PRIMI 3 MESI DI VITA"
2) Dichiarazione di Consenso a partecipare alla ricerca, per Madre del Nascituro
3) Dichiarazione di Consenso, modulo per i genitori/tutore del minore
4) INFORMATIVA E CONSENSO AL TRATTAMENTO DEI DATI PER RICERCA
5) Lettera Informativa per i genitori

RILEVATO CHE lo studio clinico
é rispettoso dei principi etici dell'istituzione;
è rispettoso della rilevante normativa vigente,

HA ESPRESSO il proprio parere favorevole allo svolgimento della Ricerca "GLI EFFETTI DELLA DEPRESSIONE MATERNA IN GRAVIDANZA SULLO SVILUPPO PSICO-BIOLOGICO DEL BAMBINO DALLA NASCITA AI PRIMI 3 MESI DI VITA" presentato dalla Ricercatrice, Dr.ssa Alessandra Frigerio.

Il parere è stato espresso all'unanimità.

ASSOCIAZIONE
la Nostra Famiglia
EUGENIO MEDEA
ISTITUTO DI RICOVERO E CURA A CARATTERE SCIENTIFICO

Comitato Etico
23842 Bosisio Parini (LC), Via Don Luigi Monza 20 - e-mail: comitato.etico@bp.lnf.it - tel +39 031 877565fax +39 031 877499

Si rilascia quindi il PARERE FAVOREVOLE all'effettuazione della ricerca, in quanto nulla osta per quanto di competenza del Comitato Etico.

Si richiede che questo Comitato Etico venga informato dell'inizio dello studio e della sua conclusione o interruzione.

Il Responsabile del progetto dovrà far pervenire al Comitato Etico, una relazione annuale sull'andamento della sperimentazione.

Si rammenta inoltre che il Comitato Etico dovrà essere informato riguardo qualsiasi emendamento dovesse rendersi necessario al progetto per relativa approvazione del Comitato e di qualsiasi evento avverso che dovesse verificarsi durante lo svolgimento.

Cordiali saluti.

Per Il Presidente, Dr. Paolo Arosio

La segretaria
Monica Castelli

APPENDIX C: Participant Information Sheet and Consent Forms

INFORMATION SHEET

THE EFFECTS OF MATERNAL DEPRESSION DURING PREGNANCY ON INFANTS' PSYCHOLOGICAL AND BIOLOGICAL DEVELOPMENT FROM BIRTH TO 3 MONTHS OF AGE.

Research Supervisors: Dr. Alessandra Frigerio & Prof. Pasco Fearon

This study has been approved by the UCL Research Ethics Committee (Project ID Number: 5617/001) and by the Eugenio Medea Scientific Institute Ethics Committee.

Dear parents,

We would like to invite you to take part in a new research project entitled "The effects of maternal depression during pregnancy on infants' psychological and biological development from birth to 3 months of age". The project is being organised by the Eugenio Medea Scientific Institute of Bosisio Parini (LC) in collaboration with the Research Department of Educational, Clinical and Health Psychology of the University College London and the hospital where you decided to give birth to your child.
We are inviting expectant mothers and fathers to take part in this study together with their babies. While mothers and babies will be actively engaged during the research sessions, we will welcome fathers' interest and we will be happy to answer their questions and share the study results.

Before you decide whether you want to take part, it is important that you read the following information carefully and discuss it with others if you wish. Please get in touch with Dr Alessandra Frigerio or with Sarah Nazzari, if there is anything unclear or you would like more information.

You should only participate if you want to. Participation in this study is voluntary. Hence, at any time, you can decide to withdraw or not to take part and this will not disadvantage you or influence the standard of care you are receiving. Should you decide to participate in the research, you will be given a copy of the present Information Sheet to keep and we will ask you to sign some Informed Consent forms.

Which is the aim of the study?
The main aim of our research is to understand how mothers' mood during pregnancy could influence infant development from birth to 3 months of age.

We know that for some women pregnancy can be a stressful time, and some mothers can experience feelings of anxiety or depression. Stress, anxiety and depression may also have physical manifestations, although little is known about how these physical aspects of stress appear among pregnant women. The bodily systems that help us deal with stress undergo significant changes during pregnancy and delivery, most likely as part of the body's preparation for birth. A key aim of this study is to further our understanding of how the physical symptoms of stress present themselves during pregnancy. A second important aim is to investigate whether these physical symptoms are associated with any differences in your baby's health and development.

Also, we know that the environment where infants grow up and, in particular, the early relationship between mother and child, plays an important role in a child's development. Therefore, we are interested in the role this plays as well, and whether the parental relationship overrides any stressful experiences during pregnancy.

We believe that a better understanding of whether and how psychological and physical factors might affect children's early development is important for helping mothers and babies have the best start in life.

Who are we looking for?
To answer all those questions, we need the participation of women during pregnancy and of their new-borns from birth to 3-months of age.

What will you be asked to do?
The study involves two phases: in *Phase I*, we will ask a large group of expectant mothers to complete some questionnaires. In *Phase 2*, we will select two smaller groups of mothers, one that is experiencing more stress and one that is experiencing less. We will invite both groups to take part in further research sessions at 36-38th week of pregnancy, within 48 hours of your babies' birth and 3 months after the delivery.

Please note that deciding to take part in the study means that you are willing to be involved during all its phases. However, you will be free to withdraw at any time without giving any reason. If this would be the case, we will delete all your personal data while we will keep the anonymous data collected until that moment, not to alter the study results.

What do the Research Sessions Involve?

Phase I

- ***Before 34th week of pregnancy***

We will ask all mothers to fill in two questionnaires about depression and anxiety and some forms to collect data on family background (e.g. age, education level), pregnancy (e.g. gestation weeks, existence of complications) and health (e.g. weight, height, possible diseases). Filling out these questionnaires usually takes 40 minutes. Mothers can decide to fill them out immediately or at home; they can discuss any questions with us personally or on the phone and, of course, they don't have to answer any questions they don't want to answer.

Data collected during Phase I will allow us to understand if this study is suitable for your family and to create two smaller groups of women, one experiencing more stress, depression or anxiety and one that is experiencing less. If this study, is not suitable for your family, you can choose to be contacted for future studies that might be a better fit (by ticking the specific box in the consent form). If this study is suitable for you, we will invite you to take part in research sessions of Phase II.

Phase II

- ***36-38th week of pregnancy***

During childbirth classes, we will give mothers some questionnaires to fill out at home about any stressful events they might have experienced recently, the strategies they use to deal with them and their social support, as well as all the material and instructions needed to collect their saliva at home.

A researcher will get in touch with you to schedule a meeting at our Institute and to ask all expectant mothers to collect their saliva at three different times (wake up, 30 minutes after wake up, before going to bed) during the two days prior to our meeting. We will remind you to collect each sample by phone or text, and we will be available to answer all your questions. The analysis of saliva will gives us information on how the bodily systems that help us deal with stress function during pregnancy. Specifically, we will analyse the levels of two markers of stress known as cortisol and alpha amylase.

During our meeting at the Medea Institute, we will interview all mothers about possible symptoms of stress, depression and anxiety, we will ask them to complete a test to assess some mental abilities during pregnancy and to return all questionnaires and saliva samples. This session will last approximately 2 hours. However, the time varies person-to-person. Please note that should mothers need it, it would be possible to take a break or schedule another meeting to complete the administration.

Possibly on an occasion of another medical examination, we will ask all mothers if they are willing to give a small blood sample that will be taken by a qualified nurse. The analysis of blood will give us information

about another important bodily system that helps us deal with stress: the immune system. Specifically, we will analyse the levels of some markers of inflammation (such as the C-Reactive Protein or Interleukin-6).

- ***Within 48 hours of your Baby's birth***

We will ask you to let us know as soon as your baby is born. Shortly after the birth, during mothers' stay in hospital, we will ask all mothers to collect their saliva according to the same procedures followed during pregnancy and to donate another small amount of blood. The analyses of both saliva and blood will help us to understand how the delivery affects the functioning of the same bodily systems and markers evaluated during pregnancy.

We will also ask all mothers to fill out a form concerning the delivery, their health and baby's health (e.g. weight, height, Apgar scores) and to complete once again the two questionnaires administered during pregnancy to evaluate symptoms of depression and anxiety.

Between the first 30 and 48 hours after your baby is born, a newborn screening test is routinely performed in the hospital. The test (known as the 'heel stick test') consists of a small blood draw from the baby's heel that is later analysed to screen for some genetic diseases. This test is done routinely for babies of this age by the hospital and is not part of this research project. However, as part of our study, we will collect your baby's saliva before the start of this procedure, and 20 and 40 minutes from the end of it, to evaluate how his/her bodily systems deal with the stress of the heel stick (as indexed by his/her levels of cortisol). To collect saliva, we will employ some small cotton swabs specifically designed for the safe collection of saliva from new-borns. We can let you do it, while one of us helps if you prefer. We will also videotape your baby's behaviour during the heel-stick in order to evaluate his/her reaction to the heel-stick.

- ***3 month-olds***

When your infant will be 3 month-olds, we will get in touch with you to plan our last two meetings.

1) During a session at our Lab, we will ask all mothers to take part in 15 minutes of free play with their child that will be videotaped. This observation will give us information on how babies interact with their moms during play. A trained researcher will then administer to your child some scales to evaluate his/her development. The researcher will show your child a number of stimuli (such as a ball, a bell, etc.) and will observe his/her reactions and behaviours. You will be present with your child throughout.

We will also give you a questionnaire on your infant's behaviour to get information about how your baby is in his/her everyday environment, a form about your baby's health and feeding, as well as a diary for noting, over the course of 24 hours, your infant's periods of sleep, wakefulness and crying. Lastly, we will ask mothers to fill out all questionnaires completed during pregnancy again, to see how things have changed since your baby's birth. You can return all questionnaires on the last session.

This session will last approximately one hour and half. Please note that we always adapt testing sessions to each child's individual needs. This means that we take as many breaks as the child needs to feed, rest or play. We will do our best to make your visit as comfortable and enjoyable as possible.

2) At the time of your child's first vaccination, which is compulsorily provided by the Government Ministry for Health at 3 months of age, we will meet you at your chosen vaccination centre. On that occasion we will collect your baby's saliva before the beginning of the vaccination and after 20 and 40 minutes from the end with the same cotton swabs employed at birth, to study how babies' bodily systems deal with a mildly painful stress (the vaccination) at 3-month-olds (as indexed by his/her levels of cortisol). We will also videotape your child's behaviour during the vaccination to see how he/she reacts to the vaccination. Lastly, we will ask you to return all the questionnaires previously filled out.

How will the biological samples be store and analysed?

All biological samples will be stored at -80° until analysed at the Biological Lab of Eugenio Medea Scientific Institute, according to scientifically approved procedures. All biological analyses will be carried out by specialized staff from the second year of the research.

Please note that:

- All biological samples will be stored anonymously and identified with a unique code.
- All biological samples will be analysed only for the present research purposes. If you are interested in having more details about the biological systems under investigation or the analyses performed, please do not hesitate to get in touch with one of the researcher.

Does the research involve any risks?

Your participation in the study does not involve any risks for you or for your child.
The procedure to collect infants' saliva is safe, quick, does not have any side effects and is not invasive. Previous studies have found that infants do not show significant distress related to this procedure. However, if your child becomes upset, we will stop the procedure. We would like to remind you that you will be present throughout the procedure and if you prefer, we could let you do it. The blood sample that we ask mothers to provide both in pregnancy and after child's birth will be carried out by a qualified nurse and will not exceed 10mL. To minimize mothers' distress, we will do our utmost to schedule to coincide with other blood test required by your hospital.

All members of our research group are qualified to perform all procedures and assessments of the protocol. Please note also that either one of you, as parents, will be always present throughout all the evaluations.

The study protocol complies with the Guidelines on Good Clinical Practice of the European Union and with the Declaration of Helsinki. This study has been approved by the Eugenio Medea Scientific Institute Ethics Committee and by UCL Research Ethics Committee.

What are the potential benefits?

Our study aims at a better understanding of the factors that, during pregnancy and in the post-partum, could influence infants' development. Your participation in this study will help us shed light on the psychological, biological and social factors that are important for infants' growth. In this way, we hope it would be possible to find new strategies to help women during pregnancy as well as their new-borns.

This is likely to mean that there are no immediate benefits for participating mothers and children, but we hope that your help will be beneficial to other mothers and children in the future. Also, we hope that you will find this experience enjoyable and interesting and we will invite you, at the end of the study, to discuss with a researcher about it.

Finally, we will give mothers a brief report of the results of the questionnaire and interview evaluating symptoms of depression and anxiety during pregnancy and we can give information about services that could offer you support. If you wish, we can also give you a brief report of the scores of your 3 month-old child on the scales we administer and on the questionnaire that you completed. We would also be happy to answer any questions you might have about this. Please note, however, that this study does not conduct diagnostic assessments of your child's development. If you had any concerns about your child's development, you would need to seek a professional opinion (e.g., from your doctor).

Lastly, if you wish, we can keep you updated about the results of this study and our future publications.

Who will have access to your data and how they will be treated?

Your data will be treated and protected according to the Italian Legislative Decree N°196/03 principles and to the English 1998 Data Protection Act principles.

We adopt strict procedures to guarantee the maximum safety and confidentiality of your personal information, specifically:

- Your personal information (like names, address and phone number) will be kept separate from all other study data.
- Biological data, questionnaires and videotapes will be identified only with a unique code.
- All data will be stored in locked file cabinets and in password protected files.

- All data will be keep as long as required for our research purposes. After that period, all data will be destroyed by use of a robust and secure method.

Please note that only the Research Supervisor, Dr Alessandra Frigerio, will access your personal data. The access to all other study data will be permitted only to authorized staff. Specifically, only the members of the research team of the Medea Scientific Institute and of the UCL, the Research Ethic Committee of both institutes, the Health Authorities and the staff deputed to verify the procedures could access the archive, without, however, being able to trace back to your personal identity.

The only exception where a breach of confidentiality might be required is if the researchers had serious concerns about your child's safety or your safety. In such a case, we have a legal duty of care to contact relevant professionals to ensure your's or your baby's safety. Wherever possible, we would discuss this with you and explain our professional duty of care before taking any action.

What happens to the findings?
The results of this study will be subject matter of a PhD thesis and of future publications. Please note that what will be reported in the thesis and any other publications are the average results of groups of participants and not individual results. No individual will be named or identified and no published information will allow in any way to trace you back.

Depending on the study results and on funds available, the research could involve, in the future, a further assessment of your child's development. You are free to decide if you wish to be contacted in the future about any follow-up studies.

How can you contact the researchers?
We are available for any further information or clarification. If you have any questions or you would like to take part in this study, please contact by phone or by email:

- Dr. Alessandra Frigerio
- Sarah Nazzari

The Research Supervisors
Dr. Alessandra Frigerio
Prof. Pasco Fearon

This study has been funded with the contribution of
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CONSENT FORM: Mother's participation

THE EFFECTS OF MATERNAL DEPRESSION DURING PREGNANCY ON INFANTS' PSYCHOLOGICAL AND BIOLOGICAL DEVELOPMENT FROM BIRTH TO 3 MONTHS OF AGE.

Research Supervisors: Dr. Alessandra Frigerio & Prof. Pasco Fearon

Please complete this form after you have read the Information Sheet and listened to an explanation about the research

This study has been approved by the UCL Research Ethics Committee (Project ID Number: 5617/001) and by the Eugenio Medea Scientific Institute Ethics Committee.

Thank you for your interest in taking part in this research. Before you agree to take part, one of the member of the research team must explain the project to you.

If you have any questions arising from the Information Sheet or explanation already given to you, please ask the researcher before you decide whether to join in. You will be given a copy of this Consent Form to keep and refer to at any time.

Participant's Statement

I

- Have read the notes written above and the Information Sheet, and understand what the study involves.
- Have received from..…..…… (Name and surname of Researcher obtaining consent) explanations about the study described in the Information Sheet and have had the possibility to ask questions and received answers to my satisfaction.
- Understand that if I decide at any time that I no longer wish to take part in this project. I can notify the researcher involved and withdraw immediately. I understand that, in this case, all personal data will be deleted while the anonymous data collected until that moment will be kept, not to alter the study results.
- Understand that some assessments will be videotaped, as described in the information sheet, and consent to processing of this material for the purposes of this research study.
- Consent to processing of my personal information for the purposes of this research study.
- Understand that such information will be treated as strictly confidential and handled in accordance with the provisions of the English Data Protection Act 1998 and Italian Italian Legislative Decree N°196/03.

Please tick (√) appropriate box:

☐ **Yes,** I would like to participate in this study

☐ **No,** I do not want to participate in this study

If **Yes,** please complete the following:

I give consent to:

☐ Give one blood sample during pregnancy and after delivery

☐ Be contacted directly by the research team at the details given by me on this form

☐ Be contacted directly by the research team at the details given by me on this form for future follow-up of the present research

☐ For the details given by me on this form to be held on participant database for future research (please leave this box blank if you only wish for us to contact you regarding the present study and possible future follow-up, but do not wish to be contacted about other studies)

☐ The videotaped material to be used for scientific and educational purposes after the end of the study

Date _____

Mother's Name _____

Mother's Signature _____

Researcher's Name_____

Date _____ **Researcher's Signature** _____

Please fill in the following details, only if you are happy to be contacted by us for taking part in the study

Address:

……

……

Telephone number ..... Mobile number……

Email

……

.

CONSENT FORM: Child's participation

THE EFFECTS OF MATERNAL DEPRESSION DURING PREGNANCY ON INFANTS' PSYCHOLOGICAL AND BIOLOGICAL DEVELOPMENT FROM BIRTH TO 3 MONTHS OF AGE.

Research Supervisors: Dr. Alessandra Frigerio & Prof. Pasco Fearon

Please complete this form after you have read the Information Sheet and listened to an explanation about the research

This study has been approved by the UCL Research Ethics Committee (Project ID Number: 5617/001) and by the Eugenio Medea Scientific Institute Ethics Committee.

Thank you for your interest in taking part in this research. Before you agree to take part, one of the member of the research team must explain the project to you.

If you have any questions arising from the Information Sheet or explanation already given to you, please ask the researcher before you decide whether to join in. You will be given a copy of this Consent Form to keep and refer to at any time.

Participant's Parents' Statement

We as parents/legal representative of

(Child's name and surname).....

- Have read the notes written above and the Information Sheet, and understand what the study involves.
- Have received from..... (Name and surname of Researcher obtaining consent) explanations about the study described in the Information Sheet and have had the possibility to ask questions and received answers to our satisfaction.
- Understand that if we decide at any time that we no longer wish to take part in this project. We can notify the researcher involved and withdraw immediately. We understand that, in this case, all personal data will be deleted while the anonymous data collected until that moment will be kept, not to alter the study results.
- Understand that some assessments will be videotaped, as described in the information sheet, and consent to processing of this material for the purposes of this research study.
- Consent to processing of our personal information and that of our child for the purposes of this research study.
- Understand that such information will be treated as strictly confidential and handled in accordance with the provisions of the English Data Protection Act 1998 and Italian Italian Legislative Decree N°196/03.

Please tick (√) appropriate box:

☐ **Yes,** We would like our child to participate in this study

☐ **No,** We do not want our child to participate in this study

If **Yes,** please complete the following:

We give consent to:

☐ Be contacted directly by the research team at the details given by us on this form

☐ Be contacted directly by the research team at the details given by us on this form for future follow-up of the present research

☐ For the details given by us on this form to be held on participant database for future research (please leave this box blank if you only wish for us to contact you regarding the present study and possible future follow-up, but do not wish to be contacted about other studies)

☐ Receive a report of the results of the scores of our 3 month-old child on the scales administered and on the questionnaire that we completed, as described on the information sheet.

☐ The videotaped material to be used for scientific and educational purposes after the end of the study

Date _____

Mother's Name _____

Mother's Signature _____

Date _____

Father's Name _____

Father's Signature _____

Researcher's Name_____

Date _____ **Researcher's Signature** _____

Please fill in the following details, only if you are happy to be contacted by us for taking part in the study

Address:

……

……

Telephone number..... Mobile number…….....

Email

……

.

APPENDIX D: Ad-hoc forms

In what follows are presented the ad-hoc forms employed throughout the current study. Specifically, the “Sociodemographic Form” and “Prenatal Form” were employed to collect demographic and pregnancy-related data at 34-36 gestational weeks. The “Postnatal Form: Birth” was employed to collect delivery and both maternal and infant health-related data at birth. Lastly, “The Postnatal Form: 3 months” was used to collect data on infant health at 3 months of age.

Socio-demographic Form

To be completed by the researcher: **Subject ID #** _____ Date |__|__| |__|__| |__|__| dd mm yy

We ask you to fill out this form to collect data regarding your family background. We ensure you that what you are going to write is subjected to the maximum confidentiality. Of course, you don't have to answer any questions you don't want to answer. Do not hesitate to get in touch with a researcher for any clarification.

DATA CONCERNING YOU

Date of birth |__|__| |__|__| |__|__| dd mm yy

Nationality ☐ italian ☐ not italian

If not italian:

Country of origin _____
Citizenship_____
Years in Italy_____ ____

Education:

- ☐ Primary school
- ☐ Junior High School
- ☐ 2/3 years of Senior High School
- ☐ Senior High School
- ☐ Some years of college
- ☐ University degree
- ☐ Master/post-graduate specialization

Actual Employment _____

☐ **Full-time** ☐ **Part-time**

Income in euro:

- ☐ 0 – 10.000
- ☐ 10.000 – 15.000
- ☐ 15.000 – 31.000
- ☐ 31.000 – 70.000
- ☐ more than 70.000

DATA CONCERNING YOUR BABY'S FATHER

Date of birth |__|__| |__|__| |__|__| dd mm yy

Nationality ☐ italian ☐ not italian

If not italian:

Country of origin _____
Citizenship_____
Years in Italy_____ ____

Education:

- ☐ Primary school
- ☐ Junior High School
- ☐ 2/3 years of Senior High School
- ☐ Senior High School
- ☐ Some years of college
- ☐ University degree
- ☐ Master/post-graduate specialization

Actual Employment _____

☐ **Full-time** ☐ **Part-time**

Income in euro:

- ☐ 0 – 10.000
- ☐ 10.000 – 15.000
- ☐ 15.000 – 31.000
- ☐ 31.000 – 70.000
- ☐ more than 70.000

Which is your marital status?

☐ Single parent ☐ Married ☐ Common-Law Partners ☐ Separated ☐ Divorced ☐ Widower

Are you expecting your first baby? ☐ No ☐ Yes

If no, which is your baby's birth order? ☐ 2 ☐ 3 ☐ 4 other: ___

Specify for all your children:
Sex ☐M ☐F Date of birth |__|__| |__|__| |__|__|
Sex ☐M ☐F Date of birth |__|__| |__|__| |__|__|
Sex ☐M ☐F Date of birth |__|__| |__|__| |__|__|

Prenatal Form

To be completed by the researcher: **Subject ID #** _____ Date |__|__| |__|__| |__|__| dd mm yy

We ask you to fill out this form to collect data regarding your pregnancy and health. We ensure you that what you are going to write is subjected to the maximum confidentiality. Of course, you don't have to answer any questions you don't want to answer. Do not hesitate to get in touch with a researcher for any clarification.

1. Current week of pregnancy: _____ 2. Due date: _____
3. Your baby's sex: ☐ M ☐ F ☐ Don't know
4. Are you anticipating a vaginal birth? ☐ Yes ☐ No
5. What was your weight before you became pregnant? _____
6. What is your actual weight? _____
7. How tall are you? _____
8. Was your baby naturally conceived? ☐ Yes ☐ No
9. Did you take any fertility treatment? ☐ Yes ☐ No **If yes**, what kind? _____
10. Have you had any complications or problems with this pregnancy? ☐ Yes ☐ No

    If yes, please check those which are applicable:

    - ☐ Bleeding
    - ☐ Cramping
    - ☐ Amniotic fluid leakage
    - ☐ Water retention
    - ☐ High Blood Pressure
    - ☐ Rapid Weight Gain
    - ☐ Uterine abnormality
    - ☐ Proteine in urine
    - ☐ High Blood Sugar
    - ☐ Vision Disturbances
    - ☐ Severe Nausea
    - ☐ Abnormal vomiting or headaches
    - ☐ Abnormal fetal growth, heart beat, movements
    - ☐ Diabetes
    - ☐ Other: _____

11. Is your pregnancy considered high risk? ☐ Yes ☐ No

    If yes, please check those which are applicable:

    - ☐ Diabetes
    - ☐ Hypertension
    - ☐ Multiple pregnancy
    - ☐ Previous complicated pregnancy
    - ☐ Ashtma
    - ☐ Rh or genetic problem
    - ☐ Under 20 or over 35 years old
    - ☐ Fetal genetic disorders
    - ☐ Exposure to hazardous materials
    - ☐ Other _____

12. Do you have any physical restrictions related to your pregnancy? ☐ Yes ☐ No

If yes, what are they and why were they given to you? _____

13. Are you currently taking any prescribed medications? ☐ Yes ☐ No

If yes, what are they and why were they given to you? _____

_____

14. Are you currently taking any over-the-counter medications including vitamins or herbal remedies?

☐ Yes ☐No

If yes, what are you taking and why? _____

_____

15. Have you took any medications during your whole pregnancy? ☐ Yes ☐ No

If yes, what are they and why were they given to you? _____

_____

16. Have you received any immunizations (shots) during your pregnancy, for example, the measles shot, a flu shot, a shot for Rh incompatibility, or any other shots? ☐ Yes ☐ No

If yes, what did you get and when? _____

17. Have you received any antibiotics, antivirals or antifungals during your pregnancy, for example for a urinary tract infection, a sinus infection or other infection? ☐ Yes ☐ No

If yes, what did you get, when and for what? _____

18. Were you tested for B Beta strep? ☐ Yes ☐ No **If yes**, result: ☐ Negative ☐ Positive

19. Are you affected by any diseases/dysfunctions not related to pregnancy? ☐ Yes ☐ No

If yes, what are they? _____

20. From the beginning of your pregnancy have you made use of:

- Cigarette ☐ No ☐ Yes **If yes**, how often _____ and in what period _____
- Alcool ☐ No ☐ Yes **If yes,** how often _____ and in what period _____
- Other substances ☐ No ☐ Yes **If yes**, what kind _____

how often _____ and in what period _____

21. Have you ever seek professional help for any kind of psychological problems? ☐ Yes ☐ No

If yes, please describe _____

22. Is there any other relevant information about your pregnancy or about your health not covered previously?

☐ Yes ☐ No **If yes**, please describe _____

Postnatal Form: BIRTH

To be completed by the researcher: **Subject ID #** _____ Date |__|__| |__|__| |__|__|
dd mm yy

We ask you to fill out this form to collect data regarding your delivery, health and your Baby's health. We ensure you that what you are going to write is subjected to the maxinum confidentiality. Of course, you don't have to answer any questions you don't want to answer. Do not hesitate to get in touch with a researcher for any clarification.

DATA ON YOUR HEALTH AND DELIVERY

1. Date of delivery: |__|__| |__|__| |__|__| 2. Time of delivery: |__|__| |__|__|
3. Labor: ☐ Yes ☐ No If yes, length of labor: _____ hours
4. Was the labor induced? ☐ Yes ☐ No
5. Method of childbirth: ☐ Vaginal delivery ☐ Caesarean section
   If Caesarean section, reasons:_____
   any complications at site of incision? ☐ Yes ☐ No
6. Assisted vaginal delivery? ☐ Yes ☐ No
   If yes, please check those which are applicable: ☐ Forceps ☐ Ventouse
7. Episiotomy? ☐ Yes ☐ No
8. Perineal tear? ☐ Yes ☐ No If yes, stitches needed? ☐ Yes ☐ No
9. Any complications at site of episiotomy/stitches? ☐ Yes ☐ No
10. Epidural anesthesia? ☐ Yes ☐ No
    If yes, any complication at site of epidural catheter? ☐ Yes ☐ No
11. Time regular contractions begun: |__|__| |__|__|
12. Ruptured membranes before delivery: ☐ Yes ☐ No If yes, time of rupture: |__|__| |__|__|
13. Fever during labor: ☐ Yes ☐ No
14. Has your midwife detected anything abnormal or remarkable during your physical assessment once arrived at the hospital? ☐ Yes ☐ No
    If yes, please describe_____

15. Any complications during delivery? ☐ Yes ☐ No

If yes, please describe _____

16. Any blood transfusions given during labor and delivery? ☐ Yes ☐ No

17. Any medications, including anesthetics, taken during Labor and Delivery: ☐ Yes ☐ No

If yes, what are they? _____

18. Meconium staining: ☐ Yes ☐ No 19. Fetal distress: ☐ Yes ☐ No

20. Any complications during hospitalization? ☐ Yes ☐ No

If yes, please describe_____

21. Expected time of your discharge: |__|__| |__|__|

22. Is there any other relevant information about your delivery or health not covered previously?

☐ Yes ☐ No

If yes, please describe_____

_____

Please continue on the next page

DATA ON YOUR BABY'S HEALTH

1. Sex: ☐ M ☐ F
2. Gestational Age: _____ (weeks)
3. Birth Weight: _____ grams
4. Length: _____ cm
5. Head circumference: _____ cm
6. 1 minute Apgar _____
7. 5 minute Apgar _____
8. Baby's fever after birth? ☐ Yes ☐ No If yes, temperature _____ °
9. Evidence of infection? ☐ Yes ☐ No
10. Evidence of any infant congenital anomaly? ☐ Yes ☐ No
    If yes, please describe: _____
11. Any medications administered after birth? ☐ Yes ☐ No
    If yes, please describe: _____
12. Any additional care or intervention provided? ☐ Yes ☐ No
    If yes, please describe: _____
13. Did you ever breastfeed or try to breastfeed? ☐ Yes ☐ No
    If yes, at what time did you first put your Baby to the breast? |__|__| |__|__|
14. In the first 48-60 hours after delivery, was your Baby given anything to drink other than breast milk?
    ☐ Yes ☐ No
    If yes, what was your Baby given to drink:
    - ☐ Infant formula, please specify the name _____
    - ☐ Plain water
    - ☐ Sugar or glucose water
    - ☐ Sugar-salt-water solution
    - ☐ Other, please describe _____
15. Last time your Baby was feed (with breast milk or formula): |__|__| |__|__|
    After that time, was your Baby given something else to drink (e.g. water, sugar water) ☐ Yes ☐ No
    If yes, at what time? |__|__| |__|__| and what _____
16. Expected time of your Baby's discharge |__|__| |__|__|
17. Any other relevant information about your Baby's health _____

Postnatal Form: 3 MONTHS OF AGE

To be completed by the researcher: **Subject ID #** _____ Date |__|__| |__|__| |__|__| dd mm yy

We ask you to fill out this form to collect data regarding your infant's health and feeding. We ensure you that what you are going to write is subjected to the maximum confidentiality. Of course, you don't have to answer any questions you don't want to answer. Do not hesitate to get in touch with a researcher for any clarification.

1. Infant's actual weight: _____ grams 2. Infant's actual length: _____ cm

3. How would you describe your infant's health today?

   ☐ Poor ☐ Moderate ☐ Good ☐ Very Good ☐ Excellent

4. Days spent in the hospital after birth: _____

5. Any disorders/diseases after birth: ☐ Yes ☐ No

   If yes, please describe _____

   _____

6. After birth, has your baby been hospitalized for any reason or taken to hospital for any outpatient procedure or sugery? ☐ Yes ☐ No

   If yes, please describe _____

7. Which of the following problems did your baby have during the past 2 weeks?

   ☐ Fever
   ☐ Diarrhea
   ☐ Vomiting
   ☐ Ear infection
   ☐ Colic
   ☐ Fussy or irritable
   ☐ Reflux
   ☐ Runny nose or cold
   ☐ Respiratory Syncytial Virus (RSV)
   ☐ Cough or wheeze
   ☐ Asthma
   ☐ Food allergy
   ☐ Eczema (atopic dermatitis)
   ☐ Other _____

8. Is your baby currently taking any prescribed medication? ☐ Yes ☐ No

   If yes, what is he/she taking and why? _____

   _____

9. Is your baby currently taking any over-the-counter medications including vitamins or herbal remedies?

   ☐ Yes ☐ No

   If yes, what is he/she taking and why? _____

10. Have you noticed any signs of teething? ☐ Yes ☐ No

11. Did you ever breastfeed or try to breastfeed your baby? ☐ Yes ☐ No

If yes, are you actually breastfeeding? ☐ Yes ☐ No If no, when did you stop breastfeeding? _____

12. Have your baby ever tried a formula feeding? ☐ Yes ☐ No

If yes, when was your Baby first fed with formula: _____

13. Which is your baby's actual feeding:

☐ Breastfeeding only

☐ Formula feeding only, please specify the name _____

☐ Mixed formula and breastfeeding, please specify the name of the formula _____

☐ Other _____

Which are the reasons?

_____

_____

14. How often are you feeding your baby during the day _____

15. How long does an average feeding session last? _____

16. At what time was your baby last fed? _____

What did your baby eat? _____

17. At what time did your baby last sleep? _____ For how long? _____

18. Is there anyone in your family who smoke? ☐ Yes ☐ No

If yes, who: _____

How often: _____

Smoke in the presence of the baby? ☐ Yes ☐ No

19. Are you doing or did you do in the past months any kind of activities with your baby (e.g. baby massage, swimming…)? ☐ Yes ☐ No

If yes, please describe _____

_____

20. Have your baby ever attended a day-care nursery? ☐ Yes ☐ No

If yes, how many times a week? _____ for how many hours? _____