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Biological mechanisms underlying psychosocial stress response: The consequences of prenatal maternal distress and childhood maltreatment on the endocrine and immune systems

Águeda Castro Quintas

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Águeda Castro Quintas

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Doctoral Program in Biomedicine - Neurosciences
Department of Evolutionary Biology, Ecology and Environmental Sciences
Faculty of Biology, Universitat de Barcelona

Director & Tutor

Lourdes Fañanás Saura
Professor UB

Doctorate student

Águeda Castro Quintas
Predoctoral Investigator UB

"Complete freedom of stress
can be expected only after death."

-Hans Selye-

"Una casa es el lugar donde uno es esperado"

-Antonio Gala-

"There are no great discoveries and advances,
as long as there is an unhappy child on earth."

- Albert Einstein-

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Abstract

During critical periods of development, psychosocial stress experiences may alter key neural networks of the human brain, with long-lasting effects on behavior and mental health. However, the specific biological mechanisms through which early life stress impacts on present and future mental health are still not well understood.

Hypothalamic-pituitary-adrenal (HPA) axis is the principal sensor and regulator of stress response. Its modulation begins early in development through fetus-placental dialogue and continues during postnatal life, from childhood to adolescence, being especially sensitive to early life's adversities. Thus, HPA axis programming during pre-postnatal developmental periods is proposed as a plausible mechanism of early sensitization that undermine future HPA axis functioning and associated mental disorders. Moreover, it should not be forgotten that the HPA axis dialogues with the immune system, exhibiting a bidirectional interaction.

In this framework, the present dissertation aimed to disentangle the impact of psychosocial stress exposure during early sensitive periods of pre-postnatal brain development on HPA axis and immunity system. Cortisol (the HPA axis ultimate effector) and secretory immunoglobulin A (s-IgA) (an immunoglobulin of the mucosal surfaces) were selected in this thesis, respectively, as putative biomarkers of the endocrine and immune response to early psychosocial stress exposure in early life.

The study of two mother/infant dyad cohorts, the *Intramural_Maternal_Epi_project* cohort and the *COGESCOV-19* cohort, enable us to explore the respectively effects of the maternal distress and SARS-CoV-2 infection during pregnancy on the newborn's early stress reactivity.

In *Intramural_Maternal_Epi_project*, we observed that the presence of depressive symptoms flattened the maternal cortisol circadian pattern, especially during the second trimester of pregnancy. Furthermore, elevated maternal cortisol levels in mid-late pregnancy were associated with poor birth outcomes, including prematurity and low weight percentile at birth. These results underscore the importance of early detection of depressive symptoms, which are often manifested as a subclinical condition during pregnancy.

Another hypothesis we tested with this cohort was based on the premise that maternal distress during pregnancy could influence DNA methylation patterns in placenta. Specifically, we studied the DNA methylation of *FKBP5*, *NR3C1* and

HSD11B2 genes in two different placental layers: maternal decidua and chorionic villi. We observed that while maternal cortisol levels in early pregnancy were associated with an increase in DNA methylation of CpG islands of *NR3C1* gene and a decrease in DNA methylation of CpG islands of *FKBP5* gene in the chorionic villi, at the level of the maternal decidua it was the increase in DNA methylation (at specific CpG sites of *FKBP5* gene) that was strongly associated with the lower gestational age of the newborn at birth. Thus, stress during the pregnancy, and its associated cortisol levels could influence placental epigenetic signatures differently depending on the time of exposure, the placental layer, and the gene of study.

Complementarily, we could explore the putative consequences of maternal exposure to SARS-CoV-2 during pregnancy in the neurodevelopmental outcomes of offspring, thanks to the *COGESCOV-19* cohort. In this regard, we observed that infants born to mothers exposed to SARS-CoV-2 (serologically positive) showed poorer responses in items related to regulation and motor system domains (NBAS-Brazelton scale) at seven weeks old. This effect was especially evident in infants whose mothers were infected in the third trimester. Considering the magnitude of COVID-19 pandemic, children born to mothers infected during pregnancy, particularly in late pregnancy, should undergo additional longitudinal screening for neurodevelopmental milestones.

On the other hand, in this thesis, we had the opportunity to explore the effect of the most severe psychosocial stress condition for a child, such as exposure to maltreatment during their early years of life. Our research was made possible thanks to *Epi_young_stress* cohort, which consists of a representative number of children and adolescents from the general population (aged between 7 and 17 years). This cohort has been recruited through the collaboration of various child and adolescent psychiatry units nationwide and includes both children with a current psychiatric diagnosis and a control group. For all subjects, the history of childhood maltreatment was thoroughly examined, and the Trier Social Stress Test for children (TSST-C) was also administered to assess the reactivity of the HPA axis to acute stress in this population.

Regarding the functioning of the HPA axis in this young population, although children with maltreatment showed higher basal cortisol levels compared to those not exposed to maltreatment, when subjected to the acute stressor (TSST-C), they exhibited a flattened cortisol response but higher perceived anxiety. Noticeably, we also observed a dose-response relationship between the frequency and severity of the maltreatment and cortisol dysregulation. Furthermore, and in relation with putative immunity biomarkers in front psychosocial stress, we described that the acute exposure to the

stress test was able to stimulate the secretion of s-IgA in young subjects after puberty. Additionally, concerning immune markers in response to acute stress, we found that exposure to the acute stressor (TSST-C) was able to stimulate the secretion of s-IgA in young subjects, but only after puberty. However, although s-IgA reactivity to acute stress was not observed in prepubescent children, when the presence of maltreatment was observed, these children had developed this immune response capability, suggesting that complex trauma could anticipates the immune maturation.

Finally, considering our previous result that salivary s-IgA quickly rises after acute stress exposure, we wanted to know if salivary s-IgA could be a new promising biomarker of psychosocial stress reactivity in young population. A systematic review of the available scientific literature revealed that s-IgA can be considered a reliable biomarker of acute stress in under 18 population. However, further research is needed to specifically determine how psychosocial stress impacts on s-IgA circadian rhythm and basal levels.

Together, the results of this thesis support the notion that psychosocial stress during prenatal and child-juvenile periods could alter endocrine and immune systems regulation, modifying early behavioral dimensions and the reactivity to stress, which might increase the risk of future mental health problems.

Sinopsis

Durante los periodos más precoces y sensibles del desarrollo del sistema nervioso central (SNC), la exposición al estrés psicosocial puede alterar los sistemas biológicos para el futuro funcionamiento adaptativo del sujeto. El cortisol (último efector del eje Hipotalámico Hipofisario Adrenal (HHA)) y la inmunoglobulina A secretora (s-IgA) se seleccionaron en esta tesis, respectivamente, como posibles biomarcadores de respuesta al estrés psicosocial en las primeras etapas de la vida.

Se pudo estudiar el papel del distrés materno durante el embarazo, y su posible asociación con en el comportamiento y la reactividad ante el estrés del recién nacido, gracias a dos cohortes de diadas madre/niño seguidas durante el embarazo y hasta las primeras semanas postnatales: la cohorte del *Intramural_Maternal_Epi_project* y la cohorte *COGESTCOV-19*.

En primer lugar, pudimos constatar que el patrón diurno de cortisol estaba desregulado en mujeres con síntomas de depresión, especialmente durante en el segundo trimestre del embarazo. Así mismo se observó que altos niveles de cortisol en esta etapa del embarazo se asociaban con prematuridad y bajo peso al nacer. Por otra parte, se pudo comprobar que los niveles altos de cortisol materno al principio del embarazo podrían asociarse con un perfil de metilación del ADN específico en genes placentarios implicados en la regulación del cortisol, incrementando el riesgo de un parto adelantado. Finalmente, se estudiaron las consecuencias de la infección materna por SARS-COV-2 durante el embarazo en las respuestas motoras y de regulación ante los estímulos del test de Brazelton en los niños a las 7 semanas de vida, constatando un mayor efecto si la infección afectaba al último trimestre de embarazo.

Por otro lado, el estudio de una cohorte de niños/as y adolescentes con y sin patología mental (cohorte del proyecto *Epi_young_stress*) nos permitió analizar el papel del maltrato infantil en la posible sensibilización temprana del eje HHA y del sistema inmunitario de los sujetos expuestos. Para testar esta posible sensibilización, en este estudio se indujo estrés psicosocial agudo a todos los sujetos de la cohorte, mediante un protocolo de laboratorio *cuasi* experimental: el test de estrés psicosocial Trier para niños (TSST-C).

Los niños/as y adolescentes expuestos a maltrato mostraron mayores niveles de cortisol basales y una hiporreactividad del eje HHA al TSST-C, a pesar de mostrar una mayor percepción de ansiedad ante estrés psicosocial agudo comparados con los niños

no expuestos a maltrato. También se observó una relación dosis-efecto entre la exposición y severidad del maltrato y la desregulación del eje HHA. Además, el estrés psicosocial estimuló la secreción de s-IgA, pero sólo después de la pubertad. Sin embargo, los niños/as expuestos a maltrato infantil mostraron secreción de la s-IgA ante estrés psicosocial de forma similar a los adolescentes, sugiriendo una anticipación de la maduración del sistema inmune en los niños maltratados. El uso de la s-IgA como posible biomarcador de estrés agudo y crónico en etapas tempranas de la vida fue estudiado y discutido mediante una revisión sistemática.

En conjunto, los resultados de esta tesis sostienen que la exposición al estrés psicosocial durante los periodos tempranos del desarrollo del SNC (prenatal, infancia y adolescencia) podrían alterar la respuesta al estrés de los sistemas endocrino e inmune, modificando algunos rasgos de la conducta temprana en el recién nacido y aumentando el riesgo de futuros problemas de salud mental.

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Abbreviations

11β-HSD2	11β-hydroxysteroid dehydrogenase type 2
ACE	Adverse Childhood Experiences
ACTH	Adrenocorticotropic Hormone
AUCg	Area Under the Curve with respect to the ground
AUCi	Area Under the Curve with respect to the increase
CAR	Cortisol Awakening Response
CECA-Q2	Childhood Experience of Care and Abuse Questionnaire
COVID-19	Coronavirus Disease 2019
CRH	Corticotropin-Releasing Hormone
CRP	C Reactive Protein
CTQ	Childhood Trauma Questionnaire
DOHaD	Developmental Origins of Health and Disease
FKBP5	FK506-binding protein 5
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Elements
HPA axis	Hypothalamic Pituitary Adrenal axis
HSD11B2	11β-hydroxysteroid dehydrogenase type 2 gene
Ig	Immunoglobulin
IL	Interleukin
MR	Mineralocorticoid Receptor
NBAS	Neonatal Behavioral Assessment Scale
NR3C1	Glucocorticoid receptor gene
pCRH	placental Corticotropin-Releasing Hormone
PNS	Parasympathetic Nervous System
s-IgA	Secretory Immunoglobulin A
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2

SNP	Single Nucleotide polymorphism
SNS	Sympathetic Nervous System
STAI-S	State-Trait Anxiety Inventory Status
TBS	Targeted Bisulfite Sequencing
TSST	Trier Social Stress Test
TSST-C	Trier Social Stress Test for Children
WHO	World Health Organization

1. Introduction

1.1. Psychosocial stress and mental disorders

The ability to react and generate responses to environmental threats or stressors is intrinsic to all live beings and has played a key role in the history of evolution. Survival has been enhanced in species displaying with rapid adaptation mechanisms to environmental stressors such as predation, natural disasters and intra or inter group competition. Therefore, evolution could have promoted a physiological alert state that would help to confront or escape from potentially endangering situations. In the early 30s, Walter Cannon coined this adaptive response as "fight or flight"(1915), characterized by the activation of different biological systems such as nervous, endocrine and immune, that produce well-orchestrated physiological changes to either "stay" and deal with a threat or to "run away" to safety.

At the present time, different social situations related to interpersonal relationships could be perceived as stressful, demanding a rapid physiological and psychological adaptation. Thus, the strain and pressure perceived during certain daily situations can also induce the fight or flight response in humans (Herr et al., 2018; Segerstrom & Miller, 2004). These situations would activate certain sympathetic nervous fibers of the autonomic nervous system, leading to the release of certain hormones by the endocrine system, initiating a rapid physiological response characterized by increase of heart rate, blood pressure and catabolism. The stress created by the situation might be helpful and helps to perform better in under daily pressure situations, such as at work or in school. However, when a stressful situation is chronic (long-lasting), a dysregulation of the stress response may occur, having detrimental effects on both body function and mental health status (Cohen et al., 2007; Druzhkova et al., 2019).

Of note, the same strategy would not be always the optimal to cope with a stressful situation and would depend mainly on the environmental cues the subject encountered during development. For this reason, life history traits and strategies tend not to be genetically fixed and evolved to show developmental plasticity (Ellis et al., 2009). Developing organisms adjust their strategic allocation choices to their local environment, following evolved rules that maximize their subsistence in different ecological conditions. For example, according to Ellis and colleagues (2009) energetic stress (e.g., malnutrition, low energy intake) would cause in the developing organism a slower growth and maturation and delayed reproduction. Contrary, unpredictable environmental situations that increase the risk of mortality would cause and accelerating sexual maturation, promoting early reproduction.

For this reason, the capacity to maintain homeostasis in front to psychosocial stress depends to a large extend on the exposition to psychosocial factors during key developmental periods of human lifespan, as prenatal stages, childhood and adolescence (Knudsen, 2004). In fact, the exposure to stressful life events from prenatal periods to late adolescence have been shown to cause not only changes on the personality and/or behavioral traits, but also lasting structural and functional brain changes (Teicher et al., 2016). Moreover, these experiences, frequently referred to as adverse childhood experiences (ACEs), have been associated with greater risk for lifetime psychopathology or different mental disorders in numerous longitudinal studies (Clark et al., 2010; Dube et al., 2003; Green et al., 2010; McLaughlin et al., 2010; McLaughlin et al., 2012). In this regard, individuals who have experienced ACEs are more likely to depression, anxiety, substance use, antisocial behavior, suicidal behavior and psychotic experiences at later points in their lives (Cohen-Woods et al., 2018; March-Llanes et al., 2017; McLaughlin et al., 2012; Oldehinkel & Ormel, 2015; Targum & Nemeroff, 2019). Furthermore, ACEs have been also associated with other health conditions in adulthood, such as ischemic heart disease, cancer, stroke, diabetes (Nelson et al., 2020) and even with premature death (Kelly-Irving et al., 2013).

Although early stages of life are periods of high vulnerability to stressful environmental conditions, the elevated brain plasticity and adaptability make them also ideal periods to protect or intervene in case of developmental issues. For example, care and nutrition of pregnant women would contribute to better offspring's growth and development throughout their life course. Interventions in the newborn period would also prevent or change the course of certain developmental delays and disabilities with prenatal origins. Childhood and adolescence also represent additional windows of opportunity, since they are critical developmental periods in which the first symptoms of many mental disorders are exhibited; moreover, intervention during childhood and adolescence could decrease the later exposition to violence and drugs, which are also risk factors for mental disorders (Clark et al., 2020).

The specific biological mechanisms through which ACEs impact on present and future health are still not well understood. This implies a lack of validated biomarkers for early psychosocial stress exposure and its association with future psychopathology. Thus, identifying the underlying biological mechanisms involved in the psychosocial stress response could contribute to understand the impact of ACEs in the development of future mental disorders and to define intervention to protect pregnant women and children at risk in order to mitigate the possible effects in their future health status.

1.2. Psychosocial stress exposure during sensitive periods on brain development

Humans (*Homo sapiens*) are essentially social subjects, and no component of the civilizations would be possible without our collective behavior. However, despite the complexity of the inferences we make about the world, many of our senses, are shared with other mammals. The difference between humans and other mammal species, including primates, may arise from the *social cognition*, the knowledge of their own minds and those of the others, which is linked to the complexity of human brain neocortex acquired during evolutionary processes (Adolphs, 2009). However, this great behavioral complexity has costs: human brain is more immature at birth; consequently, it requires a prolonged and extended postnatal period of development in which parental investment is crucial to guarantee offspring's survival until they reach reproductive age.

Structural and functional brain maturation results from complex processes that overlap over time (see Figure 1). An important part of brain development takes place in utero, although some of these processes continue during the postnatal phases that include, according to Erikson's stage theory (1968): the infancy (0-1 years old), the toddlerhood (1 to 3 years old), the early childhood (3 to 6 years old), the middle childhood (6 to 12 years old) and the adolescence (12 to 18 years old).

During the prenatal period, the main neurogenetic events -neurogenesis, neuronal migration, and the initiation of both myelination and synaptogenesis processes- occur. All of them are essential for the brain cytoarchitectural organization and the correct functionality of future cortical networks. Postnatal cerebral maturation also relies on gliogenesis, myelination and synaptic pruning which are necessary for the adequate brain connectivity. Overall, brain develops rapidly during prenatal periods and toddlerhood, and continue its maturation, although more slowly, until late adolescence.

Although brain development is under tight genetic control across the lifespan (Douet et al., 2014), it can be influenced also by environmental factors (Kolb & Gibb, 2011). These gene-environment relations allow for each child to adapt to their surroundings more quickly than they could if genes alone determined the brain's wiring (Pascual-Leone et al., 2005).

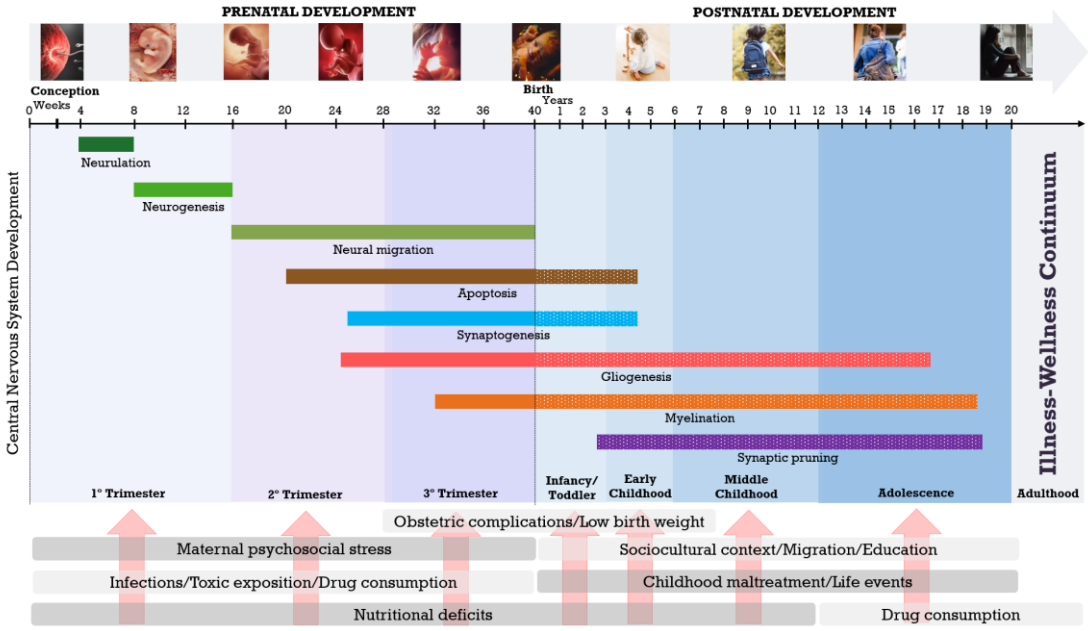

Figure 1. Human brain development from conception to adult life, and different pre and postnatal stressors associated with increased risk of mental disorders. In the first trimester of pregnancy, the formation of the neural tube (neurulation) and neurons (neurogenesis) occur. In the second trimester, the neurons begin to migrate (neural migration) and to mature by initiating the formation of synapsis (synaptogenesis) and discarding the neurons that are not going to be needed (apoptosis) until early childhood. The final stage of prenatal brain development is glial development (gliogenesis) to form myelin (myelination), processes that continue until late years of adolescence. Finally, throughout childhood and adolescence, unused connections are removed to maximize efficiency (synaptic pruning). The effects of psychosocial stressors on human brain development will depend on genetic predisposition, the time window of fetal or postnatal brain development in which exposure occurs at the time of exposure, and the strength of the chronic insult. Adapted from Knuesel and colleagues (2014).

For the correct prenatal development, the maternal environment is critical, and any perturbation could modify fetal programing, probably via epigenetic processes, increasing the risk of disease in the offspring. This relationship was suggested by David Barker in the 80s with the name of the developmental origins of health and disease (DOHaD), after observing the association between maternal cardiovascular and metabolic problems and infant weigh at birth (Barker, 2007; Barker & Osmond, 1986; Barker et al., 1989). Since then, different epidemiological studies have associated exposure to inadequate nutrition intake (Cortés-Albornoz et al., 2021), maternal exposition to psychosocial stress (Van den Bergh et al., 2017), maternal infections (San Martín-González et al., 2023) and toxins during pregnancy (Bellinger, 2013) with adverse effects across newborn's lifespan. Furthermore, higher prenatal distress would increase the vulnerability to postnatal experiences, strengthen the sensitivity to both adverse and

supportive postnatal contexts (Hartman et al., 2023). Different mechanisms have been proposed as mediators of previous associations, including neuroendocrine factors, immune regulation, and placental function (Khambadkone et al., 2020). Hence, stressors of different nature during pregnancy (psychosocial and biological) could be mainly dysregulating the same maternal-fetal communication pathways, resulting in an overlap of neurodevelopmental outcomes in the offspring.

During the postnatal period, safe and nurturing environments at home are also fundamental for an appropriate brain development. The presence of a stable adult caregiver supports children's and adolescents' overall sense of comfort. Therefore, experiences like childhood maltreatment – such as physical or emotional abuse, sexual abuse and/or chronic neglect - can undermine infant's safety, stability, and bonding and, consequently, impacting in their brain development. In fact, different structural and functional changes in brain have been associated with both physical abuse (Teicher et al., 2016) and caregiving neglect (McLaughlin et al., 2014).

Children exposed to an adverse environment would have an increased risk of psychological problems including increased risk-taking, aggressive behavior, and difficulties in interpersonal relationships (Hunt et al., 2017). Moreover, psychiatric disorders, including depression, psychosis, substance abuse and suicidal attempts are more frequent in exposed subjects (Afifi et al., 2020). Furthermore, these exposed children would have an increased risk of developing common inflammatory diseases during infancy such as viral infections, dermatitis or urinary tract infections, but also future adult clinical conditions, including cancer, asthma, diabetes, and obesity (Karlén et al., 2015). These data suggest a dysregulation of different biological systems involved in stress response (Nelson et al., 2020). In this context, it is crucial to note that although an unacceptably high number of children in our society— at least one in four— experiences childhood maltreatment while growing up, child maltreatment is often hidden (World Health Organization [WHO], 2022).

We have outlined that early developmental stages set the foundation for lifelong learning, behavior, and health. Human brain develops over the course of the prenatal period, but it will continue to go through more changes during childhood and adolescence. Although the scaffold for brain development is mainly formed by the genetic background, the interaction with the environment is fundamental for the complete brain maturation. In this context, the exposure to chronic stressors these early stages of life (pre and postnatal) could negatively impact the proper brain maturation, increasing the risk of health problems in the future.

This thesis would be focused on the study of prenatal stress exposure and childhood-adolescence maltreatment and the effects of these environmental conditions on the neuroendocrine and immune responses.

1.2.1. Prenatal brain development and maternal stressors

Every part of the fetal body forms at a specific time (Figure 2). This specific time during a body part formation is called "critical period of development", and for most body parts, it typically occurs from the week 5th to the week 14th of gestation, which is also referred as the first trimester of pregnancy. Exposure to very harmful environmental factors during very early pregnancy have the greatest risk of causing miscarriage, as they can damage most or all the cells of the fertilized egg and they also can interfere with the uterine attachment, which occurs do not occur until the embryonic period. Exposure to harmful environments during the rest of the first trimester have the greatest chance of causing major birth defects, as many important anatomic developmental changes occur during this time (Moore et al., 2013). Once a body part has formed, some exposures could still affect the growth and/or functionality of an organ (Moore et al., 2013).

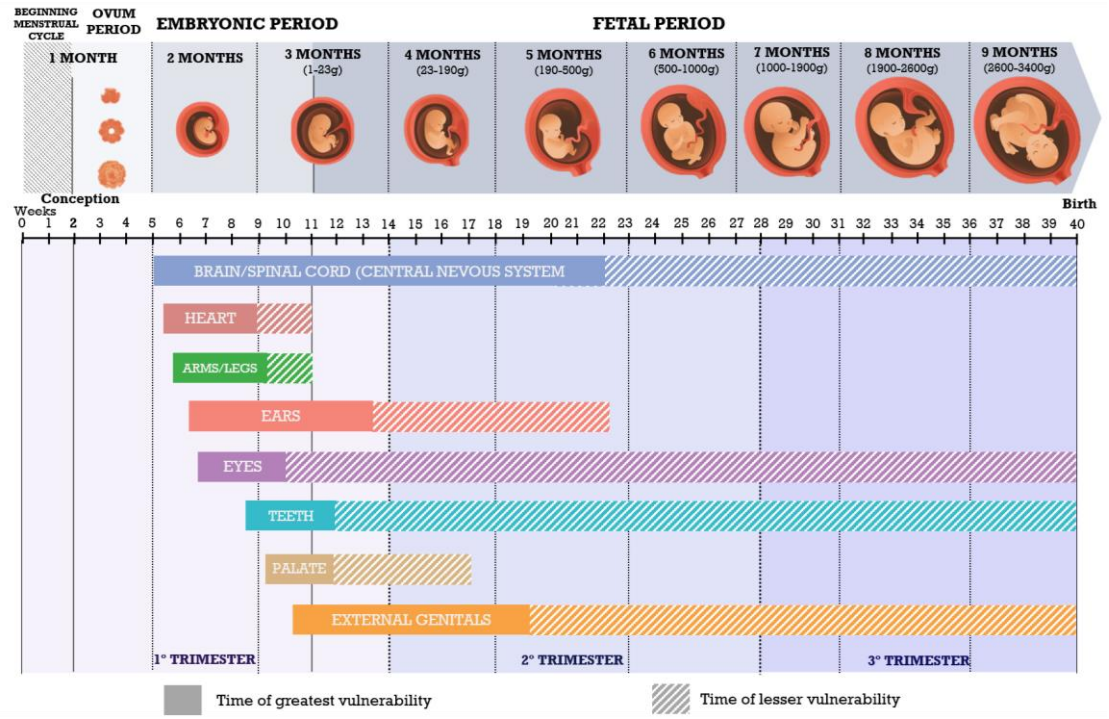

Figure 2. Prenatal development across pregnancy, from week 0 (beginning of the menstrual cycle) to week 40 (estimated birth time). The solid bars show when each part of the body is most sensitive to harmful exposures and at risk for major birth defects (structural changes). The striped bars show period when the body parts are still at risk to develop "minor" defects (functional changes). Adapted from the Organization of Teratology Information Specialists (OTIS) (2021).

Brain is the body part with the longest time of higher prenatal vulnerability - from the week 5 of pregnancy to the 22 – which means great part of the first and second trimesters. In fact, is at the end of the second trimester when some critical reflexes controlled by the brain steam are fully developed, such as the heart rate, breathing and blood pressure, so there is a possibility to survive outside the womb. Furthermore, it is also by the end of the second trimester when most neurons have reached their final destination and the functional connectome is established (Turk et al., 2019). That is why there is already an advanced maturity of the reflexes, motor, default mode, and somatosensory regions in the prenatal human brain. Hence, at birth, new-born already have neurobehavioral capabilities to cope with live, such as reflexes, functioning sense, and a capacity to learn and organized patterns of waking and sleeping (Lester & Tronick, 2004).

Finally, it is important to note that the medial frontal cortex, which is associated with social cognition, cognitive control and decision making, undergoes limited development during the prenatal period (Turk et al., 2019). Consequently, the formation and refinement of these neural networks persist well into childhood and adolescence, playing a crucial role in facilitating nuanced interaction required within the appropriate social environment in this period and also, in later adult life.

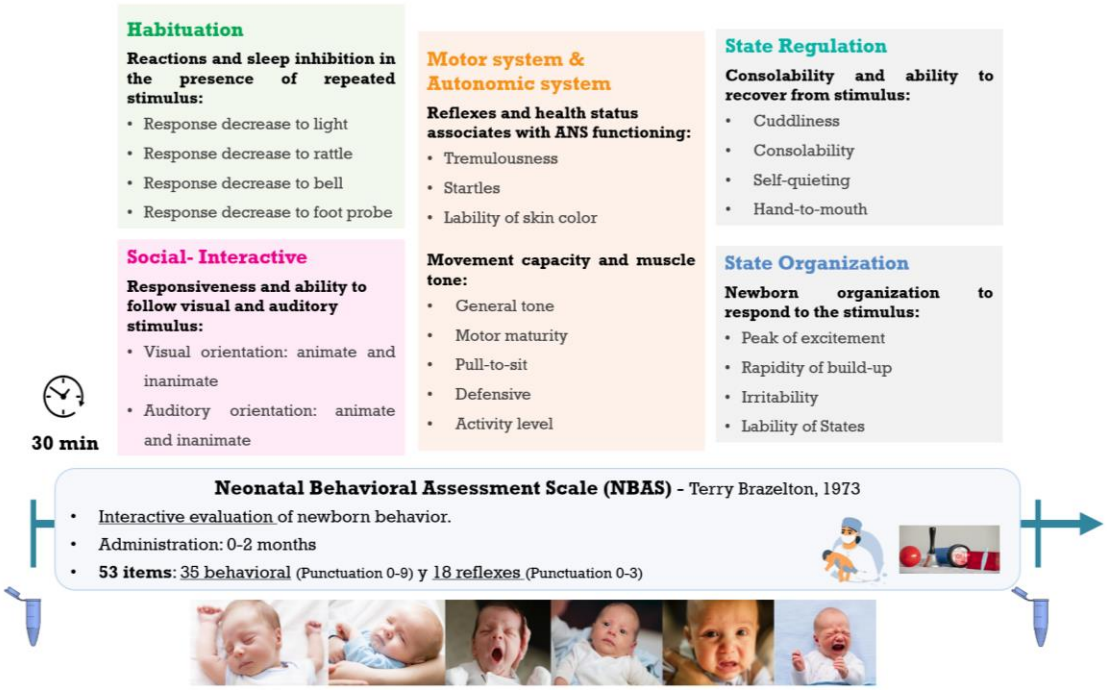

Figure 3. Neonatal Behavioral Assessment Scale (NBAS) protocol. Six relevant dimensions for neurobehavioral reactivity are included in this scale, detailed in box 1. Figure courtesy of Nerea San Martín González.

The possible neurodevelopmental consequences of prenatal environmental stressors could be already studied in the first weeks of life. This exploration can be facilitated by employing diverse scales designed to assess newborn behavior and capabilities, such as the Neonatal Behavioral Assessment Scale (NBAS) (see Figure 3) (see Box 1).

BOX 1. How to measure the newborn neurobehavioral capabilities: The Neonatal Behavioral Assessment Scale (NBAS)

At birth, newborn have certain neurobehavioral capabilities that are measurable. They present reflexes. A reflex is an involuntary response to a stimulus. There are *survival reflexes*, such as breathing reflex or eye-blink reflex and *primitive reflexes*, which are believed to be remnants of evolutionary history, such us fan the toes when the bottom of the feet is stroke (Babinski reflex). The primitive reflexes typically disappear during the early months of infancy. If these reflexes are not present at birth or they last too long in infancy there would be probably an alteration on infant's nervous system. Infants should also be able to stablish organized patterns of daily activity. They should have an organized sleep-wake pattern, to integrate biological, physiological, and psychological information (Sigelman et al., 2018).

There are different tests to measure these infant's neurobehavioral capabilities. The Neonatal behavioral Assessment Scale (NBAS), developed by Dr. Berry Brazelton and his colleagues gives one of the most comprehensive examinations of the newborn behavior (Brazelton & Nugent, 1995). The NBAS evaluates the ability of the infant to interact and respond to the environment.

The NBAS can be used with babies from birth (even premature birth, 35 weeks gestation) to around 2 months. The scale includes 53 items, 35 behavioral (punctuated between 0 to 9, generally being 9 the optimal) and 18 reflexes (punctuated between 0 to 3, generally being 2 the optimal) which are either administered or observed. The dimensions measured are Habituation (sleep protection), Social Interactive responses and capabilities, Motor and Autonomics systems, State Organization and State regulation. The duration of the test could be between 15 and 90 minutes, but usually 30 minutes. This protocol brings the opportunity to collect saliva samples before and after the procedure, enabling subsequent measurement of biological markers. See Figure 3 for detailed information.

1.2.1.1. Pregnancy and maternal psychological changes

The growth and development of a fetus during pregnancy requires complex interactions between the mother and the growing fetus. This communication between the mother and the developing embryo begins shortly after fertilization, when the maternal and embryonic signals prepare the uterus for embryo implantation and placental development, and remains constant throughout pregnancy (Norwitz et al., 2001). Furthermore, during this period there are great physiological changes not only in the fetus, but also in the mother to be, to prepare her for delivery and motherhood (Servin-Barthet et al., 2023). Thus, maternal stressors during pregnancy could be detrimental for the maternal-fetal dyad.

Factors such as previous maternal health problems (e.g., high blood pressure, obesity, diabetes, heart disorders), advance maternal age, bad lifestyle choices (e.g., smoking cigarettes, drinking alcohol, eating junk food) and obstetric conditions associated with pregnancy, could impact on maternal and fetal wellbeing leading to "high-risk pregnancies" (Badakhsh et al., 2020). The majority of pregnancies are considered "low-risk", so no maternal or fetal factors that place during gestation increased the risk for complications. However, pregnancy is never a risk-free period. For example, pregnant women had a higher risk of suffer negative daily hassles, such as job loss, intimate partner violence and poor social support (Satyanarayana et al., 2011). Socioeconomic and cultural factors, such as preference for sons over daughters, lower incomes, problems with the partner's family and parents, would be also factors of risk for pregnant woman, particularly in some regions (Chandran et al., 2002). Furthermore, they have an increased susceptibility to the infections of some organisms and an increased for severe illness from this infections (Kourtis et al., 2014). From an epidemiological perspective, these factors would impact on the health of the dyad, increasing the risk of offspring's future neurodevelopmental disorders.

Pregnant women undergo many physiological and anatomical changes in order to support fetal development and growth, birth and future lactation (see Figure 4). Many of these changes are endocrine and they are mainly mediated by hormonal secretion produced by the placenta. These changes arise in cardiovascular, pulmonary, musculoskeletal, gastrointestinal, renal, endocrine, and adipose systems, increasing blood flow, oxygenation, and energy reservations. There are also changes in the mammary glands to promote future lactation (Napso et al., 2018). These adaptations depend on the stage of the pregnancy and are associated with alterations in the metabolic

requirements of the mother versus the fetus. In normal conditions, most of these physiological changes return to pre-pregnancy condition during the postpartum period.

On the other hand, the maternal brain is also modified during pregnancy in preparation for the motherhood, involving long-lasting changes that remains for years after birth (Duarte-Guterman et al., 2019). In fact, the pregnancy and peripartum period stand out as some of the most plastic periods of a woman. The observed changes in the brain during these periods are of similar magnitude to those observed during the transition in adolescence (Carmona et al., 2019). For example, first-time mothers have brain volume reduction in several brain areas compared to nulliparous women, although this volume differences partially diminishes as the postpartum period progresses (Hoekzema et al., 2022). Furthermore, the hormonal changes of pregnancy are linked to changes in neuronal plasticity, including changes in glia, dendritic and synaptic remodeling, and neurogenesis. These changes have been observed in subcortical (e.g., hypothalamic, amygdala and nucleus accumbens) and cortical (e.g., cingulate cortex, insula and the temporoparietal junction) regions, areas that are associated with key psychological processes for motherhood such as vigilance and reward towards the infant, empathy, mentalizing and emotional regulation (Servin-Barthet et al., 2023).

The hypothalamic-pituitary-adrenal (HPA) axis, a key component in the stress responsive system and homeostasis maintenance, also suffer profound changes during pregnancy, mainly associated with placental hormones (see Figure 4 and for more details, see section 1.3.1.). Throughout pregnancy, the HPA axis final effector, cortisol, is linked to fetal maturation, promoting cell differentiation, particularly in late stages. For this reason, maternal cortisol naturally increases during pregnancy, although the placenta mediates its pass from the mother to the fetus (Krontira et al., 2020). Moreover, the additional increase of prenatal maternal cortisol by maternal psychosocial stress exposure could disrupt the protective capacity of the placenta, resulting in excessive levels of cortisol in the fetal environment. This excessive fetal exposure to cortisol has the potential to be toxic, influencing fetal brain cell differentiation and disrupting both its structure and function. Furthermore, this exposure may impact fetal HPA axis maturation, initiating a gradual desensitization process. Consequently, this could lead to a state of heightened responsiveness to stressful situations in the offspring, increasing their risk of experiencing a more adverse stress response in early life and potentially contributing to future behavioral or psychiatric problems (Davis & Narayan, 2020).

There are also changes of the maternal immune system during pregnancy (Figure 4), which for example induces anti-inflammation at the maternal-fetal interface to

prevent an unwanted immune response against the paternal antigens present in the fetus (Napso et al., 2018). However, these changes may increase woman's susceptibility to certain infections resulting in a variety of pregnancy complications. Furthermore, the susceptibility to several illness could be also increased, partially due to other physiological changes that occur during pregnancy (e.g., decreased lung capacity, changes in blood flow). Although the pass of microorganisms through the placenta is limited (as it has evolved robust mechanisms of microbial defense) some microorganisms could alter and cross this barrier, arriving to the fetus and increasing the risk of congenital infection and severe disease (Megli & Coyne, 2022). In fact, the exposure to some maternal infections in utero, such as zika, rubella, herpes simplex virus, influenza and coronaviruses have been clearly associated with risk for severe neurodevelopmental conditions but also for neuropsychiatric disorders, as schizophrenia (Zimmer et al., 2021).

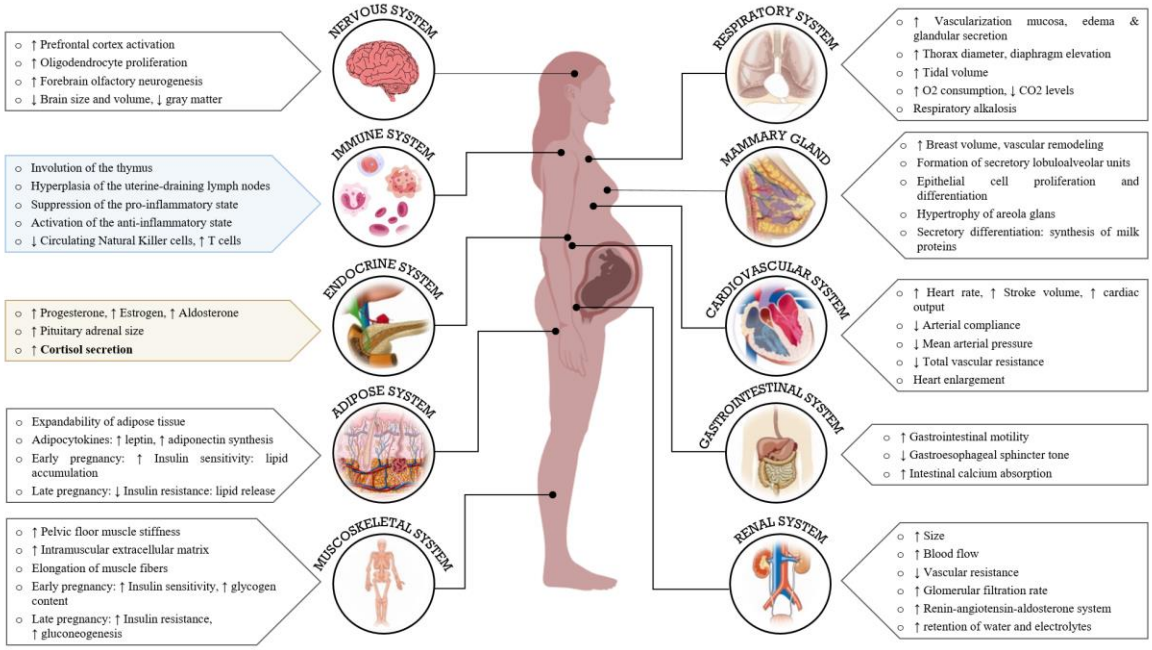

Figure 4. Scheme highlighting the main physiological modifications in pregnant women. Changes include alterations in size, morphology, function and responsiveness of tissues and organs to hormonal and metabolic cues and affect mainly the nervous system, immune system, endocrine system, adipose system, muscoeskeletal system, respiratory system, mammary gland, cardiovascular system, gastrointestinal system, and renal system. Adapted from Napso and colleagues (2018) and created with BioRender.com.

It has been recently suggested that, rather than pathogens like flu or coronavirus, a common pathway through maternal immune activation may be the link between maternal infections during pregnancy and the development of neuropsychiatric disorders in the offspring (Hall et al., 2023).

The aforementioned prenatal stressors, i.e. the maternal mental stress associated with depressive status or symptoms and infections during pregnancy are part of the focus of the present dissertation due to their putative impact on subsequent infant neurodevelopmental problems and future mental disorders. Therefore, the following sections will outline each of these stressors.

1.2.1.2. Prenatal exposure to maternal psychosocial stress and depression

It is well known in the literature that the prevalence of anxious depressive disorders during pregnancy is high. In fact, worldwide, about 12% of pregnant women experience a mental disorder, primarily depression, being this prevalence higher in low-income countries (13.1%) compared to high-income countries (11.4%) (Woody et al., 2017). This is likely attributable to the social and economic factors which may influence chronic stress during this sensitive period. Moreover, the prevalence of self-reported anxiety is around 20% during pregnancy (Dennis et al., 2017), which is also higher when compared to non-pregnant women (Dunkel Schetter & Tanner, 2012). These differences between pregnant and non-pregnant women could be associated with different factors strictly linked to pregnancy conditions. For example, if the pregnancy was planned or not, the support in the co-parenting relationship, or other demographic and financial circumstances. Furthermore, there is also a physiological vulnerability linked to the massive hormonal changes that occur, which might also unmask vulnerabilities to both physical (e.g., diabetes, hypertension) and mental (e.g., depression) health that could last even after birth. In the postpartum period, hormonal changes can coincide with various psychological and social factors. Alterations in intimate relationships, breastfeeding dynamics, and a lack of partner or social can also contribute to an increased vulnerability to psychiatric symptoms or diagnoses (Slomian et al., 2019).

The interest in the study of the effects of maternal psychological distress on infant outcomes, and its underlying biological mechanisms, has surged over the past three decades (Glover, 2015; Jeličić et al., 2022). Much of the research in this field is focused on studying the impact of postpartum depression on outcomes such maternal-infant interaction and infant temperament; however, more recent studies have also investigated the effects of different forms of maternal psychological distress during pregnancy, and their timings, on different infant outcomes, including infant development.

Maternal environmental factors of psychosocial nature during pregnancy, and in particular anxious-depressive maternal disorders, have been repeatedly associated with

poor infant outcomes (Coussons-Read, 2013; Reynolds et al., 2013; Van den Bergh et al., 2017). For example, maternal prenatal stress and depression have been associated with low birth weight and prematurity (Grote et al., 2010; Lima et al., 2018). Noticeably, antidepressant use in pregnancy has been also associated with low birth weight and prematurity (Huang et al., 2014). In this line, a meta-analysis conducted by Rogers and colleagues (2020) revealed that maternal prenatal depression and anxiety were associated with problems in socio-emotional, neurocognitive, language and motor development across infancy, childhood, and adolescence. Interestingly, they found that infant attachment difficulties were specifically associated with maternal postnatal depression, not prenatal depression. This observation aligns with prior studies suggesting that the impact of a mother's mental health on her child's well-being clearly extends beyond her psychopathological state during pregnancy.

Various biological mechanisms can underlie the impact of maternal distress during pregnancy on fetal and child brain-behavior development. Some of them are related to maternal biology, such as the activation of the maternal HPA axis or immune system, while others involve the protective role of the placenta, including epigenetic mechanisms that can alter the expression of genes associated with cortisol regulation (Monk et al., 2019). Despite the diverse effects these biological mechanisms could exert on fetal development and child outcomes, the influence of maternal psychosocial stressors during gestation on offspring can also vary based on the timing of pregnancy in which the mother to be is exposed; for instance, the first trimester poses a risk for major birth defects, and the placenta is less developed to mitigate the environmental adverse effects. However, few studies assess maternal stress conditions across pregnancy to identify one period that is more vulnerable to prenatal stress (e.g., if there is a trimester of pregnancy with higher risk for both the mother and the fetus) (Van den Bergh et al., 2017). While there are studies reporting that the effects of maternal psychosocial stressors on the infant are worst when exposed in early gestation (Davis & Sandman, 2010; Loomans et al., 2013; Zhu et al., 2013), others claim that is the exposure in the middle or late stages of pregnancy that leads to a poorer prognosis in child development (Class et al., 2011; Evans et al., 2012; Rouse & Goodman, 2014). Furthermore, some studies have found that the effect of maternal stress on offspring neurodevelopment does not vary based on the trimester of exposure (Moog et al., 2021).

The magnitude of the effect of prenatal psychosocial stress on infant outcomes also varies as a function of depression-stress measurement in pregnant women, since the presence/absence of diagnosis, and the type of diagnosis, if any, may vary depending on

the scale used to measure it (Grote et al., 2010). Moreover, despite the vast literature regarding maternal psychiatric conditions during pregnancy, a limited number of studies have undertaken a comprehensive examination of psychiatric subclinical traits to complement those solely focused on categorical diagnoses. This approach would allow us to verify, in epidemiological terms, how often pregnant women cope with moderate symptomatology within the anxious-depressive spectrum, identifying the trimester in which it could be most prevalent. Moreover, it would enable an exploration of the potential impact of these subclinical psychiatric conditions on maternal physiological stress regulatory patterns and the well-being of exposed newborns.

1.2.1.3. Prenatal exposure to maternal infection

As briefly introduced in section 1.2.1.1., during pregnancy there are multiple mediators' factors to promote health and defense against pathogens, as different signaling pathways and cytokines. However, the complex interaction between multiple host factors, including maternal infection or aberrant activation of the immune response during pregnancy, could lead to severe pregnancy complications (Kumar et al., 2022). According to the World Health Organization (WHO), around 11 women per 1000 live birth had an infection that resulted in or contributed to the death of the woman or her being near death. Furthermore, around 70 pregnant or recently-pregnant women per 1000 were found to have a maternal infection needing hospital management (WHO Global Maternal Sepsis Study (GLOSS) Research Group, 2020).

In addition, there is evidence indicating that these pregnancy complications could have a negative impact on fetal growth and development during pregnancy, increasing the risk to a variety of diseases later in life (Rahman et al., 2012). In this line, neurodevelopmental disorders as autism spectrum disorders (Jiang et al., 2016) and attention-deficit/hyperactivity disorder (Zhu et al., 2022) have been identified as consequences of exposure to gestational maternal infections. Interestingly, in 1976 Torrey and Peterson formulated the "Viral Hypothesis of Schizophrenia", claiming that prenatal viral infections produce long-lasting brain alterations, contributing to the etiology of this complex psychiatric disorder (Torrey & Peterson, 1976). Despite the difficulty in establishing such a distal relationship due to the numerous confounders present from pregnancy to the onset of psychiatric disorder (Zimmer et al., 2021), neurodevelopmental disruptions have been observed in children who later develop schizophrenia (Jones et al., 1994). Furthermore, while there is still uncertainty about whether offspring neurodevelopmental alterations depend on specific gestational timing

of exposure (Lee et al., 2020), some authors argue that exposure, particularly during the second trimester, increases the risk of future neurodevelopmental impairment (Al-Haddad et al., 2019).

There is also evidence that the consequences in the fetus depend on the type of infection to which it has been exposed. It has been clearly demonstrated that some viruses can cross the blood-brain barrier and infect neurologic tissues, such as zika virus or cytomegalovirus, causing severe morphological consequences on brain development and compromising fetal viability if transplacental transmission occurs (Gordon-Lipkin et al., 2021). However, the impact of other viral infections, such as respiratory infections, remains largely unknown to scientists, despite being the most frequently reported infections during pregnancy. In a review conducted by San Martín-González et al. (2023), it observed that common maternal respiratory infections seem to be associated with subtle alterations in motor development and attentional, behavioral/emotional minor problems, although they claimed that results were controversial.

In this context, the Coronavirus Disease 2019 (COVID-19) pandemic has caught us by surprise, and there is limited research evaluating the impact of the maternal Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) on the fetal neurodevelopment, especially considering that a significant portion of SARS-CoV-2 infections are asymptomatic and do not require medical attention. Currently, there is no strong evidence indicating that SARS-CoV-2 can cross the placental barrier (vertical transmission). However, it has been suggested that the virus could induce an exacerbated maternal inflammatory response, and that the immune mediators secretors could cross both the placental and fetal brain blood barrier, leading to neurodevelopmental and/or neuropsychiatric damages in the offspring (Granja et al., 2021). Furthermore, the COVID-19 pandemic is associated with an increased maternal perceived stress during pregnancy, which can also impact on infant's temperament (Provenzi et al., 2021). Therefore, SARS-CoV-2 infection could elevate the perceived stress of the woman during pregnancy, and the associated biological mechanisms may contribute to the impact on newborn behavioral traits reported in previous studies (Provenzi et al., 2021).

Due to the significant lack of understanding regarding the effects of the recent SARS-CoV-2 infection on the neurodevelopment of offspring, we believe it is of great relevance to study this subject in the context of the present thesis.

1.2.2. Child brain development and environmental risk factors

In the first few months of life, infants grow rapidly, gaining nearly 30 grams of weight by day. By age of two years of postnatal life, they have grown significantly, reaching about half of their eventual adult height and weighing between 12 and 14 kilograms on average (World Health Organization [WHO], 2006). In this line, during these initial two years, the brain experiences a rapid and substantial increase in size, driven by the overproduction of connections, synapses, and myelination. In fact, by the first year of postnatal life, the brain attains approximately 70 percent of its adult size, increasing to 85 percent by the age of two (Knickmeyer et al., 2008). However, it is important to note that cognitive, sensory and perceptual development in the brain do not occur simultaneously during these first years; instead, they are profoundly influenced by early life conditions and extend into early adulthood. In infancy and early childhood, there is a pronounced expansion of visual and auditory capabilities, language skills, social cognition and the ability to understand and navigate one's environment. The brain continues to undergo remarkable changes throughout childhood and adolescence, refining higher cognitive functions, emotional regulation, and decision-making processes (see Figure 5).

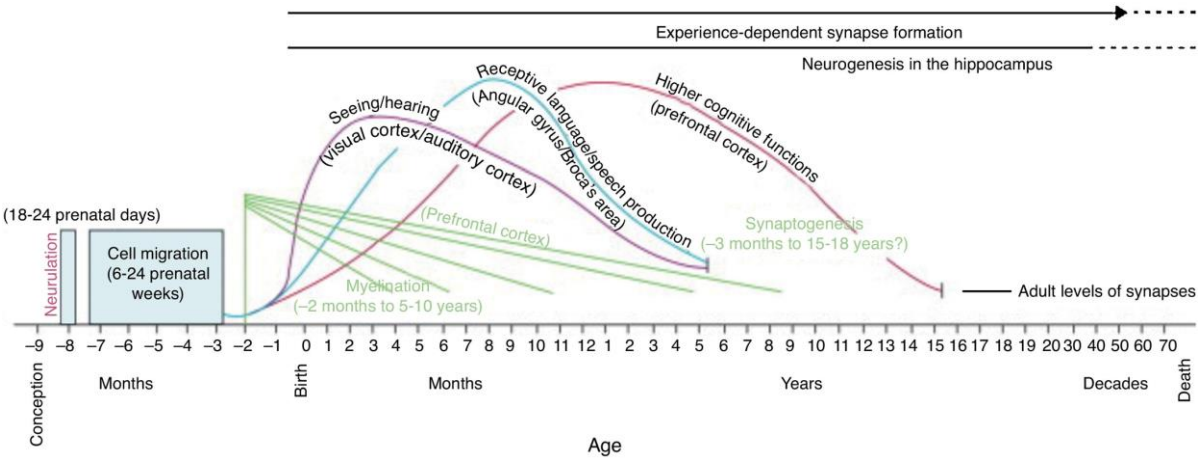

Figure 5. Human brain development. The processes included are intrauterine neuronal patterning, neurogenesis, cortical migration, gliogenesis, myelination, and experience-dependent synapse modification. Reproduced with permission from Thompson P.M. et al. Genetic influences on brain structure. Nat Neurosci. (12):1253-8 (2001).

During this period, bonding and nurturing interactions with caregivers are fundamental for infant's emotional and social development. This innate tendency of the infant to develop emotional attachment to those around them is shared with many other species and promotes adaptation, ensuring coverage of both their physical and psychological needs (Ainsworth et al., 1978). The quality of attachment will depend primarily on the type of attention and response the baby receives from the responsible adult caregiver, usually the mother.

Individuals can develop either secure or insecure attachment based on the sensitivity, availability, or predictability demonstrated by caregivers in response to their needs. Secure attachment will develop gradually as caregivers learn to interpret the signals of the child and respond promptly and appropriately, with positive, calm, and focused affection. However, if the infant grows up in an environment of violence and abuse, it is likely that an insecure attachment will form, with the repercussions that this type of bond can carry in the short and long term. It is noteworthy that numerous neurobiological mechanisms essential for the individual's future emotional regulation will be modulated through the attachment style established with the primary caregiver. Among the most relevant mechanisms, we can mentioned, for example, the secretion of neuro-hormones involved in brain plasticity and social behavior (e.g., oxytocin, dopamine) (Feldman, 2017), or, especially, the HPA activation in response to stress (Gunnar & Quevedo, 2007).

Indeed, a longitudinal study has found that institutionalization at a young age leads to severe consequences in the development of both brain morphology and behavior (Zeanah et al., 2003). The study followed three groups of children: institutionalized children (children who lived all their lives in an institutional setting of Romania), foster care children (children who were institutionalized at birth and then placed in foster care) and a never institutionalized group (children who were never institutionalized). Institutionalized children showed delayed patterns of physical and cognitive growth, and a very different pattern of brain development, characterized by a cortical hypoactivation (Marshall et al., 2004). They also showed severely impairment in IQ and manifested a variety of social and emotional disorders (Nelson et al., 2014). Interestingly, children who were placed in foster care before 2 years old showed a brain activity pattern more similar to those never institutionalized than those placed in foster care after two years old (Marshall et al., 2008). Thus, the lack of good care has detrimental effects on brain function and these effects tends to be worse when the infant is older than 2 years old at the time of exposure to the stressor.

In summary, during postnatal periods -infancy, childhood and adolescence-, the environment at home would be crucial for reaching a proper brain maturity. Specifically, adverse experiences, such as childhood maltreatment, can disrupt brain maturation, increasing the risk of emotional dysregulation, behavioral problems and psychopathology.

1.2.2.1. The relevance of early adverse events and childhood maltreatment

As mentioned at the beginning of this section, infancy is a prolonged period in the average person's expected lifespan, necessary for completing brain neurodevelopment and for integrating relevant social functions for the subject. However, infancy is not immune to psychosocial stress and maltreatment experiences. Unfortunately, these circumstances are relatively common in our societies, and their severity depends largely on the nature of these experiences (how and by whom they are carried out) as well as the manner in which they occur (intensity, frequency, and timing in development). This variability can lead to diverse impacts, both psychologically and biologically, ultimately affecting the child's physical and mental health.

Stressful life events, like the loss of a family member, a serious illness, or a separation, could lead to psychological harm, although a considerable number of them would be required to cause such damage. Contrary, experiences like childhood maltreatment, by their very nature, are inherently damaging and leave a lasting imprint on the individual, increasing the risk of developing complex trauma symptoms. The rationale for this lies in the inherent nature of humans beings, for whom experiences arising from interpersonal relationships, particularly within the familial context, are of utmost significance. Furthermore, experiences occurring in the early stages of life can exert a more profound impact on the individual, given the heightened vulnerability during that developmental phase.

Experiences of child violence can be classified based on the timing of exposure (prenatal or postnatal), the context in which they occur (within the family or outside the family), or the type of behavior involved. With regard to the latter, the most commonly used classification often distinguishes between active maltreatment (behaviors and discourse involving the use of psychological, physical, or sexual force that directly inflicts harm) and passive maltreatment (meaning mistreatment characterized by a lack of necessary interventions and/or discourse to ensure the well-being of the child). Active

maltreatment comprises emotional abuse, physical abuse and sexual abuse and passive maltreatment is composed of emotional and physical neglect.

It remains unclear how many children's lives are touched by maltreatment. Stoltenborgh and colleagues (2015) conducted a review of the available literature, primarily developed in Western countries, to estimate the prevalence rates of the different types of childhood maltreatment. The review ultimately included a total of 224 studies that utilized self-report measures. They concluded that 36.3% of individuals have experienced emotional abuse, 22.6% physical abuse, 18.4% emotional neglect, 16.3% physical neglect, and 18.0% of girls and 7.6% of boys have experienced sexual abuse. However, it is believed that these figures are underestimated. The available evidence is that only 5% of child physical abuse and 8% of child sexual abuse is reported to child protection authorities (Everson et al., 2008). Most cases are no reported, and when reported, abuse may not be officially confirmed despite being present (Mathews et al., 2016). Moreover, there is comorbidity among the types of maltreatment; in fact, it has been suggested that emotional abuse often occurs in conjunction with other forms of maltreatment (McGee et al., 1995).

Young individuals affected by different mental disorders exhibit an increased prevalence of early adverse experiences (Heim et al., 2019). In fact, maltreated individuals develop psychiatric disorder at an early age, have more comorbidities, greater symptom severity and respond less favorable to standard treatments than not maltreated individuals, due to the neurobiological changes that abuse produces at such an early age (Lippard & Nemeroff, 2020; Nederhof et al., 2015; Teicher & Samson, 2013). Additionally, the psychopathological consequences of early adversities can manifest during childhood. However, diagnosing clinical conditions in children poses significant challenges, commonly leading to the grouping of symptoms into two major spectra: internalizing (such as isolation, sadness, or fear) and externalizing (involving aggression, impulsivity, and norm-transgressing behavior). In fact, it is suggested that internalizing and externalizing symptoms in childhood may be considered prodromal states of more specific clinical conditions that emerge later in life (Fryers & Brugha, 2013).

Having reliable tools to detect childhood maltreatment while it is occurring, as well as its severity and frequency, would allow for intervention and the development of protective measures before psychiatric symptoms or risk behaviors emerge (e.g., academic failure, delinquency, drug abuse). However, there are not many protocols that assess abuse in such a thorough manner. The researcher must obtain a reliable information without causing revictimization to the subject. Additionally, there is the

difficulty that a child often does not report instances of abuse due to a lack of understanding and shame. Moreover, in many cases, it occurs within the family, so asking parents or caregivers may lead to an underestimation of the prevalence of abuse. For this reason, many studies have retrospectively assessed child abuse in adult populations. Various methods, such as semi-structured interviews and validated self-reported questionnaires, are employed to assess potential abuse situations. This becomes particularly pertinent during adolescence, a phase marked by an increased susceptibility to various forms of abuse, especially sexual abuse and bullying (Arseneault, 2018). Noteworthy instruments for this stage include the Childhood Trauma Questionnaire (CTQ) and the Childhood Experience of Care and Abuse Questionnaire (CECA-Q2) (see Box 2 for further details). Additionally, in the pediatric population, incomplete stories, drawings, or play activities can be useful procedures for exploring various instances of abuse.

BOX 2. Questionnaires to assess child abuse in adolescents

There are some questionnaires or interviews useful to assess experiences of violence or abuse in adolescents in research context. Some of these questionnaires available in Spanish include:

1) *Childhood Trauma Questionnaire short form* (CTQ-SF) (Bernstein et al., 2003). The CTQ-SF consists of 28 items, 25 measure childhood maltreatment, distributed across five subscales, each consisting of five items (physical, emotional and sexual abuse and physical and emotional neglect) and 3 items are designed to measure minimization/denial. The frequency of the maltreatment for each of the five types can be measure, since the subscales are sums of scores ranging from 'never true' (score 1) to 'very often true' (score 5); consequently, all subscales can vary between 5 and 25.

2) *Childhood Experience of Care and Abuse Questionnaire* 2nd version (CECA-Q2) (Kaess et al., 2011). The CECA-Q2 gathers information related to the quality of caregiving, experiences of abuse, and relevant details such as the presence of supportive figures, the nature of care received, and potential instances of emotional, physical, or sexual abuse. It separately assesses maternal and paternal figures, evaluating possible antipathy, neglect, physical abuse, and sexual abuse from both figures. This questionnaire considers the frequency of these abusive experiences and at what age they commenced.

Despite the challenge it poses, it is essential to explore the history of childhood maltreatment at the time it is occurring (during childhood and adolescence), employing dose-effect perspective. This approach is crucial for a better understanding of the role it will play in the neurobiological alterations that will later sustain the foundation of psychopathology and its associated physio-pathological basis.

1.3. Physiological mechanisms involved in the response to psychosocial stress

There are several biological mechanisms involved in the response to psychosocial stressors that are simultaneously activated, allowing the individual to cope with environmental threats or stressors.

The stress response begins in the brain. The amygdala is an area in the brain that process emotions, as fear, and is the first region to perceive a threat or stressor. When the amygdala interprets a situation as stressful, it instantly sends a signal to the hypothalamus, which activates multiple systems in the body, such as the sympathetic nervous system (SNS) and the Hypothalamus-Pituitary-Adrenal (HPA) (see Figures 6 and 7).

Endocrinologist Hans Seyle, the father of stress research, stablished three-stage response to exposition to the stress stimuli: alarm phase, resistance and exhaustion (Seyle, 1946). The alarm phase corresponds with the classic "fight or flight" response, characterized by the activation of the SNS. This response involves a rapid physiological adaptation, releasing catecholamines like epinephrine and norepinephrine into the bloodstream to induce alterations in blood vessels, visceral organs, glands and smooth muscles; maintaining the alertness (Godoy et al., 2018). In parallel, blood sugar and fats from temporary storage sites in the body are released to supply energy to deal with the stressor. Moreover, the SNS activates inflammatory mediators. Finally, in short stressors, the body returns to a state of balance (homeostasis) thanks to the parasympathetic nervous system (PNS), which generally has opposing effects to the SNS (see Figure 6).

If the brain continues to perceive a stressor, resistance, the second phase, begins. This phase involves the HPA axis activation, releasing in last term cortisol the blood torrent (Carrasco & Van de Kar, 2003; Godoy et al., 2018). Cortisol continue to regulate the glucose storage and utilization and moderates the magnitude and duration of inflammatory responses (Sapolsky et al., 2000). When the psychological stress is already over, homeostasis is progressively recovered, inflammation inhibited, cardiovascular parameters are restored to normal levels and HPA axis regain physiological stability thanks to cortisol negative-feedback (Hammes & Levin, 2007). In case the stressor persist or its intensity is excessive, a deregulation of these adaptive mechanisms can be produced, reaching the exhaustion phase and increasing the individual's risk of experiencing pathological conditions (Tonhajzerova & Mestanik, 2017).

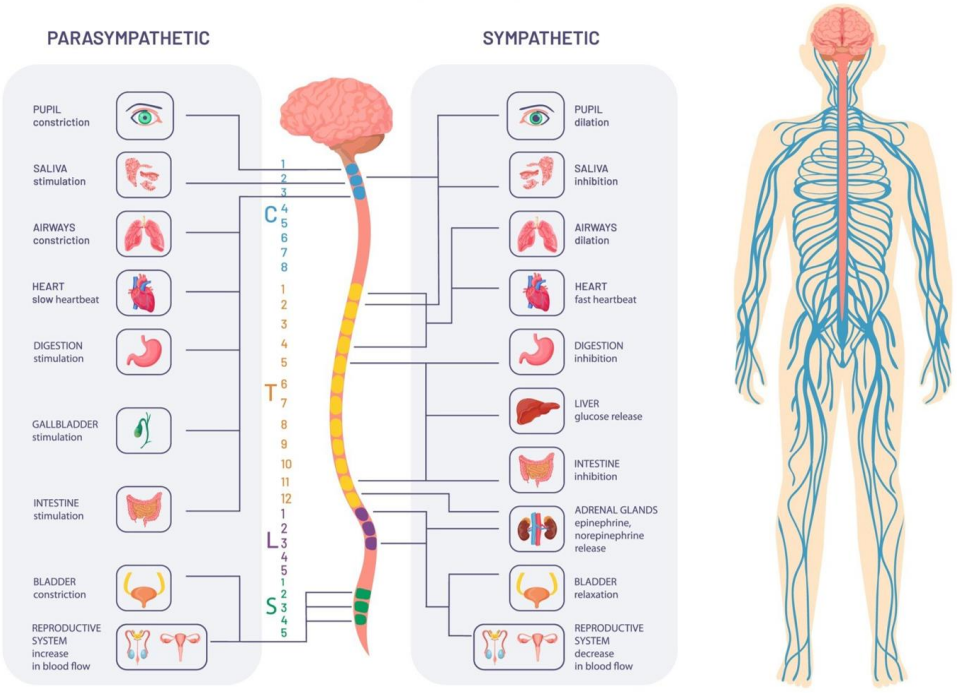

Figure 6. Overview of the autonomic nervous system. The autonomic nervous system regulates certain body processes and comprises two antagonistic sets of nerves, the sympathetic nervous system (SNS) and the parasympathetic nervous systems (PNS). The SNS involves different functions that allow a rapid physiological adaptation, while the PNS involves functions to restore the homeostasis once the stressor is over. Designed by Freepik.

It should be noted that perceiving a situation as stressful not only depends on the environmental conditions themselves, but personal experiences also play a huge role in the stress interpretation. Henceforth, the experiences that humans lived during early life stages, the emotions they perceived during their first social interactions could modulate their future physiological activation to environmental stressors (Levenson, 2003).

1.3.1. The Hypothalamic-Pituitary-Adrenal (HPA) axis

Hypothalamic-pituitary-adrenal (HPA) axis is the principal sensor and regulator of stress response in humans. Under psychological stress circumstances, HPA axis is activated releasing a cascade of hormones (see Figure 7). The hypothalamus releases corticotropin-releasing hormone (CRH), which travels to the pituitary gland, triggering the release of adrenocorticotropic hormone (ACTH). This hormone travels to the adrenal glands, prompting the release of cortisol (Godoy et al., 2018). Cortisol prepares the body to the fight-or-flight response by providing the energy required to deal with the challenge.

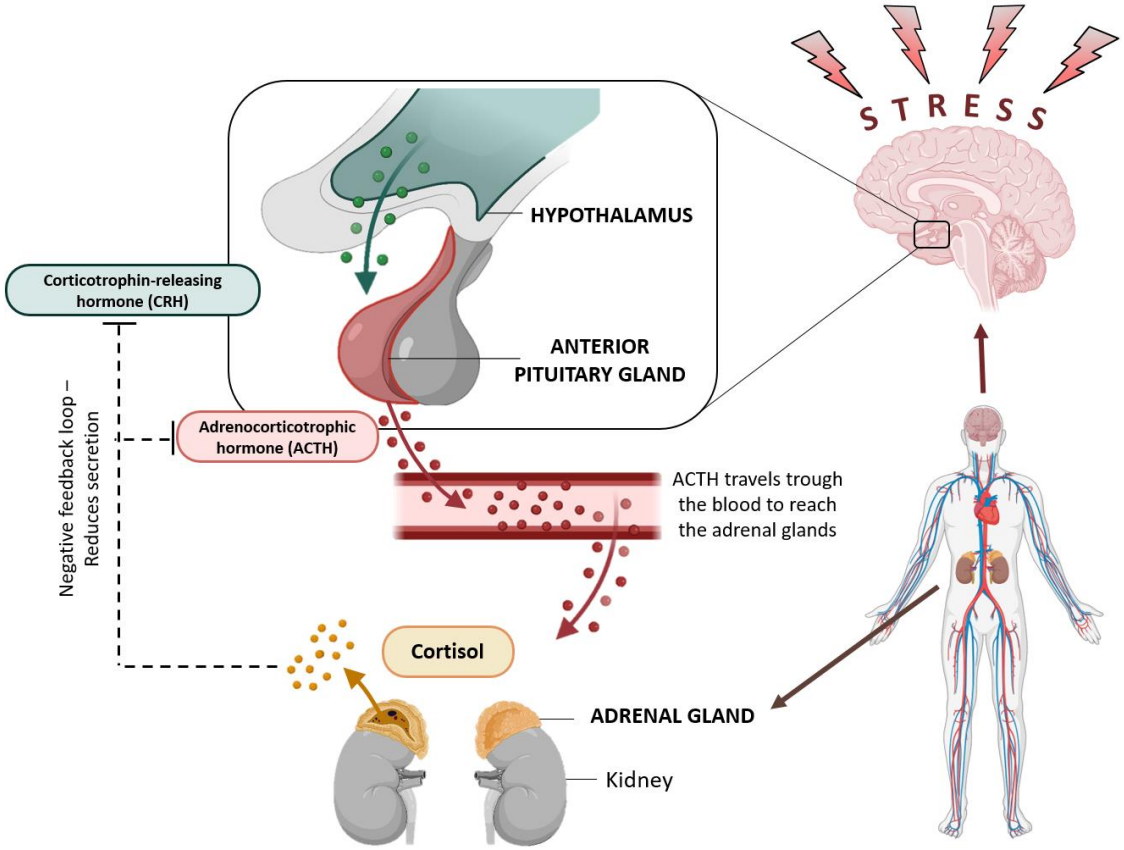

Figure 7. Hypothalamic Pituitary Adrenal (HPA) axis. When the brain interprets a situation as stressful, the hypothalamus activates the releasing of the corticotrophin-releasing hormone (CRH), in green. The CRH activates in the anterior pituitary gland the release of adrenocorticotrophic hormone (ACTH), in red. The ACTH travels through the blood to reach the adrenal glands, in the kidneys, activating the release of cortisol, in yellow. Once the stressor is over, CRH, ACTH and cortisol release would be progressively over thanks to the negative feedback. Image created with BioRender.com.

Through glucocorticoid receptor (GR), located in cell cytoplasm, cortisol induces gene expression mediating a myriad of tissue specific effects. Specifically, when bound to cortisol, the GR translocates to the nucleus where it acts both as a transcription factor and as a repressor of immune- and metabolic-related genes, among others (Nicolaides et al., 2010). This coupling enables the dissociation of a chaperone complex, of which FK506-binding protein 5 (FKBP5) is a part, preventing the nuclear translocation of GR in the absence of glucocorticoid. When the stressful situation is over, homeostasis is progressively recovered and HPA axis regains physiological stability thanks to cortisol negative-feedback via activation of the GR in the hypothalamus and pituitary gland (Hammes & Levin, 2007).

Cortisol is also responsive to normal day-to-day activities and is involved in the normative functioning of multiple physiological systems, including those responsible for glucose storage regulation and utilization, immune responses (e.g., cytokines production) magnitude and duration, and anti-inflammatory action (Spencer & Deak, 2017).

Moreover, this basal regulation is mediated mainly through mineralocorticoid receptors (MR). In healthy populations, diurnal cortisol levels follow a circadian pattern with a rapid rise at awakening in the morning followed by a decline across the day, reaching its lowest levels at around midnight (Debono et al., 2009; El-Farhan et al., 2017) (see Figure 8).

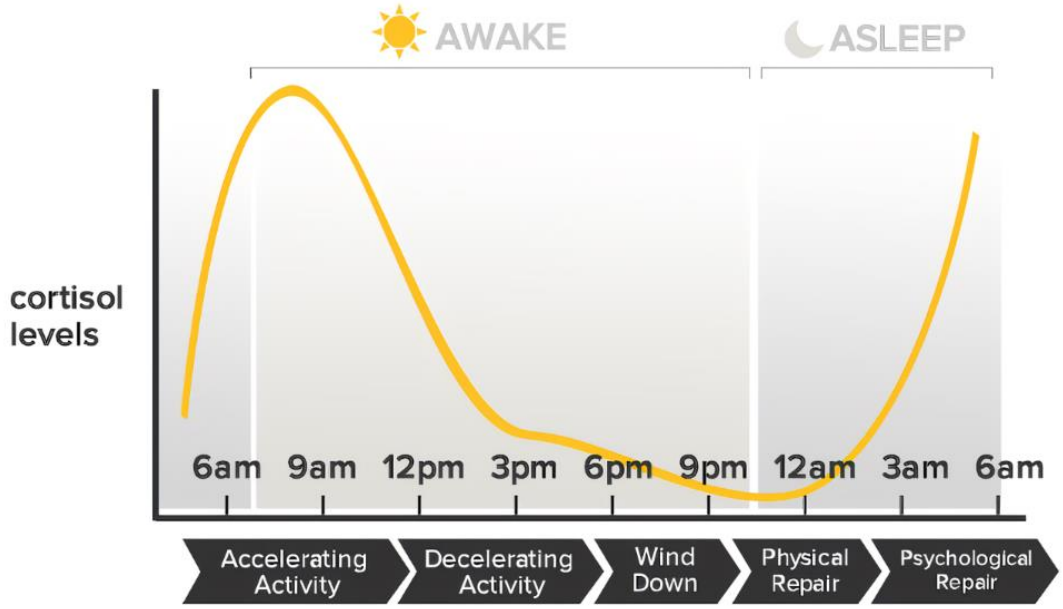

Figure 8. Cortisol circadian rhythm. Cortisol levels increase in the morning, with a peak one hour within awakening, and decrease through the day, with the lowest levels before going to sleep. Retrieved from BioRender.com

However, in case the stressor persists, or its intensity is excessive, a deregulation of these adaptive mechanisms to basal and stress circumstances can be produced. Under chronic stress a desensitization of the corticosteroid receptors – MR and GR, could occur, altering the negative feedback and basal cortisol circulatory levels. While excessively high cortisol levels are neurotoxic, altering cognitive functions, abnormally low cortisol levels are insufficient to marshal metabolic resources, necessary for optimal learning and memory consolidation (Suor et al., 2015). Furthermore, adequate cortisol levels are required for normal functioning of the SNS and the immune system (Sorrells et al., 2009). Thus, deviations of cortisol in both directions may lead to pathological outcomes in multiple bodily compartments (Tonhajzerova & Mestanik, 2017).

The modulation of the HPA axis begins early in development through maternal-placental-fetal dialogue, with placental CRH and maternal cortisol levels playing a central role in this modulation (Roettger et al., 2019). HPA axis maturation continues during postnatal life, being especially sensitive to maternal-infant bonding and early life's adversities (Bosch et al., 2012). Chronic stress situations from very early stages, even prenatally, can induce persistent changes in the ability of HPA axis to respond to stress and to regulate cortisol circadian rhythm in the long-term, which is commonly known as "desensitization".

For example, children growing up under adverse conditions required frequent response of the stress system. In these conditions, the physiological stress desensitization will lead to a hyporeactive HPA axis and, consequently, to lower blood cortisol levels under the persistent stress situations. This hyporeactivity could protect the organism from neurotoxic cortisol levels, although it would also alter the adaptive capacity of the HPA axis to respond to new acute stress situations, increasing the risk of future psychopathology in exposed children (Wesarg et al., 2020).

Different studies have associated the exposure to early stressors with blunted activity on the HPA axis, assessed using circadian or acute stress levels of cortisol, and altered behavior. For example, maternal stress and exposure to exogenous cortisol in early pregnancy have been linked with lower birthweight (Orr & Miller, 1995), increased offspring HPA axis reactivity at 6 months (Lyons-Ruth et al., 2000) and at 6 years (Gutteling et al., 2005), and neurological and cognitive developmental disturbances between 6 months and five years old (Trautman et al., 1995). In the first year of life, less sensitive parenting is associated with prolonged activations of the HPA axis to everyday stressors (Albers et al., 2008). Neglect or abuse in institutionalized children have been associated with lower basal levels of cortisol (Gunnar & Donzella, 2002). In adolescence, lower HPA axis activity has been associated with internalizing and externalizing disorders (Bae et al., 2015; Conradt et al., 2014). These altered cortisol fluctuations due early adverse experiences could be maintained during adult life and be associated with psychiatric diagnosis (Zorn et al., 2017).

In summary, HPA axis functioning can be study under both basal and acute stress circumstances trough cortisol levels. Due to the high correlation between plasma and serum cortisol levels and salivary cortisol (Hellhammer et al., 2009), HPA axis can be also studied trough salivary cortisol levels. Saliva samples are minimally invasive and can be easily taken and fixed intervals during and acute stressor or across the day, which led us to study how cortisol responses vary under stress exposure in young populations.

Study of the basal activity of HPA axis: Circadian Rhythm and the Area Under the Curve

Cortisol circadian rhythm typically shows a rise during the night, with a peak within the first hour after awakening. Then, cortisol levels drop quickly during morning hours and decline slowly through the rest of the day with a low during the night (see Figure 8). The fast-morning increase, which is known as cortisol awakening response (CAR), led to metabolic processes that gives us the energy to confront the day, while cortisol decrease during the night is related with a restful sleep. This diurnal cycle of cortisol release can be interrupted by stressors. The total diurnal cortisol output can be measured using two different approaches, the Area Under Curve with respect to the ground (AUCg) and with respect to the increase (AUCi) (see Box 3 and Figure 9).

BOX 3. Area Under the Curve with respect to the ground (AUCg) and to the increase (AUCi)

The term "area under the curve" (AUC) is frequently utilized in the context of cortisol levels over time, representing a statistical approach that integrates multiple time points to enable the detection of potential associations between repeated measures and other variables. In fact Pruessner et al. (2003) recommend using these two formulas when working with different measures of cortisol collected across different time points.

There are two main variations of AUC relevant to cortisol:

1) AUC with respect to the ground (AUCg). AUCg indicates mean cortisol levels throughout the day, encompassing both baseline cortisol levels and any fluctuations above or below the baseline over the specified time period (from awakening to before going to sleep) (see Figure 9a).

2) AUC with respect to the increase (AUCi). AUCi indicates changes of cortisol levels (increase or decrease) respect to the first measure (awakening sample) during the day. It calculates the cumulative increase in cortisol concentration above the baseline (awakening measure) throughout a defined time frame (until going to sleep) (see Figure 9b).

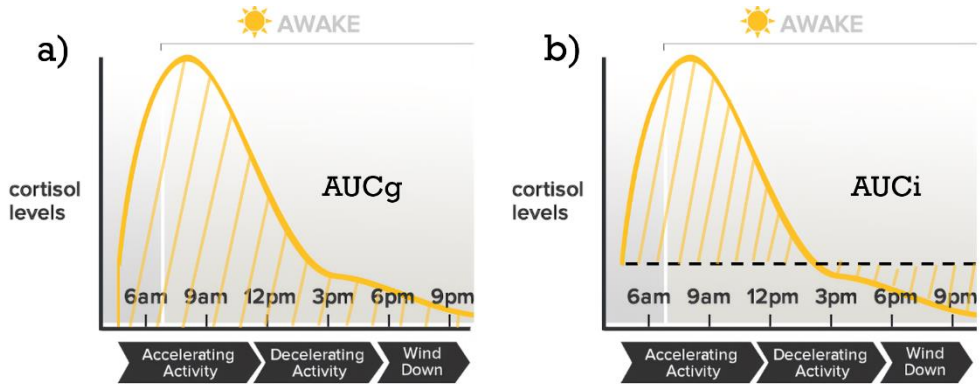

Figure 9. Area Under the Curve (AUC) formula for cortisol measures. a) AUC with respect to the ground (AUCg), indicates mean cortisol levels throughout the day. b) AUC with respect to the increase (AUCi), indicates changes of cortisol levels across the day. Adapted from BioRender.com

Cortisol circadian rhythm is present, on average, around 2 months of age (de Weerth et al., 2003), although the rhythmicity could vary due to sleeping and feeding patterns (Gunnar & White, 2001). The maturation of cortisol circadian rhythm parallels maturation of HPA axis, so environmental factors could produce lasting changes on cortisol diurnal rhythmicity (Tarullo & Gunnar, 2006). However, there is an inconsistency regarding how adverse early life experiences influence cortisol circadian rhythm. Therefore, further investigation in this field is necessary.

Study of HPA axis reactivity under acute psychosocial stress: TSST-C

Experimental paradigms that allow to assess functional changes of the HPA axis under controlled stressful situations have been developed in the last decades. This is the case of the Trier Social Stress Test (TSST) that has been widely used in infant and adult subjects, affected or not by different pathologies and chronic stress experiences (Kirschbaum et al., 2003). TSST is an interesting setting as it provides a quantitative and continuous measure of the HPA axis activity, or circulating immune cells and proinflammatory cytokines, under a controlled acute psychosocial stress situation, taking saliva samples (Allen et al., 2017) (see Box 4).

BOX 4. Trier Social Stress Test for Children (TSST-C)

Participants are scheduled in the center where the protocol would be applied, although there could be differences between studies. At arrival, participants would be in baseline rest in a neutral room, so the basal condition could be compared between subjects. After this rest period, participants go to a second room (experimental room) in which a panel of "judges" (usually a man and a woman) instruct participants the tasks they should develop. Performing each task in front of a judges introduces the element of social-evaluative threat which is enhanced by their performance being video and voice recorded. Furthermore, participants are informed that the judges are experts in behavior analyses, and they are trained to withhold positive feedback.

The first task of the TSST for adults is usually a job interview. However, in the TSST for children (TSST-C) participants are given the stem of a story for the first task and asked to complete the story in an interesting way, and that the story ending should be better than the provided for the other children that have participated. The participant has 5 minutes for prepare the story and 5 minutes to complete it. The judges ask the participant to continue if the story finishes in less than 5 minutes and withholds feedback. Finally, the participant should complete a serial subtraction task, and they are encouraged to complete it as quickly and accurate as possible. The duration of the task is around 15-20 minutes. Once the tasks are completed, participants return to the first room for recovery (see Figure 10). This protocol brings the opportunity to collect different biological and/or psychological samples across the procedure (blood and saliva samples, heart rate, stress perception, etc.) and compare them with the participant subjective perception to the stressor (cognitive test). In fact, participants are normally cited in the evening to avoid circadian rhythm variability in the collected biomarkers.

The TSST typically consist of a preparation phase, a public speaking task and a verbal arithmetic task in front of a panel of judges (see Figure 10).

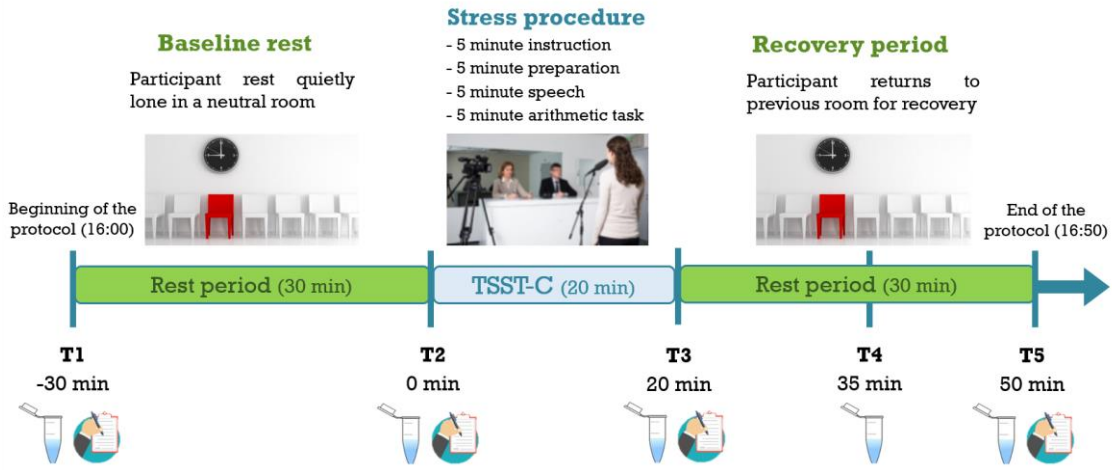

Figure 10. Trier Social Stress Test for Children (TSST-C) protocol design. Image courtesy of Laia Marqués Feixa.

The TSST was originally developed for apply to adults, although it has been adapted for the use with children - TSST for children (TSST-C) (Buske-Kirschbaum et al., 1997). However, it there are contradictory results regarding the physiological response induced by the TSST in children sample, since the developmental stage of the children and their psychopathological status could impact the effectiveness of the test (Seddon et al., 2020). It is also important to consider that the time of the day also plays a role in how different biomarkers respond to a stressor, as cortisol). Furthermore, there are few studies that use the TSST-C in child-youth population with current exposure to adverse experiences or maltreatment. Thus, more research is needed to disentangle the possible effects of childhood maltreatment on HPA axis responses to TSST-C.

1.3.2. HPA axis and pregnancy

Throughout pregnancy, there are significant alterations in the regulation of the maternal HPA axis (see Figure 11). Circulating cortisol levels increased to around threefold before pregnancy levels by the third trimester (Jung et al., 2011). This rise in cortisol levels is partly due to corticosteroid-binding globulin, which is stimulated by placental estrogens, resulting in an increase of free cortisol levels (Qureshi et al., 2007). Furthermore, During the second and third trimesters of pregnancy, the placenta releases CRH into the maternal bloodstream (Reis et al., 1999). his placental CRH prompts the maternal pituitary gland to elevate both maternal ACTH and maternal cortisol levels. Consequently, maternal cortisol further stimulates the synthesis of placental CRH, establishing a positive feedback loop (Robinson et al., 1988). The increases in maternal plasma cortisol are noted as early as the 11th week of gestation (Demey-Ponsart et al., 1982) and seems to reach a plateau in the third trimester (Nolten & Rueckert, 1981). During the course of pregnancy, the increased circulating cortisol downregulates the production of maternal CRH and, consequently, maternal HPA axis responsiveness to psychological stress is attenuated (de Weerth & Buitelaar, 2005).

Despite pregnancy entails an exponential increase in cortisol levels, high cortisol levels can be toxic for the fetus (Morsi et al., 2018). For this reason, during pregnancy, the fetus is protected from excess cortisol exposure in utero thanks to the placental enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which rises in parallel with increasing cortisol levels and metabolizes it into inactive cortisone (Murphy et al., 1974). Although a high proportion of cortisol is metabolized by the placenta during gestation (80-90%), there is a small proportion that must reach the fetus (10-20%), since cortisol is essential to the maturation of the lungs, gastrointestinal tract, liver, and central nervous system (Blackburn, 2003). For this reason, synthetic glucocorticoids are administrated in threatened preterm delivery to improve neonatal viability by accelerating fetal organ maturation (Roberts & Dalziel, 2006). Furthermore, 11β-HSD2 activity decreases at the end of pregnancy (Murphy et al., 2006), so more maternal cortisol could pass through the placenta, playing a key role on fetal HPA axis activation and in the time of parturition (Moisiadis & Matthews, 2014; Seckl, 1997). In the postpartum period, the placental CRH levels fall in the maternal circulation, leading to a gradually reduction in maternal cortisol levels and a recovery of the pre-pregnancy HPA axis function.

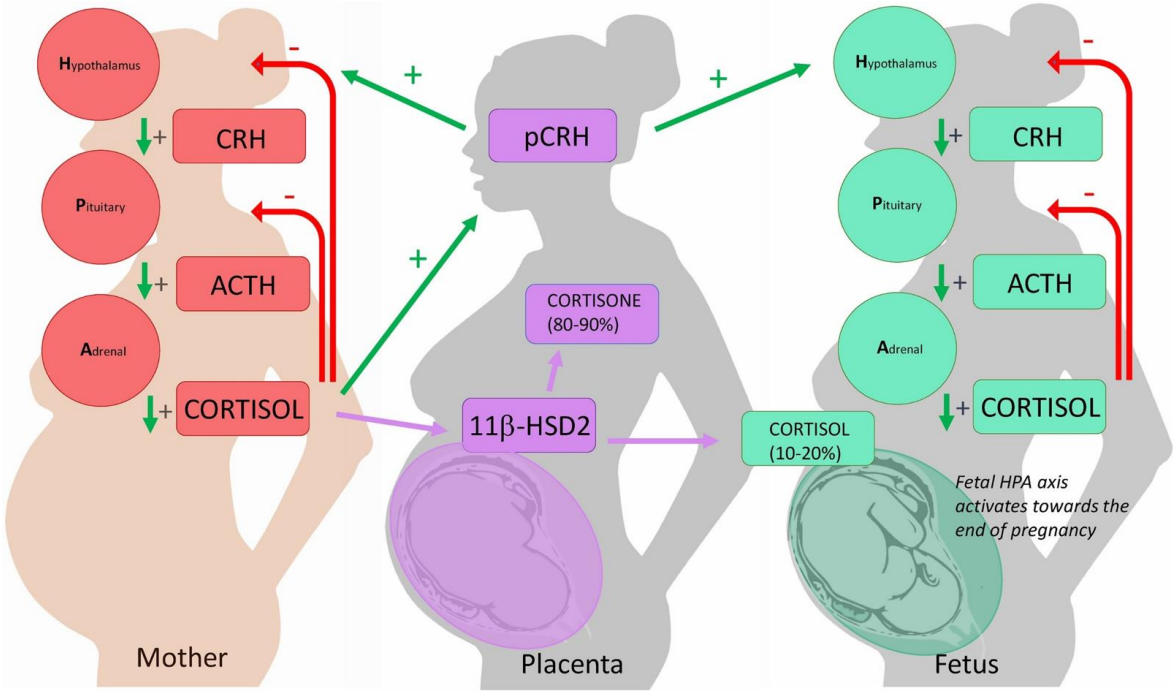

Figure 11. Functioning of the HPA axis during pregnancy. Maternal HPA axis (left) continues to function as in non-pregnant states, but with the positive feedback effects of placental CHR (pCRH) (middle), so maternal ACTH and cortisol levels increases. The fetus is protected from excessively high maternal cortisol levels thanks to the action of 11βHSD2 (middle) that metabolizes active cortisol into inactive cortisone. A small proportion of maternal cortisol (10-20%) is not metabolized by the 11βHSD2 and arrives to the fetus (left), since is necessary for organ maturation and fetal HPA axis activation. Reproduced with permission from Mustonen et al. Hair cortisol concentration (HCC) as a measure for prenatal psychological distress - A systematic review. Psychoneuroendocrinology. 92:21-28 (2018).

Despite all these natural mechanisms to minimize fetal overexposure to maternal glucocorticoids, these mechanisms fail to offer such protection during maternal stress, infection, and inflammation, thus allowing more cortisol to cross the placenta and affect the developing fetus (Cottrell & Seckl, 2009). Elevated maternal cortisol levels have been associated with increased risk of early miscarriage (Nepomnaschy et al., 2006), fetal growth retardation and premature birth (Aoki et al., 2022). Moreover, elevated maternal cortisol levels have been also linked to consequences on motor development, cognition, and temperament over the first year of newborn life (Caparros-Gonzalez et al., 2019; Davis et al., 2007). Interestingly Davis & Sandman (2010) observed that prenatal maternal cortisol had opposite effects on infant cognitive development depending on the timing of exposure. Infant had better performance on cognitive tasks at one year of age when they were exposed to low concentrations of maternal cortisol in early pregnancy and high concentration of cortisol towards the end of pregnancy. This result is consistent with the essential role that cortisol plays on some aspects of normal fetal

brain development at the end of pregnancy, but the neurotoxic effect that excessive levels of cortisol could have on neuronal structure and synapsis formation (Seckl & Meaney, 2004), processes that are already occurring in the second trimester of pregnancy.

Maternal HPA axis changes during pregnancy and increase in cortisol levels have been proposed to underlie the relationship between maternal environmental stressors and offspring disease risk. In this regard, different stressors, ranging from anxiety and depressive symptoms, natural disasters and poorer obstetric outcomes have been associated with infant poorer outcomes at birth (Talge et al., 2007). However, many of these studies did not include measures of maternal cortisol, and, if included, cortisol levels did not always correlate with maternal stress during pregnancy. This is partially due to the definition and measure of maternal stress (normally with auto-administered questionnaires), but also due to the variability of cortisol collection during pregnancy, which is related to analytical approaches. Most of the studies linking maternal adverse conditions during pregnancy and cortisol collect morning salivary samples, ignoring that cortisol follows a diurnal variation, even during pregnancy (Entringer et al., 2011), and that high evening cortisol levels have been strongly associated with psychosocial stress, particularly with depression (Adam et al., 2017). Furthermore, most of the research regarding stress and gestational cortisol have been performed in the third trimester of pregnancy (Orta et al., 2018), missing early pregnancy cortisol data. This is a critical period during which cortisol has a potentially higher neurotoxic effect on the fetus. Henceforth, more studies including maternal cortisol levels across day and in different times throughout pregnancy are required to evaluate the effect of maternal psychosocial stress on maternal gestational cortisol and offspring outcomes.

1.3.2.1. Placental function against cortisol and associated protective mechanisms for fetal development

Appropriate functioning of the placenta is crucial to fetal development. The placenta is a fetomaternal transient organ vital for transferring gases and nutrients between mother and fetus. The placental barrier limits direct contact between the embryo and maternal blood, protecting both the mother and the child from potentially harmful substances (e.g., blood cell antigens of the fetus or bacteria from the mother). Additionally, the placenta secretes hormones (e.g., CRH, estrogen, progesterone, oxytocin, prolactin) that mediate the adaptation to pregnancy, maintain pregnancy and, at the end of pregnancy, induce labor.

Early in pregnancy, after implantation, cells from the external layer of the blastocyst, referred as trophoblast, invade the maternal endometrium and remodel mother's arteries to stablish a connection between maternal blood and fetal cells.

The placenta structure changes during pregnancy, acquiring its typical round, flat appearance during week three of embryonic development, which is around the second month of pregnancy (see Figure 12). The fetal blood vessels, or villi, branch from the umbilical cord to the intervillous space of the placenta. Maternal blood in the intervillous space bathes the villi and is in contact with specialized cells known as trophoblast, which allow the transport of substances to and from the fetus.

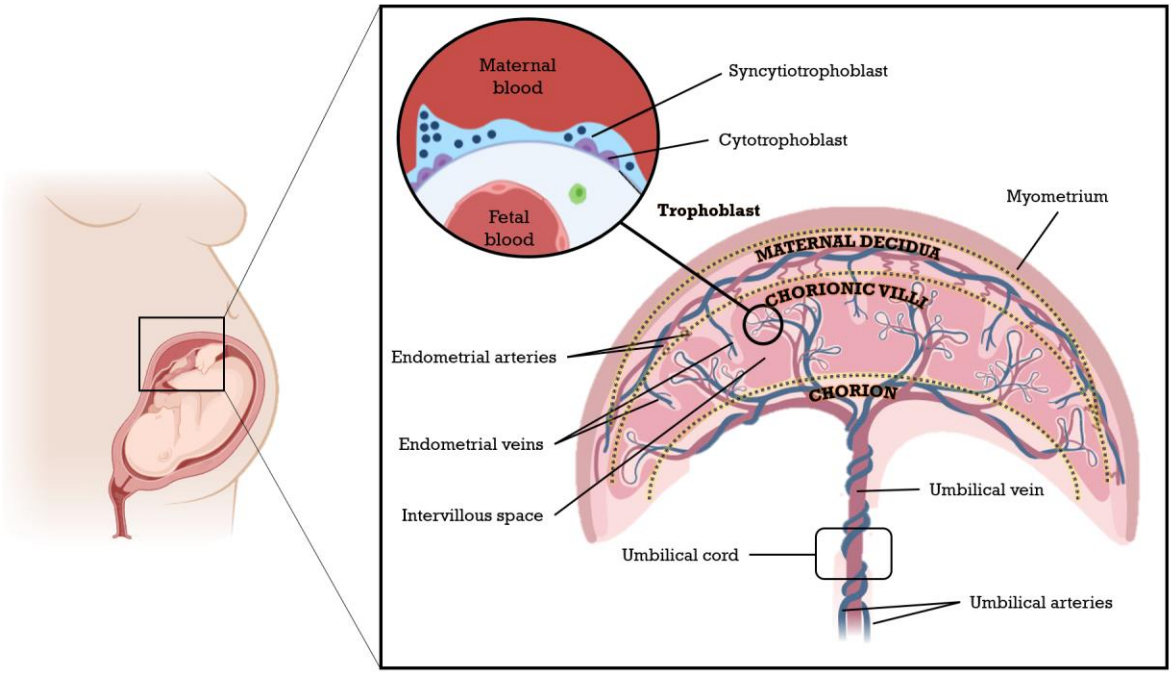

Figure 12. The structure of human placenta. The placenta acquires its typical round flat form in the second month of pregnancy. It is important to note that the placenta is composed of three differentiated layers: the chorion, the maternal decidua, and the chorionic villi. In the last two layers, but particularly in the chorionic villi, the trophoblast is present. Image created with BioRender.com.

The trophoblast layer consists of two cell types: the syncytiotrophoblast on the surface and the layer of cytotrophoblast underneath which fuse together to continuously regenerate the syncytiotrophoblast. Since the syncytiotrophoblast are in contact with maternal blood, this site is commonly referred to as de 'maternal-fetal interface' (Brady, 2021). Hence, the placenta consists in three layers: maternal decidua (mainly maternal component), chorionic villi (fetomaternal zone, intervillous space and 30-50 branching villous trees) and the chorion (fetal component). At term, the mature placenta weights around 500g and is about 2cm thick, having a diameter of 15-20cm.

Significant factors in placental development and function involve epigenetic mechanisms. The term epigenetics was coined by Waddington in 1942 to refer to the branch of biology which studies interactions between genes and their products which bring the phenotype into being (Waddington, 2012). Since then, epigenetics definition has been gradually narrowed, as it has spread into the fields that do not routinely address genetics, such ecology, physiology, and psychology. In the framework of this thesis, from the many definitions of what epigenetic means, epigenetic refers to changes in gene function, signatures that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence" (Wu & Morris, 2001).

The concept epigenetic comprises to chemical modification of DNA bases and changes of the chromosomal superstructure in which DNA is packaged, changes that can regulate gene expression despite not directly altering DNA sequence. There are four main epigenetic mechanisms including DNA methylation, histone modification, chromatin remodeling, and noncoding RNA (Figure 13).

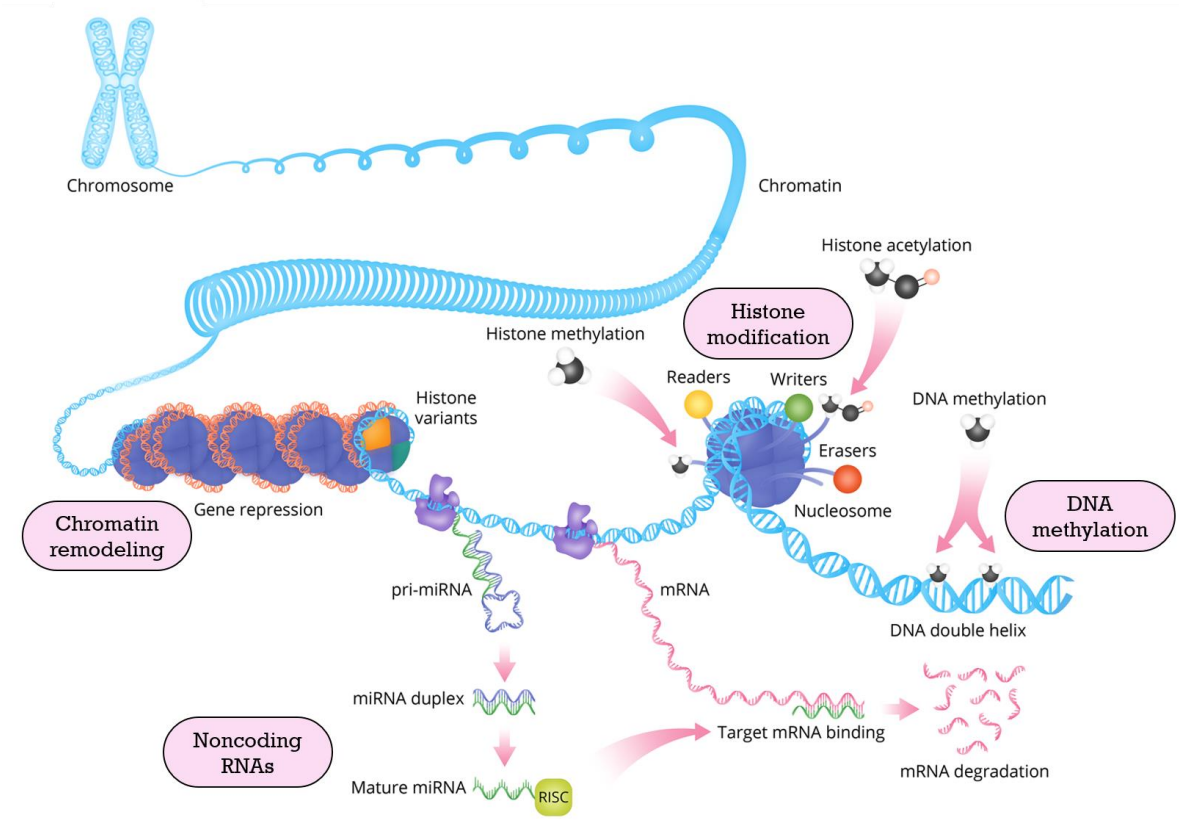

Figure 13. Scheme of the four main epigenetic processes. Adapted from Smigielski and colleagues (2020).

Among all epigenetic modifications, DNA methylation is the best characterized one. In humans, the addition of this methyl group occurs predominantly on cytosine that precede a guanosine in DNA sequence, generally named as CpG dinucleotide (Herman & Baylin, 2003). These dinucleotides can be clustered in small sequences of DNA, known as CpG islands, which are generally in promoter regions. Most CpG islands in gene promoters are unmethylated, allowing gene transcription (Herman & Baylin, 2003).

In placenta, methylation plays an important role in the differentiation of the embryonic and extraembryonic lineages, in other words, epigenetic mechanisms are necessary for the differentiation of the cells that will give rise to the placenta and the fetus respectively (Nelissen et al., 2011). Besides, DNA methylation also affects placenta physiology, being essential in the regulation of the functioning of glucocorticoid-related genes and, consequently, on infant exposure to cortisol and/or stress (Paquette et al., 2015).

As mentioned above (see Figure 11), 11βHSD2 is one of the main regulators of cortisol in the placenta, playing a pivotal role in controlling fetal exposure to maternal cortisol by converting cortisol into its inactive metabolite cortisone. This enzyme is predominantly expressed in the syncytiotrophoblast cells (Krozowski et al., 1995), the site of fetal-maternal exchange (see Figure 14 for more details), but also in the epithelial cells of maternal decidua (Zhu et al., 2019).

The presence of this enzyme is directly modulated by cortisol or indirectly by placental CRH (Fahlbusch et al., 2012) or GR (van Beek et al., 2004). The methylation of particular CpG sites of the gene encoding 11βHSD2, known as *HSD11B2* gene, has been associated with prenatal adversity (Appleton et al., 2015), reduced newborn quality of movement (Marsit et al., 2012) and with lower muscle tone in newborns (Conradt et al., 2013). However, it is still not clear whether maternal prenatal distress associated with depressive symptoms can influence fetal neurobehavioral via placental *HSD11B2* DNA methylation.

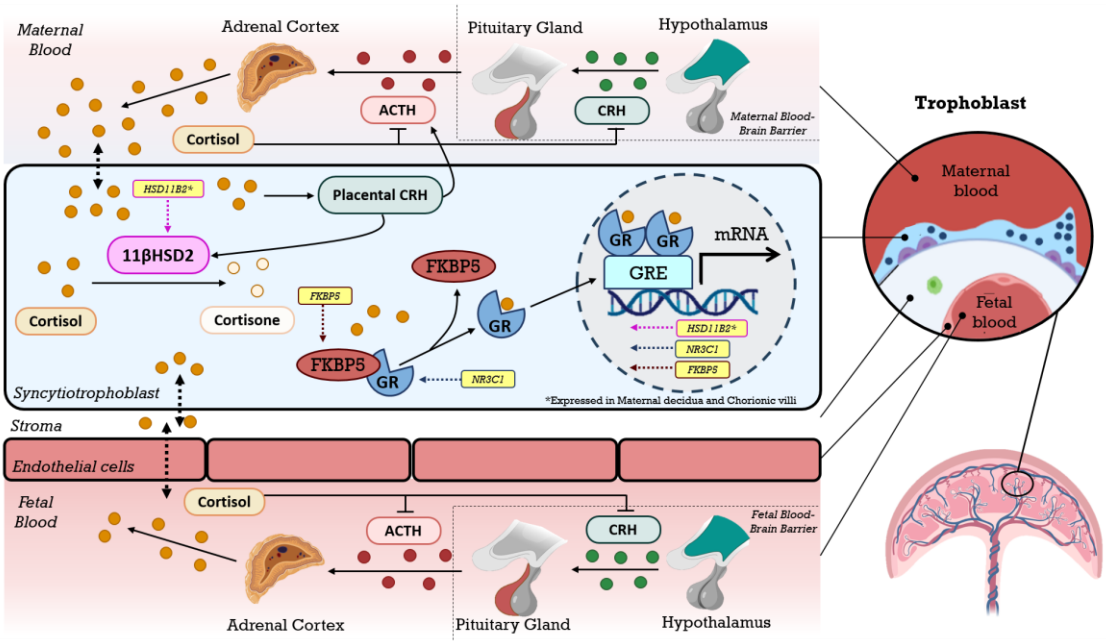

Figure 14. Cortisol function and regulation during pregnancy and candidate genes. Maternal cortisol can diffuse trough the placenta across syncytiotrophoblast. Once there, maternal cortisol stimulates placental corticotrophin-releasing hormone (CHR) expression, which in turn increased maternal adrenocorticotropic hormone (ACTH) and subsequently cortisol levels in blood. Placental CRH also regulates the expression of 11 beta-hydroxysteroid dehydrogenase-2 (11βHSD2) (pink), which converts cortisol into its inactive metabolite, cortisone, protecting the fetus from the high maternal cortisol levels. 11βHSD2 is codified by *HSD11B2* gene (yellow). Furthermore, cortisol regulates gene expression via glucocorticoid receptor (GR) (blue). When FK506 binding protein 51 (FKBP5) (red) is bound to the GR, the receptor has lower affinity for its ligand and is retained in the cytoplasm; upon cortisol binding, GR dissociates from FKBP5, and translocates into the nucleus. In the nucleus, GR binds glucocorticoid responsive elements (GRE), activating DNA transcription. GR and FKBP5 are codified by *NR3C1* and *FKBP5* genes respectively (yellow). Figure adapted from Chatuphonprasert and colleagues (2018) and created with BioRender.com.

The glucocorticoid receptor (GR), which is the cytoplasmic receptor for cortisol, also plays an important role in mediating the actions of cortisol through the regulation of gene transcription in the nucleus. As can be seen in Figure 14, this mediation is regulated by a large chaperone protein complex that includes the FKBP5. When FKBP5 is bound to the GR, the receptor has lower affinity for its ligand and is retained in the cytoplasm; upon cortisol binding, GR dissociates from FKBP5, and translocates into the nucleus (Binder, 2009). The DNA methylation of genes encoding for the GR (*NR3C1*) and the FKBP5 protein (*FKBP5*) has been observed to be relevant in the context of studies exploring early stress and its association with current psychopathology in lymphocyte DNA (Binder, 2009; Palma-Gudiel, Cordova-Palomera, et al., 2015), although in placenta is not well characterized.

HSD11B2, *NR3C1* and *FKBP5* genes are essential for cortisol regulation and homeostasis in placenta. However, despite the DNA methylation of these genes have been studied in other tissues, there is scarce literature regarding DNA methylation of these genes in placenta. The study of the epigenetic signatures of these genes in placenta could help to better understand the putative mechanisms underlying the association between maternal adverse experiences during pregnancy and increased risk of psychopathology in the offspring. It is noteworthy that the three layers of the placenta, maternal decidua, chorionic villi and chorion could have a completely different methylation profile (Robinson & Price, 2015) and that *HSD11B2* is only expressed in maternal decidua and chorionic villi (Zhu et al., 2019). For this reason, the study of DNA methylation in *HSD11B2*, *NR3C1*, and *FKBP5* will be conducted in this thesis across two distinct placental layers: maternal decidua and chorionic villi. This will contribute to a better understanding of placental stress regulation and its consequences for fetal and newborn outcomes.

HSD11B2

11βHSD2 is encoded by the *HSD11BD* gene, located in the chromosome 16q22. *HSD11BD* gene is comprised of 5 exons separated from each other by small introns. The gene contains four CpG islands, two located in the promoter region and between the promoter and the exon 1, one in the exon 5 and one in the downstream region (Figure 15). The methylation levels of the CpG islands in *HSD11B2* promoter-first exon are low in human placental tissue (Alikhani-Koopaei et al., 2004). Low methylation in the promoter region of the placental *HSD11B2* has been associated with increased expression of the gene and, subsequently, increasing the expression of the enzyme 11βHSD2 (Green et al., 2015; Jahnke et al., 2021). These results would explain why placental syncytiotrophoblast at the chorionic villi can maintain a high level of 11βHSD2 expression.

Accumulating evidence suggest that increased methylation of *HSD11B2* gene, particularly in CpG islands of the promoter region, is associated with adverse conditions in pregnancy, resulting in poor infant outcomes. Maternal anxiety and perceived stress during pregnancy have been associated with higher placental *HSD11B2* DNA methylation in the promoter region in previous literature (Conradt et al., 2013; Monk et al., 2016) and lower expression of placental 11βHSD2 (O'Donnell et al., 2012) compared to women without anxiety. Decreased 11βHSD2 has been also associated with increased maternal cortisol levels (Jahnke et al., 2021) and preterm birth (Majchrzak-Celińska et al., 2017). Thus, high and sustained maternal stress, and its associated excessively high

levels of cortisol, may increase DNA methylation of CpG islands covering the promoter region of *HSD11B2* in the placenta, diminishing the expression of the gene and decreasing the concentration of the enzyme. Therefore, more cortisol would be transferred from the mother to the fetus trough the placenta, promoting the early maturation of vital organs for its survival in extra-uterine life.

Moreover, according with Monk and colleagues (2016) abnormally increased cortisol levels in utero would contribute to an altered fetal brain maturation and to the sensitization of the fetal HPA axis, thereby increasing the risk of neurobehavioral issues and affecting his/her future stress reactivity.

However, there are inconsistences regarding placental *HSD11B2* methylation and offspring outcomes. Marsit and colleagues (2012) identified an inverse relationship between DNA methylation levels of the placental *HSD11B2* promoter and infant quality of movement, but a positive correlation between *HSD11B2* methylation and the capacity of attention of these infants. In this line, Paquette and colleagues (2015) observed that increased DNA methylation of the placental *HSD11B2* promoter was associated with a less reactive and better regulated profile. Furthermore, Liu and colleagues (2021) observed that increased *HSD11B2* DNA methylation in the promoter was associated with less risk of emotional symptoms and hyperactivity.

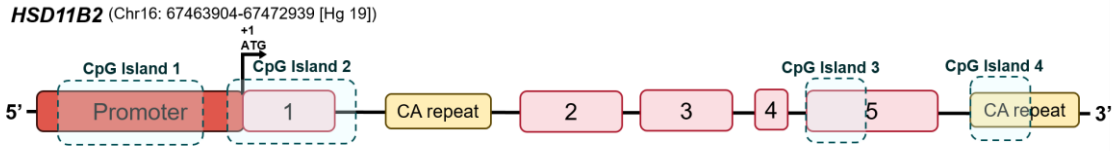

Figure 15. Gene structure of human *HSD11B2*. This gene is located in the chromosome 16q22. Solid black line boxes with a number and colored in pink represent the different exons and the 5'–3' orientation goes from left to right. The *HSD11B2* gene contains five exons (pink), which are separated from each other by small introns, and two tandems of repeats of cytosine-adenine (2 microsatellite CA) one located in the intron 1 and the other in the 3' untranslated region (yellow). Additionally, the *HSD11B2* has four GC islands (blue), the Island 1 located in the promoter, the Island 2 spanning the promoter and exon 1, the Island 3 in exon 5 and the Island 4 in the downstream region.

Thus, more research is needed to disentangle how placental *HSD11B2* DNA methylation interacts with maternal and infant health. Of note, in most of the research studying placental *HSD11B2* DNA methylation, only 4 islands of the promoter region have been studied. A more accurate methodological approach for assessing DNA methylation would be necessary to cover the elevated number of GpC sites between islands 1 and 2 of *HSD11B2* (almost 120 CpG sites) that are susceptible for methylation.

NR3C1

Glucocorticoid receptor is encoded by the nuclear receptor subfamily 3 group C member – *NR3C1* – gene, located in chromosome 5q31. *NR3C1* gene is comprised by 8 coding exons and 9 alternate non-coding first exons (1_A, 1_B, 1_C, 1_D, 1_E, 1_F, 1_I and 1_J). Non-coding exons are located in the gene promoter region and are transcribed into different mRNA variants. In fact, each of the non-coding exons transcript seem to display a characteristic tissue distribution (Turner & Muller, 2005). Seven of these nine exons are clustered along the same CpG island (Figure 16).

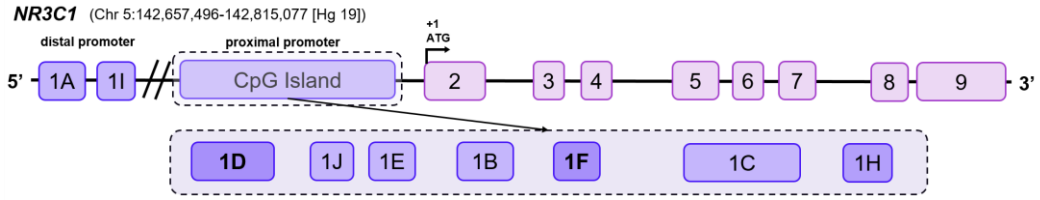

Figure 16. Gene structure of human *NR3C1*. This gene is located in the chromosome 5. Solid black line boxes with a number represent the different exons and the 5'–3' orientation goes from left to right. The *NR3C1* gene is composed for multiple exons: two distal in the promoter region (1A and 1I), seven in the proximal promoter (1D, 1J, 1E, 1B, 1F, 1C, 1H) (violet), and nine common exons (lavender). CpG islands located at the proximal promoter region are marked in grey.

GR has an essential role in the downregulation of HPA axis activity after a stressor has ended. The exposure to excessive cortisol levels due to chronic stressor (e.g., major depression) could led to an impairment of this negative feedback mechanism, which is known as GR resistance, disrupting body homeostasis and leading to inflammation and hypercotisolemia (Pariante, 2017).

Due to the essential role of GR in the regulation of HPA axis functioning, epigenetic modifications of the *NR3C1* gene have been deeply studied in the context of early life stress and psychopathology. In this regard, two decades ago, Weaver et al. (2004) found that rat pups raised by mothers that exhibited more nursing behavior (more pup licking and grooming) showed remarkable hypomethylation at a specific CpG sites of the promoter, located at a transcription factor. Since thus pioneering work, several studies have focused on exon 1_F of the *NR3C1* to disentangle the possible epigenetic repercussions of maternal pre and postnatal experiences on the fetus and childhood outcomes. Palma-Gudiel et al. (2015) revised the literature regarding severe stress during pregnancy and NR3C1 methylation status in the cord blood of the newborn. They observed that 20 of the 23 articles included considered DNA methylation exon 1_F and, in particular, of the small cluster of CpG sites preceding exon 1F (the region that was

reported to be epigenetically modified by Weaver et al. (2004)). In newborn blood, hypermethylation of this specific region of *NR3C1* has been associated with negative prenatal early-life environments (Palma-Gudiel, Córdova-Palomera, et al., 2015). However, little attention has been paid to the methylation at the first exons other than 1_F.

In fact, methylation at a functional GRE site, situated in exon 1_D of *NR3C1* was linked to anxious-depressive psychopathology and hippocampal connectivity in a cohort of concordant and discordant monozygotic twins, whereas exon 1_F exhibited uniform hypomethylation across the entire sample (Palma-Gudiel et al., 2018). Interestingly, exon 1_F and 1_D have been reported to be expressed in placenta (Hogg et al., 2013; Paquette et al., 2015). Hogg and colleagues (2013) reported that placental exon 1_D methylation was increased in women suffering early pre-eclamsia during pregnancy, when compared to healthy pregnant women. In this line, increased placental *NR3C1* methylation was associated with maternal distress during pregnancy (Capron et al., 2018; Monk et al., 2016). Contrary, lower placental DNA methylation of exon 1_F of the *NR3C1* was associated with a more reactive and worse regulated profile in newborns after the first 24 hours of life (Paquette et al., 2015) and poor motor development in infants at 3 months old (van Dokkum et al., 2022).

The complexity of the NR3C1 promoter region, the enrichment in CpG sites of the seven exons and the expression of exons 1_D and 1_F in placenta suggest that placental DNA methylation plays an important role in prenatal and postnatal stress response and the associated infant development.

FKBP5

FKBP5 binding protein 51 is encoded by the *FKBP5* gene, located in the short arm of human chromosome 6 (chromosome 6p21.31). *FKBP5* consist of 13 exons, 2 alternative and 11 common exons (see Figure 17). *FKBP5* expression shows a robust induction by GR. In fact, it has been shown that GR exerts long-range regulation of FKBP5 via very distal intronic and intergenic sites; since glucocorticoid response elements (GREs) have been located in a region of the promoter, but also in introns 1, 2, 5 and 7 (Paakinaho et al., 2010).

It has been found that GR resistance, and consequent homeostasis disruption, leading to hypercortisolism and inflammation, is in part conferred by an overexpression of FKBP5 (Binder, 2009). Common single nucleotide polymorphisms (SNPs) in *FKBP5* have been associated with differential induction of FKBP5 expression by GR activation

under chronic adverse experiences; particularly, one SNP 200 pb away from a GRE element in intron 2 (Binder et al., 2008; Binder et al., 2004). Furthermore, changes in DNA demethylation in intron 7 increases this differential responsiveness of FKBP5 to GR activation in individuals with the allele of risk exposed to childhood trauma (Klengel et al., 2013). This result suggests that DNA methylation in other introns of *FKBP5* that contain GREs could be also modulating the relationship between early psychosocial stress and HPA axis alterations. However, only few CpG sites located in enhancers within *FKBP5* are covered by the EPIC array and, therefore, they are understudied.

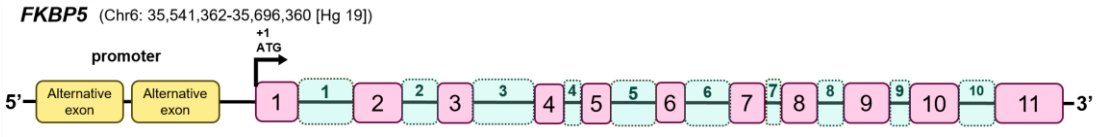

Figure 17. Gene structure of human *FKBP5*. This gene is located in the chromosome 6. Solid black line boxes with a number represent the different exons and the 5'–3' orientation goes from left to right. The *FKBP5* gene is composed for two alternative exons in the proximal promoter region (yellow), eleven common exons (pink) and ten introns (blue), which usually contain CpG sites.

To our knowledge, only four studies have conducted placental *FKBP5* methylation analyses in humans. Monk et al. (2016) reported that higher maternal distress was associated with increased placental DNA methylation of *FKBP5*, which in turn predicted less fetal movements. In consonance with these result, Paquette and colleagues (2014) observed that higher methylation of intron 7 of *FKBP5* was associate with increased risk for a higher arousal in the offspring in their first 24 hours of life. Conversely, Liu et al. (2021) reported that maternal prenatal anxiety was associated with decreased methylation of placental *FKBP5* gene, but this was only detected in preschool-aged boys, although they did not observe any associations between methylation and neurobehavioral outcomes. Finally, Czamara et al (2021) observed that exposure to exogenous glucocorticoids during pregnancy was associated with lower methylation in a region proximal to GRE element of intron 7. Interestingly, in this last study, DNA methylation was assessed using a novel technique called Targeted Bisulfite Sequencing (TBS), which allows a fine-mapping of loci of interest.

Studying DNA methylation in placental *FKBP5* would lead to a better understanding of the possible links between maternal distress and infant poor outcomes at birth. A thorough investigation of regions containing GRE elements, such as introns 1, 5, and 7 (given the pivotal role of GR in regulating FKBP5 expression), could contribute to a better understanding of the *FKBP5* gene in stress situations.

1.3.3. Immune response to stress

From an evolutionary perspective, the body initiates an immune response against threats, as traditionally, these represented risks of wounds, injuries, and infections (Miller & Raison, 2016). Therefore, similarly to cortisol, various inflammatory proteins are released into the bloodstream and mucosal surfaces under endangering situations, facilitating the rapid healing of wounds and preventing infections (Godoy et al., 2018).

At the present time, several studies suggest that psychosocial stress can also induce these psychobiological changes, rapidly activating both the HPA axis and the immune system. In fact, these two systems exhibited a bidirectional interaction. For example, cortisol has a potent immunosuppressive function as the majority of immune cells possess glucocorticoid receptors (Baschant & Tuckermann, 2010). Moreover, different immune mediators, such as cytokines, can cross the brain blood barrier and activate the HPA axis (Dunn, 2007). Thus, the downregulation of cortisol receptors under chronic stress reduces immunity cell capacity to respond to anti-inflammatory signals resulting in the exacerbation of cytokine-mediated inflammatory processes, such as the over activation of the HPA axis. This could elucidate the reason behind the apparent dysregulation of these two systems in different psychiatric disorders, but particularly in major depression and psychosis (Garcia-Bueno et al., 2014; Hassamal, 2023; Pariante, 2009).

The immune system provides defense to several pathogens in a short time, minutes to hours after infection, thanks to innate immunity. In case of injury, immune cells congregate in the damaged tissue and fight the invaders, releasing different molecules such as cytokines or the complement factor (Segerstrom & Miller, 2004). These pro-inflammatory cytokines (e.g. Interleukins) play and important signaling role, regulating responsiveness of immune cells (Maier & Watkins, 1998). Adaptive immunity is activated a few days after infection. Lymphocytes have specific surface receptors responding to a single antigen, as bacteria or viruses. Upon activation, lymphocytes start to divide giving rise to a population of cells with the same antigen-specificity (Segerstrom & Miller, 2004). Activated lymphocytes release cytokines and immunoglobulins (Ig), Y-shaped proteins that specifically bind to the antigen, inducing phagocytosis and preventing disease development.

The immune system undergoes a process of maturation from birth and continues to develop throughout a lifetime, adapting to various external challenges encountered from childhood through adolescence, young and mature adulthood (including

pregnancy), until the onset of old age (Simon et al., 2015). In utero, fetal environment demands that the immune system remains tolerant to the maternal alloantigens; while, after birth, the exposure to environmental antigens calls for a rapid change to make distinct immune responses appropriate for early life.

As introduced in section 1.2.1.1., the maternal immune system changes during pregnancy, increasing woman susceptibility for maternal infections. Some infections can cross the placental barrier while others might have an effect on the developing fetal brain trough maternal antibodies and/or proinflammatory molecules, dysregulating maternal and infant neuroendocrine and immune responses (Khandaker et al., 2014). Thus, maternal viral infections during pregnancy, and the associated immune and endocrinological responses, may play a key role on fetal neurodevelopment and on offspring's behavior and future psychopathology (Knuesel et al., 2014). Considering the recent COVID-19 pandemic and knowing that, in all likelihood, all pregnant women have been exposed to the possibility of infection during this period, we consider of great interest to study early neurodevelopmental signs in children born to infected mothers and to include this hypothesis among the hypotheses explored in this thesis.

During infancy, the immune system gradually matures. The first major exposure to pathogens is during the passage through birth canal and then as soon as he/she makes skin and respiratory contact with the exterior In fact, critical early protection against many infection will be provided by the mother trough maternal milk antibodies (Rio-Aige et al., 2021). As the child develops, intercurrent infections and vaccinations contribute to shaping the immune repertoire. Most childhood infections happen only once and then protection is lifelong.

However, early adverse experiences can also influence immune system maturation, leading to disruptions that may persist even into adulthood. In this respect, increased concentration of interleukin (IL)-6 and C reactive protein (CRP) were found in adults with a history of maltreatment when they were exposed to a psychosocial stressors in adulthood (Carpenter et al., 2010; Danese et al., 2007; Hostinar et al., 2017). Likewise, in 12 year old children with a history of physical abuse, and with a current depression diagnosis, significantly elevated levels of CRP were detected when compared to control children of the same age (Danese et al., 2011). Moreover, Miller and Cole (2012) have described the association of depression and inflammation in a group of adolescents exposed to childhood maltreatment while growing up, suggesting that the inflammatory status observed in some patients with depression might be present (and therefore be liable to answering to a specific treatment) only in those subjects exposed

to adversity early in life. This data suggests that in those young patients there is already a dysregulation of the stress response that is evidenced by an alteration of the inflammatory status associated, not only to the stress inherent to the mental disorder, but also to the previous experiences of early life stress or childhood maltreatment.

1.3.3.1. The secretory immunoglobulin A (s-IgA)

There are five existing types of Igs, each characterized by unique biological features, structural attributes, target specificity, and distribution within the human body. Among these Igs, immunoglobulin A (IgA), particularly its secretory form, emerges as a particularly promising inflammatory stress biomarker. This is attributed to its role as the primary immunoglobulin in mucosal secretions, enabling secretory IgA (s-IgA) to rapidly activate in response to stress and allowing for non-invasive collection (Bosch et al., 2002) (see Figure 18).

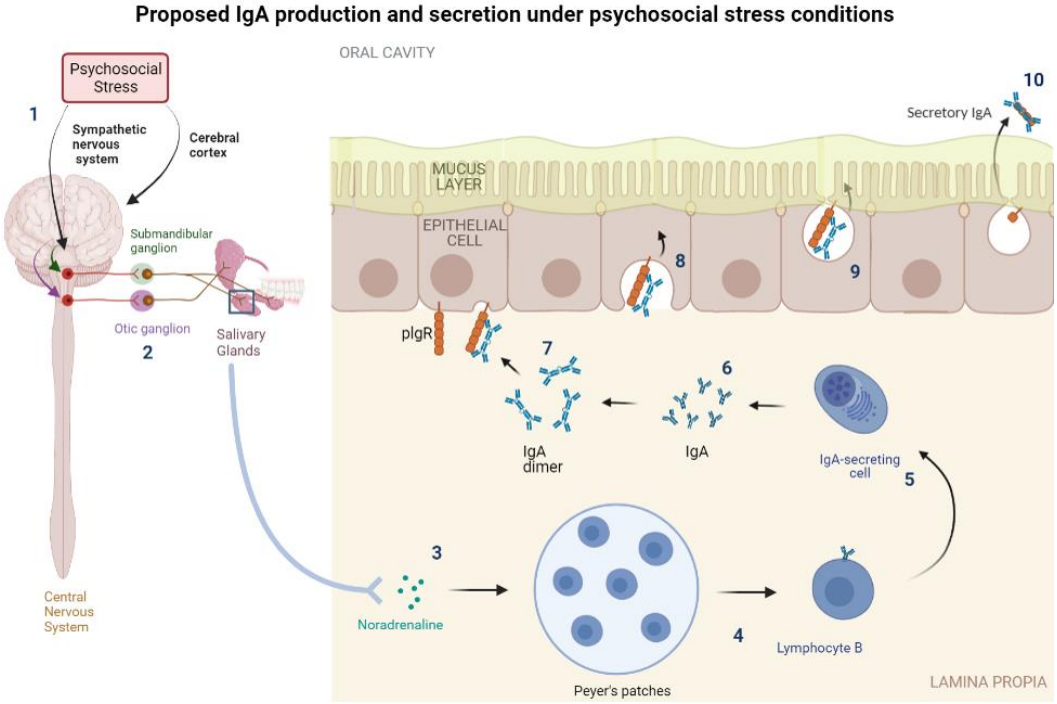

Figure 18. Proposed s-IgA production and secretion under acute stress conditions. Under psychosocial stress, noradrenaline in the salivary glands triggers the immune response in Peyer's patches, activating B lymphocytes that will induce IgA secretion. The s-IgA is composed of two IgA molecules and a secretory component. This component is added when dimeric IgA translocates across the epithelium before release into the mucosal lumen. Reproduced with permission from Castro-Quintas et al. Salivary secretory immunoglobulin A as a potential biomarker of psychosocial stress response during the first stages of life: A systematic review. Frontiers in Neurosciences. 71:101083 (2023).

IgA acts as an important first line of defense against infections, preventing the passage of foreign substances into the circulatory system. While IgA can be detected in serum, the majority of the IgA produced in response to a threat can be found in mucosal fluids, particularly in saliva. In fact, the concentration of IgA at various mucosal surfaces, such as the oral cavity, remains independent of circulating IgA concentrations. This autonomy is attributed to the presence of pre-existing and stored s-IgA in the lamina propia of the mucosa. In response to a threat, s-IgA undergoes a structural transformation, adopting a dimeric configuration.

As illustrated in Figure 18, under stressful conditions, the SNS releases noradrenaline in the lamina propia, triggering the activation of IgA-secreting cells derived from B lymphocytes, which release monomeric IgA. During their transit to the oral cavity, two polypeptides are added to two monomeric IgA: the joining chain, formed in IgA secreting cells, and the secretory component, produced in epithelial cells (de Sousa-Pereira & Woof, 2019). The secretory component serves to stabilize s-IgA, rendering it resistant to degradation by both host and microbial proteases (Corthésy, 2013). Hence, IgA is transported through the epithelium and released to saliva as s-IgA, where it proves more effective in neutralizing toxins and binding to invading organisms compared to serum IgA (Tomasi, 1992).

Under basal conditions s-IgA levels vary during the day following a circadian rhythm with a peak just after awakening, then decreasing to the next 4 hours and remaining relatively constant toward early evening (Shirakawa et al., 2004). In a similar way, under acute stress, s-IgA has been shown to be under strong neuroendocrine control. The autonomic nerves that innervate the salivary glands robustly impact salivary s-IgA secretion, whereby activation of the sympathetic nerves enhanced s-IgA rapid output (Proctor & Carpenter, 2014).

Despite the potential interest of s-IgA as a reliable marker for immune system function in response to psychosocial stress, only one study appears in the literature exploring its reactivity to acute psychosocial stress in the child and adolescent population (Laurent et al., 2015). However, the authors did not include a variable of utmost interest in their design, as it is the history of prior maltreatment. For this reason, investigating this marker was deemed highly relevant, considering salivary s-IgA levels as a potential new biomarker of interest in the realm of biological consequences associated with child maltreatment. Furthermore, we found it relevant to investigate whether this immunoglobulin could be a possible biomarker of altered psychosocial stress immunity response in infants, children, and adolescents.

1.4. Psychosocial stress across lifespan and endocrine and immunity putative consequences during brain development in the context of this PhD project

In this introduction, we have highlighted that early adverse experiences, especially related to psychosocial stress in both prenatal and postnatal phases, have the potential to exert a chronic and enduring influence on an individual's well-being. This impact is notably substantial on mental health due to the ongoing development of the brain until young adulthood. Factors like maternal infections or stress during pregnancy, caregiving, and the social environment during childhood and adolescence can compromise mental health. Unfortunately, these experiences, often involving significant maltreatment, commonly occur simultaneously, increasing susceptibility to psychopathological outcomes. In adulthood, experiences such as divorce, job loss, and relocation can induce stress, although to a lesser extent than stressors in earlier stages (Figure 19).

One of the primary mediators influencing the impact of early psychosocial stressors on future mental health is cortisol, the principal effector of the HPA axis. In response to stressful circumstances, cortisol levels rise. However, chronic stress experiences can lead to dysregulation of the HPA axis, resulting in elevated circulatory cortisol levels, which can be neurotoxic. Maternal stress during prenatal stages, like depression, can disrupt the maternal HPA axis, affecting the protective mechanisms of the placental barrier and potentially causing neurodevelopmental alterations in offspring. After birth, the infant's own chronic stress experiences can further dysregulate these immature stress response systems. In this regard, it is important to note that cortisol has a significant interaction with the immune system.

During pregnancy, changes in the immune system increase vulnerability to infections or other medical conditions. Maternal infection or its combined impact with associated stress activates various biological pathways in the mother, influencing fetal development and elevating the risk of neurodevelopmental impairment in offspring. In the postnatal period, continuously elevated cortisol levels can trigger peripheral inflammation, leading to neuroinflammation and an increased risk of psychopathology.

Nevertheless, the precise manner in which exposure to psychosocial stress during the early sensitive periods of prenatal and postnatal development impacts the HPA axis and immune system remains unclear, and this is the puzzle we aim to unravel in this thesis.

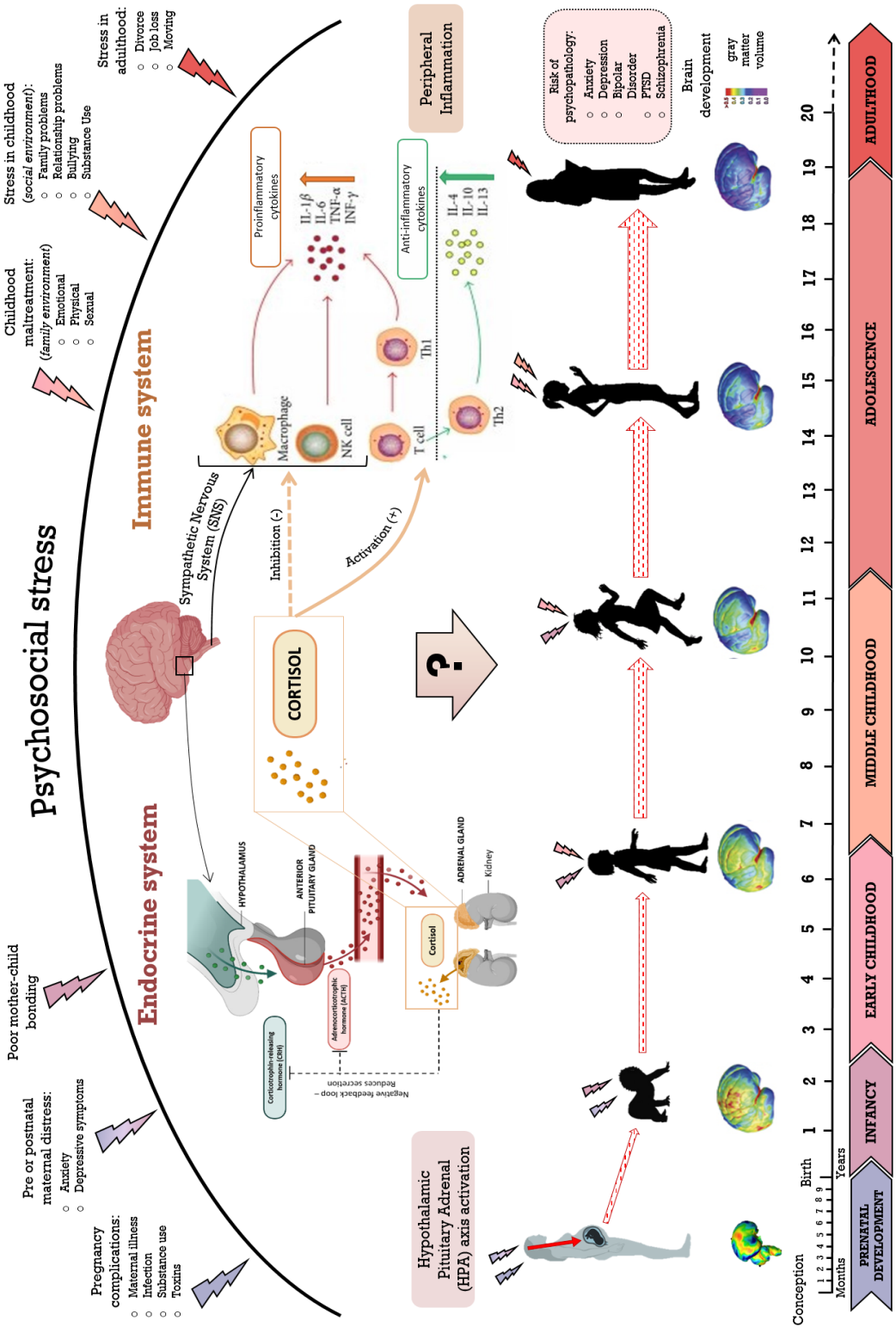

Figure 19. Framework of the present thesis. Exposure to various psychosocial stressors persistently during the early stages of development (from prenatal to early adulthood) may modify various biological mechanisms involved in stress response, such as the hypothalamic-pituitary-adrenal (HPA) axis and the immune system. These alterations are often marked by an increase in cortisol and inflammation, both of which can be detrimental to the developing brain, thereby heightening the risk of psychopathology.

2. Hypothesis and objectives

General hypothesis

Exposure to psychosocial stress during prenatal, childhood, and adolescence periods can induce endocrine and immune sensitization, crucial for responding to later stressors and contributing to the development of mental disorders.

Particularly the following hypotheses were proposed:

Hypothesis I: Maternal stress exposure during pregnancy, such as experiencing depressive symptoms, increases the risk for poorer offspring outcomes. Specifically:

i) Maternal depressive symptoms during pregnancy may alter the maternal Hypothalamic-Pituitary-Adrenal (HPA) axis, leading to increased circulatory cortisol levels and affecting newborn outcomes at delivery.
ii) Cortisol changes associated with depressive conditions could alter placental buffering through epigenetic signatures that affect genes involved in cortisol regulation at different placental layers and potentially influencing the neurodevelopmental trajectories of the newborn.
iii) Maternal infection to SARS-CoV-2 during pregnancy would also increase the risk of early neurodevelopmental impairment in the offspring.

Hypothesis II: Childhood maltreatment is associated with alterations in biological systems involved in basal diurnal rhythmicity and acute stress response, particularly in i) the HPA axis and of ii) the s-IgA of the immune system.

Specific objectives

In order to explore these hypotheses, the following specific objectives, divided in two sections and six scientific articles, were developed:

Section I – Maternal distress, cortisol regulation and early offspring outcomes

1. In the context of the *Intramural_Maternal_Epi_project* cohort, to explore the functioning of the HPA axis, in a sample of primiparous women from the general population, with or without of depressive symptoms. The analyses included the study of: i) cortisol diurnal rhythmicity, ii) overall cortisol levels, and iii) the

potential effects of these cortisol fluctuations on gestational age and newborn weight at birth.

2. Within the framework of the previous cohort, to understand the role that maternal depressive symptomatology and HPA axis functioning play in on the methylation pattern of placental genes involved in cortisol regulation, including *HSD11B2*, *NR3C1* and *FKBP5* genes. To analyze these signatures at two placental layers: placental layers: the maternal decidua and the chorionic villi. Furthermore, exploring whether there is a relationship between placental methylation patterns and newborn birth outcomes and early neurobehavioral profiles at 6-8 weeks old, according with the NBAS scale measures, and the HPA axis reactivity through this scale.

3. To analyze neurodevelopmental milestones at 7 weeks, using the NBAS scale, in infants born to mothers infected and non-infected with SARS-CoV-2 during pregnancy in the context of the *COGESCOV-19* cohort.

Section II – Childhood maltreatment, psychosocial stress and responses of the endocrine and immune systems

4. In the context of the *Epi_young_stress* cohort, to analyze the impact of childhood maltreatment on HPA axis functioning and on the perception of anxiety, in a sample of infant-juvenile subjects (with and without current psychiatric diagnosis; with and without history of childhood maltreatment) by studying i) their cortisol diurnal secretion, ii) their cortisol reactivity in front a laboratory induced psychosocial acute stressor the TSST for children (TSST-C), iii) their anxiety levels through measures of the State-Trait Anxiety Inventory Status (STAI-S) taken during the TSST-C procedure.

5. Exploring the reactivity of the secretory immunoglobulin A (s-IgA) in front of the TSST-C, disentangling the role that pubertal status (childhood vs adolescence) plays in s-IgA reactivity in accordance with the history of childhood maltreatment.

6. Reviewing the scientific literature to study the utility of s-IgA as a putative new biomarker of psychosocial stress response in infancy, childhood, and adolescence.

3. Publications

Supervisor's report on impact factor

The doctoral thesis "Biological mechanisms underlying psychosocial stress response: The consequences of prenatal maternal distress and childhood maltreatment on the endocrine and immune systems" is based on the original results obtained by Águeda Castro Quintas, in collaboration with her research group of reference placed in the Evolutionary Biology, Ecology and Environmental Scienced (BEECA) department of the Universitat de Barcelona.

These results are based on the endocrine, immunological and epigenetic analysis in i) a sample encompassing primiparous pregnant women from the general population (from 18 to 40 years) and their offspring (n=150 dyads), exhaustively researched in terms of psychopathology, neurodevelopment and behavior (*Intramural_Maternal_Epi_project*), ii) a sample of 8 weeks old infants whose mothers were infected or non-infected with SARS-CoV-2 during pregnancy (*COGESCOV-19*) (n=100) and iii) a sample of 187 infant-juvenile subjects (from 7 to 17 years old) with current psychiatric diagnosis, exposed and non-exposed to childhood maltreatment, and in a matched control group without psychiatric diagnosis exposed and non-exposed to childhood maltreatment (*Epi_young_stress* project), studied with the Trier Social Stress Test (TSST) a well-established paradigm that induces acute psychosocial stress in laboratory conditions.

Complementary, this doctoral thesis also comprises a systematic review performed by Águeda Castro Quintas which aided the construction of a conceptual framework to determine a putative new immunity biomarker of response to early psychosocial stress based on s-IgA reactivity. These results and review have been published or have been submitted to international peer review journals. The impact factors of these journals demonstrate the quality of the research conducted, and are as following:

1. **Diurnal cortisol throughout pregnancy and its association with maternal depressive symptoms and birth outcomes**, Castro-Quintas et al. (2023), published in *Psychoneuroendocrinology*. This journal publishes papers dealing with the interrelated disciplines of psychology, neurobiology, endocrinology, immunology, neurology, and psychiatry, with an emphasis on multidisciplinary studies aiming at integrating these disciplines in terms of either basic research or clinical implication. This journal is indexed in Journal Citation Reports 2022 with a current impact factor of 3.7 and classified in the second quartile of the area Neurosciences (ranking: 113/272).

2. **Distress during pregnancy and epigenetic signatures at glucocorticoid related genes in placental chorionic villi and maternal decidua layers: a pilot study in Spanish primiparous women**, Castro-Quintas et al., submitted to *Clinical Epigenetics*. This journal is devoted to the study of epigenetic principles and mechanisms as applied to human development, disease, diagnosis and treatment. This journal is indexed in Journal Citation Reports 2022 with a current impact factor of 5.7 and classified in the first quartile of the area Genetics & Heredity (ranking: 25/171).

3. **Exploring the impact of COVID-19 on newborn neurodevelopment: a pilot study**, Ayesa-Arriola* & Castro-Quintas* et al. (2023), published in *Scientific Reports*. This journal published research from across nature sciences, psychology, medicine and engineering. This journal is indexed in Journal Citation Reports 2022 with a current impact factor of 4.6 and classified in the second quartile of the area Multidisciplinary Sciences (ranking: 22/73).

4. **Childhood maltreatment disrupts HPA-axis activity under basal and stress conditions in a dose-response relationship in children and adolescents**, Marques-Feixa et al. (2021), published in *Psychological Medicine*. This journal is in the fields of psychiatry, clinical psychology and the related basic sciences and its main focus is to publish research focused on understanding the causes of mental health problems and improving clinical practice. This journal is indexed in Journal Citation Reports 2021 with a current impact factor of 10.592 and classified in the first decile of the area Psychology (ranking: 7/80) and in the first quartile of the area Psychiatry (18/155).

5. **Secretory immunoglobulin A (s-IgA) reactivity to acute psychosocial stress in children and adolescents: the influence of pubertal development and history of maltreatment**, Marques-Feixa* & Castro-Quintas* et al. (2022)**,** published in *Brain Behavior and Immunity*. This journal publishes basic, experimental, and clinical studies dealing with behavioral, neural, endocrine, and immune system interactions in humans and animals. It is an international, interdisciplinary journal devoted to original research in neuroscience, immunology, integrative physiology, behavioral biology, psychiatry, psychology, and clinical medicine and is inclusive of research at the molecular, cellular, social, and whole organism level. This journal is indexed in Journal Citation Reports 2022 with a current impact factor of 15.1 and

classified in the first decile of the areas Neurosciences (ranking: 8/272), Psychiatry (6/155) and Immunology (10/161).

6. **Salivary secretory immunoglobulin A as a putative biomarker of psychosocial stress response during the first stages of life: A systematic review**, Castro Quintas et al. (2023), published in *Frontiers in neuroendocrinology*. This journal is comprised of comprehensive review articles, systematic reviews, opinion pieces, and meta-analyses. Their research areas of interest include neuroendocrine and immune interactions and their links to behavior and disease, among others. This journal is indexed in Journal Citation Reports 2022 with a current impact factor of 7.4 and classified in the first decile of the area Neurosciences (ranking: 30/272) and on the first quartile of the area Endocrinology and Metabolism (19/145).

Accordingly, I confirm the quality of the published and submitted articles.

Signed by Prof. Lourdes Fañanás,

Barcelona, 5th December 2023

Section I - Maternal distress, cortisol regulation and early offspring outcomes

3.1. Diurnal cortisol throughout pregnancy and its association with maternal depressive symptoms and birth outcomes

Águeda Castro-Quintas, Elisenda Eixarch, Nerea San Martin-Gonzalez, Maria Daura-Corral, Laia Marques Feixa, Helena Palma-Gudiel, Mireia Rocavert-Barranco, Alba Miguel-Valero, Jose Luis Monteserin-García, Lorena de La Fuente Tomás, Fátima Crispi, Bárbara Arias, Maria Paz Garcia-Portilla, Lourdes Fañanas

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Diurnal cortisol throughout pregnancy and its association with maternal depressive symptoms and birth outcomes

Águeda Castro-Quintas [a,b,c], Elisenda Eixarch [d,e,f], Nerea San Martin-Gonzalez [a,b,c], Maria Daura-Corral [a], Laia Marques-Feixa [a,b,c], Helena Palma-Gudiel [c,g], Mireia Rocavert-Barranco [h], Alba Miguel-Valero [a], Jose Luis Monteserín-García [a,b,c], Lorena de la Fuente-Tomás [c,i], Fátima Crispi [d,e,f], Barbara Arias [a,b,c], María Paz García-Portilla [c,i,j], Lourdes Fañanás [a,b,c,*,1]

[a] Department of Evolutionary Biology, Ecology and Environmental Sciences, Faculty of Biology, University of Barcelona, Barcelona, Spain
[b] Biomedicine Institute of the University of Barcelona (IBUB), Barcelona, Spain
[c] Network Centre for Biomedical Research in Mental Health (CIBER of Mental Health, CIBERSAM), Institute of Health Carlos III, Madrid, Spain
[d] BCNatal - Barcelona Center for Maternal-Fetal and Neonatal Medicine (Hospital Clínic and Hospital Sant Joan de Déu)
[e] Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Universitat de Barcelona, Barcelona, Spain
[f] Network Centre for Biomedical Research on Rare Diseases (CIBER of Rare Diseases, CIBERER), Institute of Health Carlos III, Madrid, Spain
[g] College of Public Health and Health Professions, Department of Epidemiology, University of Florida, United States
[h] Faculty of Health and Medical Sciences, University of Copenhagen, Denmark
[i] Department of Psychiatry, University of Oviedo, Asturias, Spain
[j] Servicio de Salud del Principado de Asturias (SESPA), Oviedo, Spain

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ABSTRACT

Background: Depression during pregnancy is a common complication that can negatively affect fetal health and birth outcomes. Cortisol is believed to be a key mediator of this association. Although pregnancy entails a natural increase in cortisol levels, preclinical depression could alter its circadian rhythm, producing excessively high overall diurnal cortisol levels that might be harmful for the fetus and future offspring development.
Objectives: Using a prospective longitudinal design, we aimed to study (i) trimestral cortisol circadian rhythm and its overall levels throughout pregnancy in healthy women, (ii) the extent to which maternal depressive symptoms influence both cortisol rhythmicity and overall levels, and (iii) the possible adverse consequences of elevated maternal cortisol on the offspring's weight and gestational age at birth.
Study design: 112 healthy pregnant women from the general Spanish population were recruited before their first pregnancy. To assess cortisol circadian rhythm, participants provided four saliva samples at each trimester of pregnancy (at awakening, 30 min after awakening, before lunch and before going to bed). Overall cortisol levels were calculated with AUCg approximation. Depressive symptoms were evaluated in each trimester and defined according to EPDS cut-off values (1st trimester, EPDS $\geq$ 11; 2nd and 3rd trimesters, EPDS $\geq$ 10). At birth, the risk for low weight, prematurity and weight birth percentile was retrieved for 100 infants. Mixed models and simple effects were employed to study changes of maternal cortisol circadian rhythm and overall levels throughout pregnancy and the possible influence of maternal depressive symptoms. Finally, logistic regressions were performed to assess the associations between maternal overall cortisol levels in each trimester of pregnancy and birth anthropometrics.
Results: Although overall diurnal cortisol levels increase throughout pregnancy, cortisol circadian rhythm is preserved in all trimesters [1st ($F(3110)= 92.565$, $p < .001$), 2nd ($F(3,85)= 46.828$, $p < .001$) and 3rd ($F(3,90)= 65.555$, $p < .001$)]. However, women with depressive symptoms showed a flattened cortisol circadian pattern only during the second trimester, characterized by a blunted awakening peak and reduced evening decline ($F(3,85)= 4.136$, $p = .009$), but not during the first ($F(3,11)= 1.676$, $p = .176$) or the third ($F(3,90)= 1.089$, $p = .358$) trimesters. Additionally, they did not show a cortisol increase from second to third trimester ($p = .636$).

* Correspondence to: Faculty of Biology, University of Barcelona, Diagonal Avenue, 643, 2n A, Barcelona, Spain.
E-mail address: lfananas@ub.edu (L. Fañanás).
[1] +34934021461

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Finally, higher maternal cortisol levels in second and third trimesters seemed to be associated with increased risk of prematurity (adjusted OR −0.371, 95% CI 0.490–0.972, p = .034) and low birth weight percentile (adjusted OR −0.612, 95% CI 0.348–0.846, p = .007) respectively.

Conclusion: Maternal cortisol levels increased throughout pregnancy, although cortisol circadian rhythm was preserved in all trimesters of pregnancy. However, prenatal depressive symptoms were associated with flattened maternal cortisol circadian rhythm in mid-pregnancy. Therefore, it seems that women with depressive symptoms tended to increase less gradually their cortisol levels from mid to late pregnancy. Finally, higher maternal cortisol levels in mid and late-pregnancy seem to be associated with poorer birth anthropometrics Early detection of depressive symptoms in general population could help to prevent putative obstetrical and birth adverse outcomes.

1. Introduction

Pregnancy is a sensitive period for the expectant mother in which multiple psychological and physiological changes occur. During this period, it is common that women experience depressive symptoms. In fact, while major depression is present in up to 11.9% of pregnant women, the prevalence of subclinical depressive symptomatology is higher, although it often remains undetected (Woody et al., 2017; Yonkers et al., 2009). Depression during pregnancy has been associated with an increased risk of adverse birth and child outcomes, including prematurity, low birth weight, neurodevelopmental delays and adult psychiatric disorders (Davis et al., 2007; Deave et al., 2008; Glover, 2011; Grigoriadis et al., 2013; Osborne et al., 2022; Plant et al., 2015). A key potential mediator linking maternal depression and offspring's developmental alterations is the flow of abnormally high levels of maternal cortisol, which can cross the placental barrier and compromise fetal development (Buss et al., 2012; Reynolds, 2013; Vlenterie et al., 2022).

Cortisol is the end effector of the Hypothalamic-Pituitary-Adrenal (HPA) axis and it plays a crucial role in homeostatic regulation during stress situations (Godoy et al., 2018). Cortisol is also responsive to normal daily activities and is involved in the proper functioning of multiple physiological systems (Spencer and Deak, 2017). Cortisol secretion follows a circadian rhythm in healthy individuals, with a maximum peak approximately 30 min after awakening, followed by a decline across the day, reaching the lowest levels at night (Debono et al., 2009; Weitzman et al., 1971). Over the course of pregnancy, maternal cortisol increases two to four-fold due to the release of other hormones such as estrogen and placental corticotrophin-releasing hormone (CRH), that interact with maternal HPA axis stimulating cortisol secretion (Jung et al., 2011; Qureshi et al., 2007; Sandman et al., 2006). Despite the increasing circulating levels of cortisol, its circadian rhythmicity seems to be maintained throughout pregnancy (Entringer et al., 2011).

In utero, the fetus is partly protected from excessive cortisol exposure thanks to the placental enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which metabolizes cortisol into inactive cortisone (Murphy et al., 1974). Although 11β-HSD2 rises in parallel with maternal cortisol during pregnancy, about 10–20% of maternal cortisol still makes it through the placenta, reaching fetal circulation and playing a role in fetal development and organ maturation (Murphy et al., 2006; Trainer, 2002). In addition to these naturally occurring maternal endocrine fluctuations, it has been shown that maternal depression can cause a short term increase in cortisol levels, further increasing fetal exposure to this hormone (Duthie and Reynolds, 2013). In that sense, excessive cortisol levels could influence in infant birth outcomes, reducing birth weight by inducing the vasoconstriction of the uterine artery, diminishing the flow of nutrients and oxygen, and shortening the gestation by stimulating the production of placental CRH (Field and Diego, 2008). Moreover, according to Krontira et al. (2020), an elevated glucocorticoid signaling in utero may dysregulate fetal HPA axis through alterations on the epigenetic landscape, predisposing children to worse emotional outcomes (Krontira et al., 2020; Monk et al., 2016; Morsi et al., 2018).

The evidence of an association between maternal depression and cortisol levels has been controversial, partially due to the variability of cortisol collection during pregnancy and analytic approaches, but also to the definition of maternal depressive condition (Dunkel Schetter, 2011; Orta et al., 2018). Although cortisol can be measured in different tissues (e.g.: blood, hair, and urine), it has been commonly measured in saliva due to its ability to identify subtle fluctuations in concentrations across the day, and its non-invasive easy access. Despite multiple samples are needed to capture cortisol diurnal patterns, most of the studies collect salivary samples only in the morning, which does not allow to assess diurnal variation (Orta et al., 2018; Vlenterie et al., 2021). Furthermore, most of the studies linking maternal adverse conditions and cortisol have been performed in the third trimester of pregnancy (Bublitz et al., 2019; Fassaie and McAloon, 2020; Orta et al., 2018). To the best of our knowledge, only three articles have assessed cortisol levels at different time points throughout the day and in different moments of pregnancy, showing contradictory results. While two studies observed that cortisol diurnal pattern was flatter in depressed women in all trimesters (Murphy et al., 2022; O'Connor et al., 2014), the other study did not show any association between maternal depressive symptoms and cortisol circadian rhythm (van den Heuvel et al., 2018). Additionally, a recent review indicates that maternal depression correlates with cortisol concentrations in only 24 out of 47 studies reviewed (Seth et al., 2016). In this line, an independent meta-analysis found no consistent relationships between self-reported stress or depression and hair cortisol levels in pregnant women (Stalder et al., 2017).

In order to clarify the association between maternal depression, cortisol and birth anthropometrics, the aims of the current research were: (1) to study how cortisol diurnal patterns and overall levels vary over the three trimesters of pregnancy, (2) to explore how maternal depressive symptoms impacts on her cortisol rhythmicity and overall levels, and (3) to study the influence of overall maternal cortisol levels on infant's weight, weight percentile and gestational age at birth. Based on previous literature, we hypothesized that diurnal cortisol levels would increase throughout pregnancy, especially at the evening. Additionally, we expected that pregnant women with depressive symptoms would show higher overall cortisol concentrations since the beginning of the pregnancy, when compared to control women. Finally, the offspring of women with higher cortisol levels will have and increased risk of low weight, prematurity and/or low weight for gestational age.

2. Materials and methods

The current sample includes 112 healthy primiparous pregnant women from the general Spanish population, recruited from May 2016 to March 2020 in the ongoing "Intramural Maternal Epi-Project". Participants were recruited before their first ultrasound from the maternity units of two public Spanish general hospitals (Hospital Clinic of Barcelona and Hospital Universitario Central de Asturias). Inclusion criteria included: (1) less than thirteen weeks of pregnancy, (2) age between 18–40 years, (3) first pregnancy, (4) no history of physical, neurological, or mental diseases, and (5) singleton pregnancy. Multiple pregnancies and health conditions were discarded to avoid possible biases. This study was approved by the medical ethical committee of the local hospitals and was conducted in full compliance with Helsinki declaration.

After providing their written informed consent, participants were interviewed by a trained psychologist three times during pregnancy, coinciding with the ultrasounds offered by the Spanish Public Healthcare system: between 11th–13th weeks (first trimester), between 20th–22nd weeks (second trimester) and between 32nd–34th weeks (third trimester). At the end of each interview, participants were instructed to collect four salivary samples at home. At birth, perinatal outcomes of 100 infants were obtained.

The protocol of the project is summarized in Fig. 1.

2.1. Data collection

2.1.1. Depressive symptoms

The Edinburgh (Postnatal) Depression Scale E(P)DS (Cox et al., 1987) is a screening scale for depressive symptoms during pregnancy and postpartum (Cox et al., 1996). This hetero-administered questionnaire consists of 10 questions, measured on a Likert scale from 0 to 3, regarding woman's emotions on the previous 7 days. The total score ranges between 0 and 30. Following Bergink et al. (2011) recommendations, the cutoff-values for depressive symptoms considered for this study were 11 or greater in the first trimester and 10 or greater in second and third trimesters. Depressive symptoms through pregnancy were defined as the presence of depressive symptoms at least in one trimester.

2.1.2. Body Mass Index (BMI)

maternal BMI was calculated using self-reported measures of weight and height at first trimester of pregnancy.

2.1.3. Cortisol circadian rhythm measures

Participants were given four specially designed test-tubes (Salivette®, Sarstedt, Germany) at the end of each trimester's interview for self-saliva collection, following a protocol highly used and accepted in the literature(Sørensen et al., 2021). Moreover, to avoid possible biases, clear written instructions for salivary self-collection were given (Section 1.1, Supplementary material). Samples must be taken immediately after awakening (B1), 30 min after awakening (B2), before lunch (B3), and before going to bed (B4). Participants were instructed to store their saliva samples in their freezer until they delivered them to the research center in the next trimestral appointment and then samples were stored at −25 ºC until analysis.

Salivary cortisol was determined using a highly sensitive enzyme-linked immunosorbent assay (ELISA) (commercial kit Salimetrics, LLC, State College, PA; Table S1, Supplementary material).

2.1.4. Birth anthropometrics

Weight at birth, gestational age at birth and offspring's birth weight percentile were retrieved. The cutoff-value for low weight was <2.500 kg, for preterm birth was <37 0/7 weeks according with the World Health Organization (2014) and the American College of Obstetricians and Gynecologists(American College of Obstetricians and Gynecologists, 2013), respectively. Offspring birth weight percentile was calculated with an online tool based on the clinical growth charts developed by the World Health Organization (WHO) for infants and children ages 0 to 2 years of age (Kiserud et al., 2017). Weight percentile was calculated according to gestational age (in days) and offspring sex. The total score ranges between 1 and 99 and the cutoff-value for low weight percentile was <10.

2.2. Statistical analysis

Analyses were conducted using SPSS 26.0 (IBM, Chicago, Illinois, USA) for Windows. Salivary cortisol concentrations were log10 transformed to fulfill the requirements for normal distribution.

Three set of analyses were conducted: (i) The DIURNAL-set examined maternal cortisol circadian rhythm, independently in each trimester, and the possible influence of maternal depressive symptoms. Thus, three mixed-effects models (one per trimester) were employed, in which the main factor time had four categories corresponding to the diurnal saliva collection moments (B1, B2, B3 and B4).

(ii) The PREGNANCY-set examined changes on daily cortisol secretion throughout pregnancy and explored if these changes differed regarding depressive symptoms. To have a measure of the total cortisol output during a day, the area under the curve with respect to ground (AUCg) was calculated for each trimester using the four log10 transformed cortisol concentrations. For this PREGNANCY-set, only one mixed-effect model was executed. The main factor time had three categories corresponding to each trimester of pregnancy.

For all mixed models, a random effect of intercept and a random

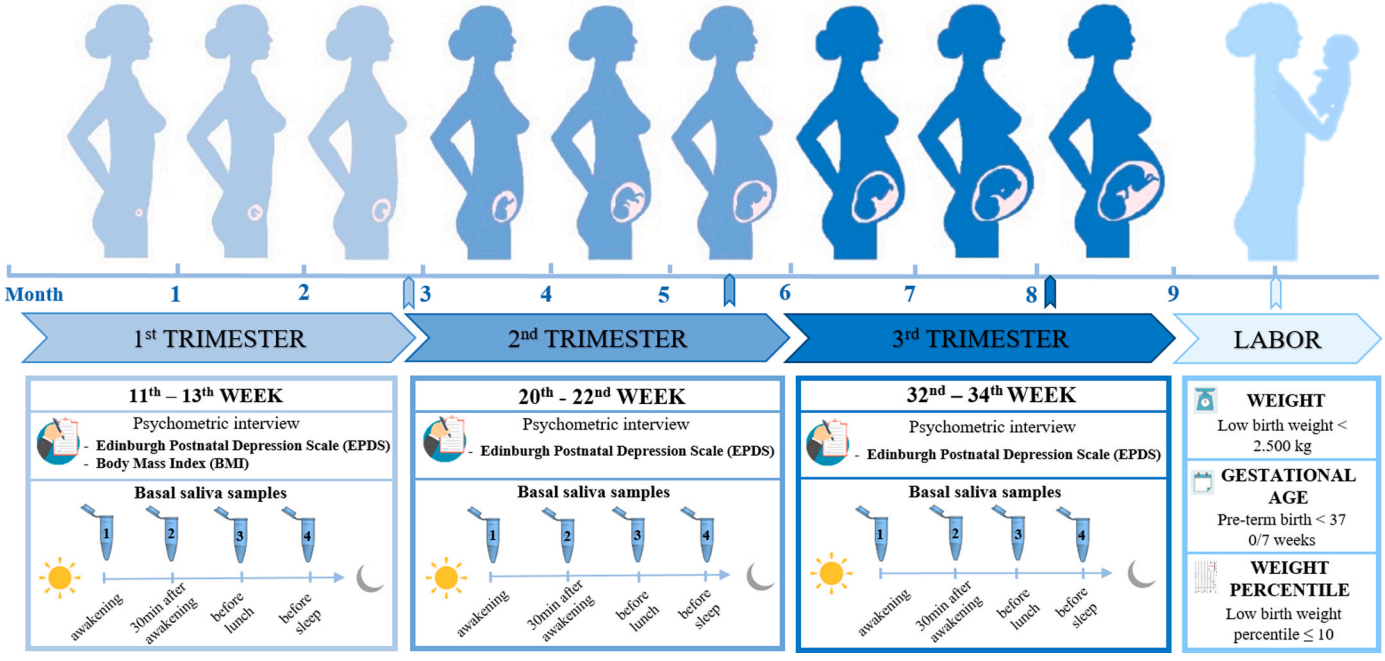

Fig. 1. Summary of the study protocol; EPDS, Edinburgh prenatal depression scale.

slope of time were employed to account for within-subject correlations, as recommended by Heisig & and Schaeffer (2019). The main effect of interest was the interaction between depressive symptoms and time, defined as four moments throughout one day for the DIURNAL-set and three trimesters for de PREGNANCY-set. Simple effects were also calculated to evaluate the specific time-point effect between groups. To account for possible confounders, maternal age, the gestational week in which salivary samples were collected, the time of first cortisol sample (awakening, B1) and maternal BMI were included as covariates for DIURNAL-set and maternal age for PREGNANCY-set.

(iii) The BIRTH-set explored the effect of diurnal total cortisol output (AUCg) in each gestational trimester in low weight, preterm birth and offspring birth weight percentile. For this set, logistic regressions were performed, crude and adjusting β coefficients with 95% confidence intervals. The following confounders were included: maternal age, the gestational week in which saliva samples were collected, maternal BMI and offspring's sex. The effect of maternal depressive symptoms (EPDS) in each gestational trimester in low weigh, preterm birth and low offspring birth weight percentile were also analyzed (see Section 2.6, Supplementary material).

All tests were two-tailed with significance defined as p-value <0.05. To correct for the testing of three different trimesters of pregnancy (DIURNAL-set) and the overall cortisol model (AUCg; PREGNANCY-set), a Bonferroni correction was applied in Table 2, by dividing the original alpha level ($p < .05$) by 4 (3 + 1) and obtained a new significance level of $p < .013$. Furthermore, to correct for the testing the three trimesters of pregnancy with birth anthropometrics (BIRTH-set), a Bonferroni correction was applied by dividing the original alpha level ($p < .05$) by 3 and obtained a new significance level of $p < .017$.

3. Results

Out of 112 pregnant women initially recruited, 5 women dropped out of the study in the 1st trimester, 3 women dropped out in the 2nd trimester and 4 dropped out in the 3rd trimester of pregnancy (i.e., no cortisol and no questionnaire data available). Finally, birth information was retrieved for 100 dyads. For more information regarding missing data, see Section 2.1, Supplementary material.

When comparing the participants who dropped out the study and those included in the analysis, there were no significant differences in either maternal age ($p = .578$) and overall cortisol values in first trimester (AUCg) ($p = .634$). However, excluded participants exhibited significantly higher depressive symptoms than those included ($t = 3.90$ $p = .048$).

Maternal and neonatal characteristics are presented in Table 1 and information regarding cortisol samples is detailed in supplementary (Table S1). As expected, having depressive symptoms in the first trimester correlates with having depressive symptoms during the second ($r = .51$, $p < .001$) and third trimesters ($r = .67$, $p < .001$), indicating a time-stability of this subclinical condition.

3.1. Cortisol circadian rhythm across trimesters and maternal depressive symptoms

The DIURNAL-set indicated that cortisol levels fluctuated significantly throughout the day, showing a circadian rhythm in all trimesters [1st ($F(3110) = 92.565$, $p < .001$), 2nd ($F(3,85) = 46.828$, $p < .001$) and 3rd ($F(3,90) = 65.555$, $p < .001$)] (see Fig. 2).

Regarding maternal depressive symptoms, the second trimester-mixed model indicated that cortisol diurnal pattern differs between both groups ($F(3,85) = 4.136$, $p = .009$), surviving Bonferroni correction. While women without depressive symptoms showed significant differences between all the diurnal time-points ($p < .001$ between B1-B2, B2-B3, and B3-B4), women with depressive symptoms did not show the expected morning cortisol increase (between B1-B2) ($p = .214$) nor the evening decline (between B3-B4) ($p = .292$). These observations were more accentuated in women with severe depressive symptoms (see supplementary material). Cortisol diurnal pattern did not significantly differ between both groups in the first ($F(3110) = 1.676$, $p = .176$) and the third trimester ($F(3,90) = 1.089$, $p = .358$). However simple effects indicated that in the 1st trimester both morning cortisol increase, and evening decline were slightly blunted in women with depressive symptoms ($p = .224$ between B1-B2; $p = .145$ between B3-B4). (see Table 2 and Fig. 3).

For more details about simple effects and covariates, see Tables S3

Table 1
Demographics of the recruited sample (n pregnant women = 112, n offspring = 100).

Variables		Total sample
Maternal age (years) (M, S.D., range)		32.24 (4.43) [21–40]
Country of birth	Spain (*n, %*)	83 (74.1%)
	Other[a] (*n, %*)	29 (25.9%)
Level of education[b]	Low (*n, %*)	15 (13.4%)
	Moderate	31 (27.7%)
	High	66 (58.9%)
Pre-pregnancy BMI (kg/m^2) (M, S.D., range)		23.71 (4.24) [17.29-39.02]
EPDS 1st Trimester[c,d] (M, S.D., range)		6.87 (4.50) [0–19]
	Absence of depressive symptoms (*n, %*)	76 (71.0%)
	With depressive symptoms (*n, %*)	31 (29.0%)
EPDS 2nd Trimester[c,d] (M, S.D., range)		6.13 (5.16) [0–24]
	Absence of depressive symptoms (*n, %*)	79 (79.8%)
	With depressive symptoms (*n, %*)	20 (20.2%)
EPDS 3rd Trimester[c,d] (M, S.D., range)		6.25 (6.25) [0–23]
	Absence of depressive symptoms (*n, %*)	75 (77.3%)
	With depressive symptoms (*n, %*)	22 (22.7%)
Infant birth weight (kg)[e] (M, S.D., range)		3.29 (0.51) [1.80-4.51]
	Normal weight (*n, %*)	93 (93.0%)
	Low weight (*n, %*)	7 (7.0%)
Gestational Age at delivery (weeks)[f] (M, S.D., range)		39.75 (1.49) [32.86-42.57]
	Full term (*n, %*)	93 (93.0%)
	Pre-term (*n, %*)	7 (7.0%)
Offspring birth weight percentile[g] (M, S.D., range)		10.71 (28.42) [1–99]
	Appropriate for gestational age (*n, %*)	74 (74.0%)
	Low for gestational age (*n, %*)	16 (16.0%)
Infant sex	Male (*n, %*)	48 (48.0%)
	Female (*n, %*)	52 (52.0%)

Note: BMI, Body mass index; EPDS, Edinburgh prenatal depression scale

[a] Other includes Latin American (19.0%), Western Europe (5.7%) and Eastern Europe (1.6%)

[b] Low level of education includes no education, lower general secondary education and intermediate vocational education, Moderate level of education includes higher general secondary education, pre-university education and higher vocational education, High level of education includes university (degree, master or doctorate)

[c] Absence of depressive symptoms includes EPDS scores lower than 11 for 1st and lower than 10 for 2nd and 3rd trimester of gestation while depressive symptoms included EPDS scores equal or higher to 11 for 1st and equal or higher than 10 for 2nd and 3rd trimester of gestation

[d] n EPDS: 1st trimester (weeks 11–13) = 107, 2nd trimester (weeks 20–22) = 99, 3rd trimester (weeks 32–34) = 97

[e] The cutoff-value for low birth weight was <2.500 kg

[f] The cutoff-value for pre-term birth was <37 0/7 weeks

[g] The cutoff-value for low offspring birth weight percentile was <10

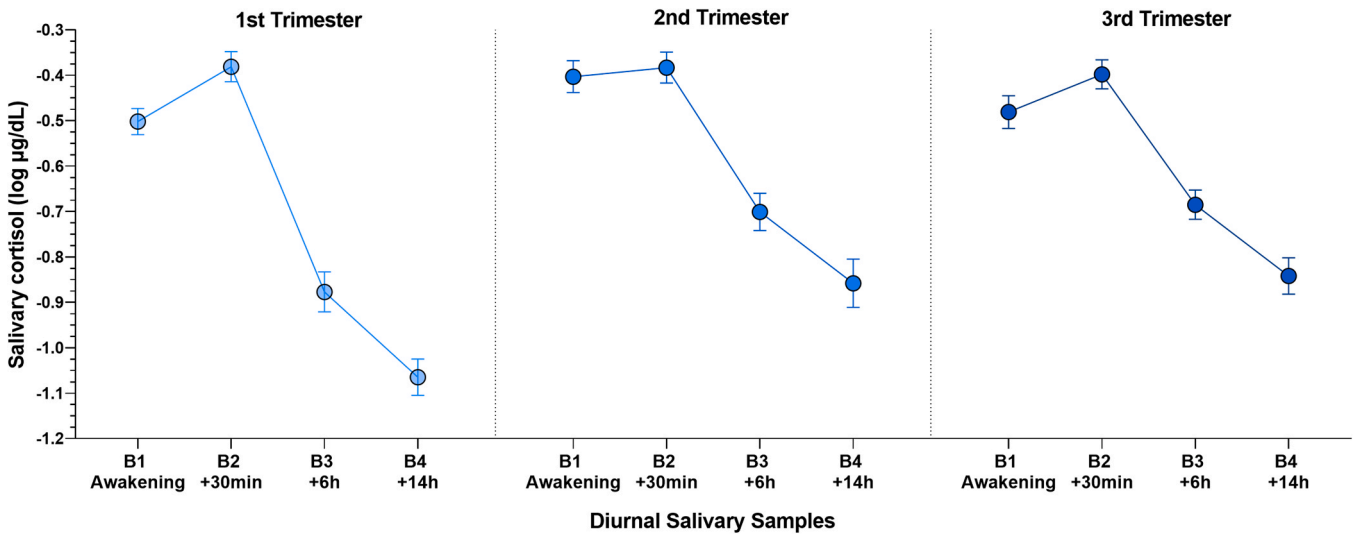

Fig. 2. Maternal salivary cortisol fluctuations throughout a day in each of the three trimesters of pregnancy (error bars SE). n analyses: T1 (weeks 11–13) = 93, T2 (weeks 20–22)= 91, T3 (weeks 32–34)= 81. Results of the DIURNAL-model indicate that cortisol significantly fluctuates during a day in all the three trimesters (1st trimester, F = 111.358, p < .001; 2nd trimester, F = 52.933, p < .001; 3rd trimester, F = 71.889, p < .001).

and 4, Supplementary material.

3.2. Overall cortisol levels throughout pregnancy and maternal depressive symptoms

The AUCg mixed model showed that overall diurnal cortisol varies across the three trimesters of gestation (*F(2101)= 15.393, p < .001*). When exploring simple effects, overall cortisol levels showed a significant increase from first to second trimester (*p < .001*) but did not vary from second to third trimester (*p = .551*), indicating a special increase in the first stages of pregnancy.

Interestingly, no significant differences between women with depressive symptoms and controls were found for overall diurnal cortisol throughout pregnancy (*F(1,87)= 0.948, p = .333*). However, simple analysis revealed that women with depressive symptoms only increased cortisol levels from first to second trimester, while control women tended to increase their cortisol levels gradually during pregnancy (see Table 2) (see Fig. S1 and Table S4, Supplementary material).

Regarding covariates, while maternal BMI did not significantly influence the AUCg (F(1,22)= 0.360, p = .360), maternal age was associated with higher AUCg throughout pregnancy (F(1,92)= 1.036, p = .024).

3.3. Maternal cortisol levels during pregnancy and birth outcomes

Regarding BIRTH-set, in the second trimester a significant association was observed between overall maternal cortisol levels (AUCg) and prematurity (adjusted odds ratio −0.371, 95% confidence interval 0.490–0.972, p = .034), although this result did not survive the conservative Bonferroni correction. In the 3rd trimester, higher maternal AUCg was associated with lower offspring weight birth percentile (adjusted odds ratio −0.612, 95% confidence interval 0.348–0.846, p = .007), surviving Bonferroni correction.

However, no associations were observed between maternal cortisol and birth anthropometrics in the 1st (See section 2.5, Supplementary material) and between covariates and birth anthropometrics in 1st, 2nd and 3rd trimester (See Table S5, Supplementary material). Interestingly, no associations were observed between maternal depressive symptoms in 1st, 2nd and 3rd trimesters and birth anthropometrics (See section 2.6., Supplementary material).

4. Discussion

The present study provides a deeper insight into the relationship between gestational maternal depressive symptoms, maternal cortisol fluctuations and birth anthropometrics. According to our results, cortisol circadian rhythm was preserved in all trimesters of gestation. However, women with depressive symptoms showed an attenuated cortisol circadian profile, presenting blunted morning cortisol increase and evening decline. These effects were apparent in the first trimester and significantly detectable in the second one, although not observable in the third. As expected, overall diurnal cortisol levels significantly increased during pregnancy in all women, even though women with depressive symptoms tended to present a milder increase from second to third trimester. Furthermore, higher cortisol levels in mid and late pregnancy seemed to be associated with prematurity and low weight percentile in newborns.

Previous studies have also linked HPA axis alterations and hypercortisolemia to clinical depression, in both pregnant women (Orta et al., 2018; Seth et al., 2016) and non-pregnant population (Mikulska et al., 2021; Nandam et al., 2020). Despite EPDS scale is used in all the above-mentioned studies, different cut-offs have been adopted, which might influence the number of depression cases identified and results interpretation. Particularly, our findings suggest that depressive symptoms are related to a flattening of the cortisol circadian rhythm in early-mid pregnancy, indicating that women with depressive symptoms (although not diagnosed) could be exposed to lowered morning and heightened evening cortisol levels in early mid pregnancy compared to their healthy peers. Moreover, we observed a significant effect between groups of severity, showing that 2nd trimester cortisol circadian rhythm flattening was more accentuated in women with severe depressive symptoms (see Fig. S2, Supplementary material). This is in line with previous literature (Murphy et al., 2022; O'Connor et al., 2014), reporting flatter diurnal cortisol patterns across all trimesters among pregnant women with anxious depressive disorders or exposed to early adverse life events. Moreover, Heuvel and colleagues (2018) identify a blunted cortisol awakening response (CAR) among pregnant women with somatic anxiety symptoms compared to controls; however, they did not observe associations regarding maternal diurnal rhythmicity and depression. Contrary, Osborne et al. (2022) did not find differences in cortisol diurnal rhythmicity nor in CAR regarding preconceptional depression diagnosis. To the best of our knowledge, our study is the first to analyze each trimester independently, while previous research

Table 2
Cortisol values log10 transformed in the three trimesters of pregnancy and cortisol secretion (AUCg) throughout pregnancy of the total sample and according to the presence of depressive symptoms.

		Total sample (Mean ± SD)	F (p)[a]	F (p)[b]	Depressive symptoms		
					Without depressive symptoms (Mean ± SD)	With depressive symptoms (Mean ± SD)	F (p)[c]
1st TRIMESTER (μg/dL Log transformed)	B1-T1st	-.502 ± .030	109.827 (<.001 ***)▲	0.688 (.409)	-.507 ± .026	-.498 ± .055	1.676 (.176)
	B2-T1st	-.396 ± .036			-.371 ± .031	-.421 ± .064	
	B3-T1st	-.881 ± .048			-.918 ± .042	-.844 ± .086	
	B4-T1st	-1.067 ± .043			-1.142 ± .037	-.993 ± .078	
2nd TRIMESTER (μg/dL Log transformed)	B1-T2nd	-.410 ± .037	84.603 (<.001 ***)▲	1.185 (.279)	-.455 ± .031	-.365 ± .067	4.136 (.009 **)▲
	B2-T2nd	-.395 ± .035			-.358 ± .029	-.433 ± .064	B1-B2 * **▲
	B3-T2nd	-.705 ± .043			-.753 ± .036	-.657 ± .077	B3-B4 * **▲
	B4-T2nd	-.859 ± .057			-.949 ± .047	-.769 ± .104	
3rd TRIMESTER (μg/dL Log transformed)	B1-T3rd	-.500 ± .037	89.710 (<.001 ***)▲	1.233 (.271)	-.431 ± .035	-.569 ± .065	1.089 (.358)
	B2-T3rd	-.412 ± .034			-.374 ± .032	-.450 ± .059	
	B3-T3rd	-.700 ± .034			-.679 ± .032	-.722 ± .060	
	B4-T3rd	-.851 ± .042			-.846 ± .040	-.857 ± .074	
AUCg throughout pregnancy	T1st	-12.086 ± 0.438	101.175 (<.001 ***)▲	0.948 (.333)	-12.291 ± 0.499	-11.882 ± 0.717	1.036 (.359)
	T2nd	-9.862 ± 0.366			-10.481 ± 0.416	-9.242 ± 0.599	
	T3rd	-9.515 ± 0.388			-9.661 ± 0.426	-9.569 ± 0.644	

Mixed-model analyses for cortisol diurnal slopes in the three trimesters of pregnancy and cortisol secretion (AUCg) throughout pregnancy according to maternal depressive symptoms.
Note: AUCg, area under the curve with respect to ground (indicating the total cortisol output); B1: basal sample at awakening, B2: basal sample 30 min after awakening, B3: basal sample before lunch, B4, basal sample before going to bed, T1st: 1st trimester, T2nd: 2nd trimester, T3rd: 3rd trimester
Mean time for saliva sample collection: 1st trimester [week 15.43 ± 3.26 (8-21)], 7:54 ± 1:09 (5:48–11:50) (B1-T1st); 8:28 ± 1:09 (6:30–12:30) (B2-T1st); 14:13 ± 1:10 (11:48–16:20) (B3-T1st); and 22:37 ± 1:40 [20:10–1:40(+1 day)] (B4-T1st); 2nd trimester [week 26.08 ± 4.50 (18-32)], 8:06 ± 1:08 (6:10–11:50) (B1-T2nd); 8:48 ± 1:19 (6:40–12:30) (B2-T2nd); 14:14 ± 1:03 (12:00–16:30) (B3-T2nd); and 22:40 ± 1:38 [20:00–1:48(+1 day)] (B4-T2nd); 3rd trimester [week 35.52 ± 2.81 (30-41)], 8:33 ± 1:08 (6:18–11:30) (B1-T3rd); 9:09 ± 1:07 (6:48–12:01) (B2-T3rd); 14:17 ± 0:47 (12:10–16:10) (B3-T3rd); and 22:41 ± 1:18 [19:00–1:30(+1 day)] (B4-T3rd)
Absence of depressive symptoms includes EPDS scores lower than 11 for 1st and lower than 10 for 2nd and 3rd trimester of gestation while depressive symptoms included EPDS scores equal or higher to 11 for 1st and equal or higher than 10 for 2nd and 3rd trimester of gestation
n depressive analysis (T1 = 93, Absence of symptoms = 70, With symptoms = 23; T2 = 91, Absence of symptoms = 71, With symptoms =20, T3 = 81, Absence of symptoms = 59, With symptoms = 22; Total pregnancy = 73, Absence of symptoms = 53, With symptoms = 20)
[a] Mixed-effects model for time
[b] Mixed-effects model for depressive risk
[c] Mixed-effects model for interaction between depressive risk and time. Values in superscript (B1-B2 and B3-B4) indicate the samples with significant differences in cortisol fluctuation regarding depressive symptoms in the simple effects test in the context of mixed-effect model.
The analyses include the following covariates: maternal age, weeks of gestation and time of awakening (for single cortisol measurements in all trimesters) and maternal age (for AUCg).
p values: **p < .01, and ***p < .001. ▲p ≤ .017 (as the Bonferroni-corrected level of significance for multiple testing (.05/4 = 0.013).

included the data of all trimesters in the same statistical analysis.

Although the presence of depressive symptoms, moderate or severe, seem to be associated with subtle alterations on cortisol rhythmicity, flattening diurnal and evening responses, depressive symptoms are not able to completely dysregulate HPA axis functioning in early pregnancy. Furthermore, in late pregnancy no differences on cortisol circadian rhythm regarding maternal depressive symptoms were observed. This result is consistent with previous evidence reporting no differences in CAR among depressed and non-depressed pregnant women in late pregnancy (Hellgren et al., 2013). Of note, placental CRH increases especially during the last 6–3 weeks of pregnancy (Kammerer et al., 2006; Mastorakos and Ilias, 2003)[i], inducing the desensitization of pituitary CRH receptors (Thomson, 2013), and preventing women from hypercortisolemia (Grammatopoulos, 2007). In fact, Entringer et al. (2010) observed that cortisol responses to acute stress are attenuated only in the third trimester, suggesting that HPA axis functioning in late pregnancy may have a greater influence of hormonal perinatal environment rather than maternal stress conditions.

In accordance with previous studies, our results indicate that maternal overall cortisol levels significantly increase during pregnancy (Buss et al., 2012; Entringer et al., 2011; Orta et al., 2019; van den Heuvel et al., 2018). Interestingly, while control women show a gradual increase, women with depressive symptoms show a more pronounced increase of cortisol levels in mid-pregnancy. A pregnant woman goes throughout different metabolic, endocrine and immunological changes during the transition to motherhood. However, these changes are not completely gradual across pregnancy. Noticeably, the placenta is not fully formed until the second trimester. Thus, many changes associated

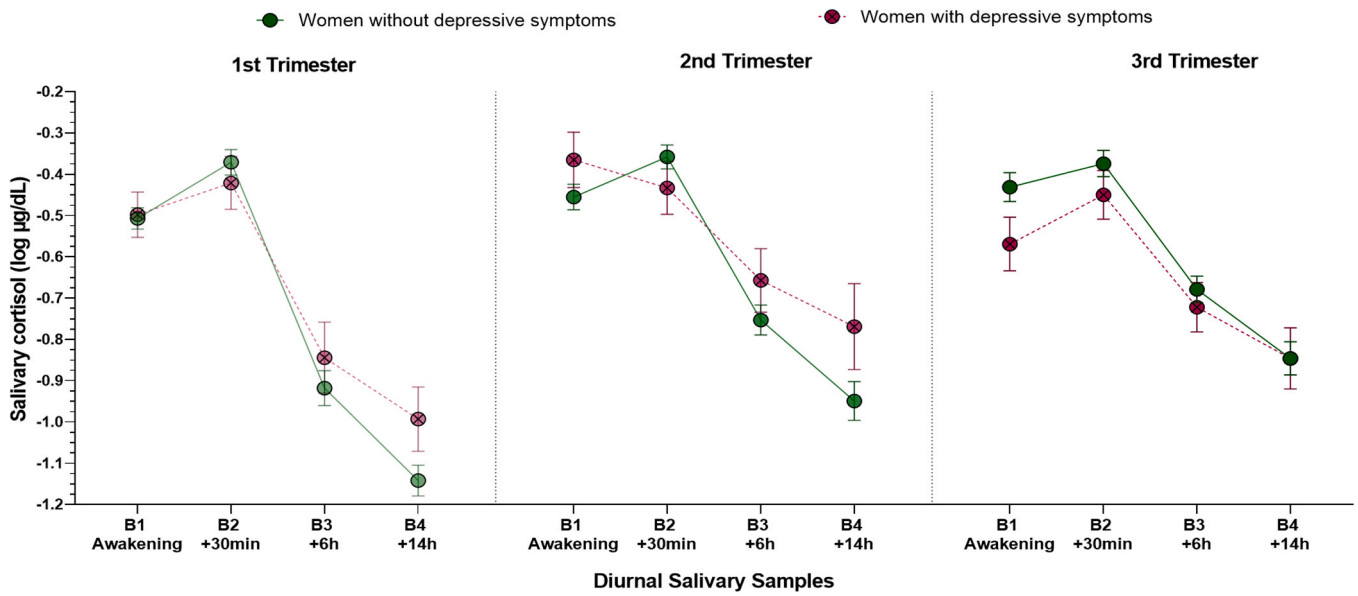

Fig. 3. Maternal salivary cortisol fluctuations throughout day in the three trimesters of pregnancy according to depressive symptomatology (error bars SE). Pregnant women with depressive symptoms (EPDS cut-off value ≥11 for 1st and ≥10 for 2nd and 3rd trimesters) are represented with dashed-pink lines and women with no depressive symptoms are represented with solid-green lines. n analyses: T1 (weeks 11–13) = 93 (Absence of symptoms = 70, With symptoms = 23), T2 (weeks 20–22) = 91 (Absence of symptoms = 71, With symptoms = 20), T3 (weeks 32–34)= 81 (Absence of symptoms = 59, With symptoms = 22). Results of the DIURNAL-model indicate that cortisol fluctuations during a day significantly differ between women with and without depressive symptoms only in second trimester (1st trimester, $F(3,11) = 1.676$, $p = .176$; 2nd trimester, $F(3,85) = 4.136$, $p = .009$,; 3rd trimester, $F(3,90) = 1.089$, $p = .358$).

to placental endocrine signals occur in this trimester, such as the increase in blood flow volume and maternal insulin resistance (Mayer and Joseph, 2013). Furthermore, hormonal fluctuations associated to placenta might be playing a role on the maternal brain reorganization occurring in pregnancy, increasing maternal susceptibility to depression (Servin-Barthet et al., 2023). Remarkably, although depressive symptoms seem to alter cortisol diurnal rhythmicity in mid-pregnancy, cortisol overall levels did not significantly differ between women with and without depressive symptoms. Although it seems contradictory, flatter diurnal cortisol contains elements of hypo and hyper-cortisolism (including low morning and high evening cortisol levels) which might explain this discrepancy and reinforces the idea that diurnal cortisol sampling protocol should include sampling at different time-points across the day.

Higher cortisol levels in mid-late pregnancy seemed to increase the risk of low birth weight percentile and prematurity. These results suggest that the increment of cortisol in mid-pregnancy, associated with maternal depressive symptoms, could lead to poorer birth outcomes. Noticeably, cortisol is necessary for fetal organ's maturation in late pregnancy (Austin and Leader, 2000), as demonstrated by the use of corticosteroids in cases of growth restriction (McGoldrick et al., 2020). Nevertheless, an excess of maternal cortisol during early to mid-gestation has been reported to cause structural and functional changes on fetal nervous system and HPA axis and worse birth outcomes (Davis and Sandman, 2010; Howland et al., 2017; Vlenterie et al., 2022). Although the association between maternal cortisol levels during 3rd trimester and infant's weight percentile at birth was strong, the weak association observed in the 2nd trimester suggests that other routes could be playing a role in this association. For example, alterations in the diurnal cortisol slope haves been strongly associated with immune and inflammatory outcomes (Adam et al., 2017). The maternal immune system is adapted during pregnancy to protect the mother and the fetus from pathogens while to avoid detrimental immune responses against the fetus (Abu-Raya et al., 2020). However, perinatal maternal depression is associated with increases in peripheral inflammatory cytokines (IL-6, IL-1β), which might affect both maternal and fetal health. Pro-inflammatory activity might be responsible on functional and morphological changes in maternal brain structure (Servin-Barthet et al., 2023). Meanwhile, the activation of maternal inflammation could activate the inflammatory process in placenta, compromising fetal environment. Moreover, cortisol diurnal disruption might alter the pass of maternal cortisol to the fetus trough the placenta by altering epigenetic mechanisms associated with the gene encoding the enzyme 11β-HSD2 or other glucocorticoid pathway genes, such as *NR3C1* or *FKBP5* (Monk et al., 2016). Thus, might alter the functioning of this enzyme and, subsequently, the fetus could be exposed to higher cortisol levels although maternal cortisol levels are not considered excessively high.

According to a recent systematic review, there is a lack of consensus across European countries regarding the clinical recommendations for screening, diagnosing and managing peripartum depression (Motrico et al., 2022). In our cohort, the prevalence of depressive symptoms was about 22%, which almost doubles the number of pregnant women with a depression diagnoses (Woody et al., 2017). Considering the high prevalence of depressive symptoms and the putative maternal and fetal implications, it seems necessary to implement adequate strategies to detect depressive symptoms at least once at each trimester. This is especially important in early and mid-pregnancy since depressive symptoms seem to modify cortisol functioning, which might impact on birth anthropometrics. Moreover, this study opens up the possibility to implement cortisol as a universal biomarker for screening mothers at risk, particularly measuring its diurnal rhythmicity or at least morning and evening levels.

Future studies exploring depression in pregnant women should include not just categorical diagnosis but also the assessment of depressive symptoms, preferably explored in different moments along pregnancy. Although the role of cortisol is not yet completely understood in the earliest weeks of pregnancy nor the postpartum, we strongly recommend studying these time-points, preferably by the collection of at least four daily salivary measures. Given the tendency to low birth weight and prematurity among children exposed to higher prenatal cortisol levels in mid pregnancy, future studies with higher sample size could confirm this association and explore possible neurodevelopmental impairments in the offspring.

A major strength of our study was interviewing in different time-points along pregnancy and analyzing them separately to get a better insight of time sensitive windows for intervention. Additionally, we explored a healthy population in which depression screening is uncommon. Finally, four salivary samples were collected to capture different aspects of the cortisol circadian rhythm.

Regarding limitations, not all women did the interview and the collection of saliva in the same week, therefore some first trimester samples were collected at the beginning of the second trimester. To avoid bias, analyses were adjusted for the exact week of collection. Moreover, the assessment of cortisol concentrations just after conception would have provided valuable information about changes in the first stages of pregnancy. Additionally, attrition bias analysis revealed that women with depressive symptoms had a greater tendency to drop out the study. Dropouts are very common in longitudinal psychiatry studies and should be consider, as their data could impact in the final results (Mazumdar et al., 2007). When comparing cortisol patterns across trimesters, it should be also considered that not all participants completed the hole protocol in all the trimesters. Finally, combining EPDS administration and a diagnostic interview would have allowed to better characterize the severity of depressive symptoms.

5. Conclusions

Maternal cortisol levels gradually increased during pregnancy, although cortisol circadian rhythm was preserved in all trimesters of pregnancy. Depressive symptoms seem to be associated with blunted cortisol rhythmicity in mid-pregnancy, although there might be a physiological readjustment of cortisol levels in late pregnancy. Finally, maternal cortisol levels in mid and late-pregnancy could be associated with increased risk of prematurity and low gestational age at birth. This study highlights the importance of screening for symptoms of mental health issues throughout pregnancy, regardless the past or current history of psychiatric disorder, since it might have an impact on infant health.

CRediT authorship contribution statement

Conceptualization, A.C.-Q., E.E., F.C., M.P.G-P and L.F.; methodology, A.C.-Q., M.D-C, M.R.-B., A.M.-V., L.dlF.-T. and J.L.M.-G.; software, A.C.-Q.; validation, A.C.-Q., M.R.-B. and A.M.-V.; formal analysis, A.C.-Q.; investigation, A.C.-Q., H.P.-G., E.E., B.A., M.P.G.-P and L.F.; resources, E.E., F.C., M.P.G.-P.G. and L.F.; data curation, A.C.-Q. and J.L.M.-G.; writing—original draft preparation, A.C.-Q., M.R.-B., A.M.-V. and B.A.; writing—review and editing, A.C.-Q.,E.E., N.S.M-G., L.M.-F., H.P.-G., M.R.-B., A.M.-V., B.A., M.P.G.-P and L.F.; visualization, A.C.-Q. and L.F.; supervision, A.C.-Q., E.E., B.A., M.P.G.-P. and L.F.; project administration, A.C.-Q., E.E., M.P.G.-P and L.F.; funding acquisition, E.E., F.C., M.P.G.-P and L.F. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

Authors do not have any conflict of interest regarding the publication of this manuscript.

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Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.psyneuen.2023.106930.

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UNIVERSITAT DE BARCELONA

Dr. Lourdes Fañanás Saura
Section of Zoology and Biological Anthropology
Dept. Evolutionary Biology, Ecology and Environmental Sciences
Faculty of Biologia, Universitat de Barcelona

Supervisor's report on the contribution of the PhD applicant to the article.

Prof. Dr. Lourdes Fañanás Saura, full professor at the Department of Evolutionary Biology, Ecology and Environmental Sciences of the Faculty of Biology (Universitat de Barcelona) and supervisor of the present doctoral thesis by Águeda Castro Quintas, hereby certifies that the participation of the PhD applicant in the article "*Diurnal cortisol throughout pregnancy and its association with maternal depressive symptoms and birth outcomes*" included the following tasks:

- Participation in the conception and design of the study
- Recruitment and evaluation of the included participants
- Coordination of the project
- Laboratory analyses
- Data preparation
- Statistical analyses and data interpretation
- Writing of the first manuscript draft
- Critical revision of the article for intellectual content

Signed by Prof. Lourdes Fañanás

Barcelona, 5th December 2023

3.2. Distress during pregnancy and epigenetic signatures at glucocorticoid related genes in placental chorionic villi and maternal decidua layers: a pilot study in Spanish primiparous women

Agueda Castro-Quintas, Helena Palma-Gudiel, Elisenda Eixarch, Nerea San Martin González, Simone Röh, Sussan Sauer, Monika Rex-Haffner, Jose Luis Monteserin-Garcia, Fatima Crispi, Maria Paz Garcia Portilla, Elisabeth B. Binder*, Lourdes Fañanas*

Clinical Epigenetics (submitted)

Distress during pregnancy and epigenetic signatures at glucocorticoid related genes in placental chorionic villi and maternal decidua layers: a pilot study in Spanish primiparous women

Agueda Castro-Quintas[1,2], Helena Palma-Gudiel[2], Elisenda Eixarch[3,4], Nerea San Martin González[1,2], Simone Röh[5], Sussan Sauer[5], Monika Rex-Haffner[5], Jose Luis Monteserin-Garcia[1,2], Fatima Crispi[3,4], Maria Paz Garcia Portilla[2,6,7], Elisabeth B. Binder[5]☆, Lourdes Fañanas[1,2*]☆

Abstract

Background Maternal stress during pregnancy can impact offspring health, increasing the risk of neuropsychiatric disorders. The human placenta plays a crucial role in understanding this effect, as it may influence fetal programming. We hypothesize that maternal stress influences children's outcomes through placental DNA methylation in genes involved in cortisol regulation and functioning. These genes would include *NR3C1*, *FKBP5*, and *HSD11B2*.

Potential bias from previous studies exploring placental DNA methylation and maternal stress can arise from, both, the differential effect of stress depending on the trimester of pregnancy and, the putative different reactivity of the placental layers in front to maternal stress, given their distinct origins. We believe that assessing maternal stress condition throughout pregnancy and separately analyzing the methylation signatures of diverse placental layers could clarify the role of maternal stress in placental epigenetics.

Methods In this pilot study, we analyzed the layers of chorionic villi and maternal decidua in the placenta from 45 mother-infant dyads, which come from a broader Spanish cohort, the *Intramural_Maternal-Epi Project*. The selected women represent both high-risk and low-risk cases for maternal distress, measured by depressive symptoms, childhood violence, and high perceived stress during pregnancy. All dyads were monitored from early pregnancy to two months post-birth. Both, mothers and newborns, underwent comprehensive assessments of psychological and physiological stress. Maternal depressive symptoms were evaluated in all trimesters, and saliva samples were collected four times a day to estimate a diurnal cortisol functioning measure. Newborn neurodevelopment was assessed at 7 weeks using the Neonatal Behavioral Assessment Scale (NBAS), and saliva samples were collected before and after the test to measure cortisol levels. Placental layers were collected within 15 minutes after birth and frozen at -80ºC. Targeted bisulfite sequencing was employed to finely map CGs of the selected placental genes, ensuring uniform processing to avoid technical biases.

Results Preliminary results revealed that increased maternal diurnal cortisol levels in the first trimester of pregnancy were significantly associated with an increased DNA methylation at exon 1_D of the *NR3C1* gene and lower DNA methylation at intron 7 of the *FKBP5* gene of the chorionic villi. Interestingly, an elevated DNA methylation at intron 1 and at intron 7 of *FKBP5* at the placental maternal decidua was strongly associated with an anticipated delivery. However, the percentage of methylation observed in the promoter region of *HSD11B2* was unexpectedly low and homogeneous in all analyzed placental samples, including those from mothers exposed to elevated levels of stress.

Conclusions Elevated maternal cortisol levels at the beginning of pregnancy appear to be associated with differential placental methylation in chorionic villi in the *NR3C1* and *FKBP5* genes. Furthermore, higher methylation of *FKBP5* in the maternal decidua was associated with an anticipated delivery. These results underscore the importance of exploring layer-specific methylation differences at specific moments during pregnancy, highlighting the complex interplay between maternal stress, placental epigenetic modifications, and fetus during all the prenatal periods.

Keywords Maternal stress, pregnancy, cortisol, placenta, DNA methylation, *FKBP5*, *HSD11B2*, *NR3C1*, infant, Neonatal Behavioral Assessment Scale (NBAS) scale

$ Elisabeth B. Binder and Lourdes Fañanás contributed equally to this work.

* Correspondence
Lourdes Fañanás
lfananas@ub.edu

[1] Department of Evolutionary Biology, Ecology and Environmental Sciences, Faculty of Biology and Biomedicine Institute of the University of Barcelona (IBUB), University of Barcelona, Barcelona, Spain

[2] Health Institut Carlos III, Network Centre for Biomedical Research in Mental Health (CIBER of Mental Health, CIBERSAM), Madrid, Spain

[3] BCNatal - Barcelona Center for Maternal-Fetal and Neonatal Medicine (Hospital Clínic and Hospital Sant Joan de Déu), Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Universitat de Barcelona, Barcelona, Spain

[4] Health Institut Carlos III, Network Centre for Biomedical Research on Rare Diseases (CIBER of Rare Diseases, CIBERER), Madrid, Spain

[5] Department of Translational Psychiatry, Max Planck Institute of Psychiatry, Munich, Germany

[6] Department of Psychiatry, University of Oviedo, Oviedo, Spain

[7] Servicio de Salud del Principado de Asturias (SESPA), Oviedo, Spain

Introduction

The hypothesis that the environment surrounding a developing fetus can influence health and disease in later life has been conceptualized as the Developmental Origin of Health and Disease (DOHAD) (Barker, 2007). According to this hypothesis, maternal stress during pregnancy has been linked to a range of adverse outcomes in the offspring including cardiovascular, endocrine, metabolic and psychiatric disorders (Reynolds, 2013). Even though the mechanisms linking this relationship are not yet fully understood, the placenta could be a key organ in unraveling the pathways and mechanisms responsible of these associations.

Current research highlights the placenta as the primary regulator of the *in-utero* environment, mediating the impact of maternal insults on offspring health and development (Bronson & Bale, 2016; Ursini et al., 2018; Vuppaladhadiam et al., 2021). This temporary organ regulates the exchange of nutrients, gas, endocrine signals, cytokines and growth factors between the mother and the fetus (Fowden et al., 2009). Adapting to fetal growth demands, the placenta undergoes structural, compositional, and gene expression changes, partly attributed to placental epigenetic related mechanisms (Sferruzzi-Perri et al., 2023). As gestation progresses, the placental DNA methylation landscape undergoes significant changes, responding both to its ontogenic development and to changes in utero conditions (Novakovic et al., 2011; Robinson & Price, 2015). Disruption of these adaptive processes can be linked to adverse birth outcomes, highlighting the role of placental DNA methylation signatures as potential *in utero* markers of prenatal stress with crucial consequences on fetal development and late newborn health and behavioral conditions (Hodyl et al., 2017; Xiao et al., 2016).

Recent interest has focused on understanding the intricate network of epigenetic changes in placenta and the psychosocial stress experienced by mothers during pregnancy. According to Krontira and colleagues (2020), elevated glucocorticoid signaling *in utero* might be one of the key mediators of prenatal stress effects on the offspring (Krontira et al., 2020). In this regard, the potential dysregulation of the placental epigenetic landscape by glucocorticoids, and its impact on the fetal Hypothalamic-Pituitary-Adrenal (HPA) axis, is considered a point of significant interest.

Pregnancy is a sensitive and vulnerable period for expectant mothers, marked by multiple biological and physiological changes. Specifically, depressive symptoms are highly common during this period, with a prevalence of 11.9% (Woody et al., 2017). Furthermore, the prevalence of subclinical depressive symptomatology is even higher and often goes undetected (Yonkers et al., 2009). Moreover, depression during pregnancy has been associated with an increased risk of adverse delivery complications and newborn outcomes, including prematurity, low birth weight, neurodevelopmental delays, and adult psychiatric disorders (Grigoriadis et al., 2013; Osborne et al., 2022; Plant et al., 2015). A key potential mediator linking maternal stress associated with depressive status and fetal conditions would be the maternal HPA axis dysregulation. In fact, stress during pregnancy can lead to maternal HPA axis dysregulation and abnormally increased levels of maternal circulatory cortisol, potentially resulting in adverse effects on fetal development, including the putative sensitization of the fetal HPA axis (Castro-Quintas, Eixarch, et al., 2023; Entringer et al., 2011; Seth et al., 2016).

Within the complex system to protect the fetus from excessive cortisol levels, the placental enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2), primarily expressed in the maternal decidua and chorionic villi layers (Zhu et al., 2019), acts as a safeguard. This enzyme converts active maternal cortisol into inactive cortisone to maintain the necessary balance of fetal cortisol for adequate neurodevelopment (Murphy et al., 1974). However, excessive high and sustained maternal cortisol levels associated with stress could increase the methylation in the promoter region of the *HSD11B2* gene reducing placental enzyme production and elevating fetal cortisol levels along with their associated risks (Alikhani-Koopaei et al., 2004). Within this framework, maternal anxiety and perceived stress, during mid-late pregnancy, have previously been associated with higher placental *HSD11B2* DNA methylation and decreased 11βHSD2 expression, when compared to non-anxious counterparts (Conradt et al., 2013; Monk et al., 2016; O'Donnell et al., 2012). Moreover, decreased 11βHSD2 placental expression has been linked to elevated maternal cortisol levels and lower birth-weight (Jahnke et al.,

2021; Marsit et al., 2012). However, little is known about placental *HSD11B2* methylation signatures and their association with offspring neurobehavioral outcomes and the existing data are contradictory. Some studies indicate a positive association between placental *HSD11B2* methylation and certain neurobehavioral aspects in offspring such a better response to the environment (Conradt et al., 2013; Marsit et al., 2012). However, other studies propose a negative correlation (Liu et al., 2021; Paquette et al., 2015). Though, in most of the mentioned studies the particular layer of the placenta selected and studied is not specified. This lack of specification is known to exert a significant influence on the epigenetic profile due to the distinct embryological origin of each layer (Robinson & Price, 2015).

While the methylation of the *HSD11B2* gene in the placenta has been explored in relation to maternal stress and fetal outcomes, the analysis of other cortisol-related genes in placental tissue in the context of maternal stress has been much less investigated. This includes the glucocorticoid receptor gene (*NR3C1*) and the FK506 binding protein gene (*FKBP5*), which interact to crucially modulate the cellular effects of cortisol, playing integral roles in the intricate regulatory network governing the stress response. More specifically, FKBP5 acts as a cochaperone, adjusting the glucocorticoid receptor (GR)'s sensitivity to cortisol in the cytoplasm. The glucocorticoid receptor, reciprocally, influences gene expression including the *FKBP5* gene. Notably, maternal anxious-depressive psychopathology has been previously associated with hypermethylation of *NR3C1* in offspring blood(Palma-Gudiel et al., 2018; Palma-Gudiel, Córdova-Palomera, et al., 2015). Some authors also suggest that specific methylation signatures in *FKBP5* lead to gene overexpression, associated with hypercortisolism and inflammation, in individuals exposed to extreme psychosocial stress such as childhood trauma (Binder, 2009; Binder et al., 2008). In placental tissue, elevated maternal distress and preeclampsia have been associated with increased methylation of *NR3C1* and *FKBP5* sites (Capron et al., 2018; Hogg et al., 2013; Monk et al., 2016). However, in other study, higher exogenous maternal glucocorticoids during pregnancy were associated with lower methylation of placental *FKBP5* (Czamara et al., 2021). Additionally, Paquette and colleagues (2015) observed a reduced DNA methylation of placental *NR3C1* and heightened *FKBP5* methylation in newborns with neurodevelopmental alterations (Paquette et al., 2015).

In summary, while prior studies appear to suggest associations between maternal stress, placental methylation at the *HSD11B2*, *NR3C1*, and *FKBP5* genes and fetal and newborn's outcomes, the overall results are unclear. Therefore, a thorough examination of the methylation patterns of these genes in distinctly layered placenta samples holds promise for providing a more comprehensive understanding of these interactions. Additionally, adopting a longitudinal approach with multiple measures throughout pregnancy is crucial to capture the dynamic nature of the epigenetic mechanisms underpinning the ongoing active processes of placental adaptation to maternal stress during pregnancy.

Taken together, the aim of this study was to investigate, in a representative sample of primiparous women from the general population, (i) whether maternal stress, assessed through depressive symptoms throughout pregnancy, and the associated cortisol levels, could influence DNA methylation patterns at the *FKBP5*, *NR3C1*, and *HSD11B2* genes in two distinct placental layers: the maternal decidua and the chorionic villi. Additionally, (ii) to assess whether such epigenetic patterns were correlated with gestational age at birth or birth weight, as well as the newborn's neurobehavioral response at 7 weeks of age using the Neonatal Behavioral Assessment Scale (NBAS) and their cortisol levels before and after this test.

Results

Sample characteristics

This pilot study, involving 45 primiparous women from the general Spanish population, aimed to explore how maternal stress, assessed through depressive symptoms (EPDS), and the associated cortisol levels assessed by measuring AUCg and AUCi, influence various placental epigenetic signatures. Additionally, we explored the subsequent impact of these placental epigenetic signatures on newborn birth outcomes and infant neurodevelopment at 7 weeks old. The NBAS scale was used for the latter, and salivary measures before and after this test were collected for future cortisol measures.

Maternal and infant demographic, psychological and biological characteristics are detailed in Table 1.

Maternal age ranged from 21 to 40 years old (mean ± SD = 32.47 ± 4.47) and the vast majority of participants (77.8%) were born in Spain. The majority had high level of education (67%), defined as university education (degree, master, or doctorate). Mothers were classified as high or low stress risk based on scores of depressive symptoms from the EPDS scale. Moreover, cortisol diurnal levels were calculated in each trimester of pregnancy using the Area Under the Curve with respect to the ground (AUCg) and the Area Under the Curve with respect to the increase (AUCi). AUCg indicates the mean cortisol levels throughout the day and AUCi indicates changes of cortisol levels across the day.

The average gestational age was in our sample 39 weeks and 6 days, the average birth weight was 3.36 kg and almost one third of the infants (28.90%) were male. Cortisol levels in the child were measured both before and after the NBAS neurodevelopment test, with the calculation of basal cortisol (pre NBAS measure), AUCg (mean cortisol levels throughout the test) and AUCi (changes in cortisol levels in response to the test) based on salivary measures collected before and after the NBAS.

1. Comparative methylation signatures between chorionic villi and maternal decidua

As observed in Table 2, the mean methylation percentages at the analyzed CpG sites for each region of the included genes did not significantly differ between placental sides. Furthermore, all the CpG methylation signatures in the maternal side were positively correlated with CpG methylation signatures of the chorionic villi for all the studied regions (FKBP5 intron 1 (β = 0.489, p < .001, 95% CI: 0.246, 0.732), FKBP5 intron 5 (β = 0.843, p < .001, 95% CI: 0.572, 1.114), FKBP5 intron 7 (β = 0.534, p < .001, 95% CI: 0.249, 0.819), NR3C1 exon 1D (β = 0.787, p < .001, 95% CI: 0.584, 990), HSD11B2 island 1 (β = 0.423, p = .005, 95% CI: 0.134, 0.713) and HSD11B2 island 2 (β = 0.420, p = .005, 95% CI: 0.135, 0.705).

However, taking into account the intra-individual range of variation observed for the *FKBP5* intron 1 and *NR3C1* exon 1_D signatures, it was deemed worthwhile to analyze separately for each placental layer the possible associations between the studied methylation signatures at these genes and the variables of interest. Due to the overall hypomethylation observed for all *HSD11B2* gene regions analyzed (in islands 1 and 2) these regions were excluded from analyses (see table 2 for details).

2. Maternal depressive symptoms, cortisol levels and placental gene methylation

The results of all adjusted linear regression models are shown in Table 3.

In our analyses, maternal depressive symptoms were not significantly associated with differences in DNA methylation at *FKBP5* introns 1, 5 and 7 and in *NR3C1* exon 1_D, either in the maternal side or in the chorionic villi of the placenta (all p values > 0.05).

Regarding maternal diurnal cortisol throughout pregnancy, overall cortisol levels and rhythmicity (AUCg and AUCi) in any trimester were not associated with DNA methylation levels of the explored genes at the maternal side of the placenta. In regard to the chorionic villi, both maternal diurnal cortisol and rhythmicity during the first trimester was associated with DNA methylation profiles at both the *FKBP5* intron 7 and *NR3C1* exon 1D. Specifically, high maternal diurnal cortisol levels (AUCg) were associated with higher DNA % methylation in *NR3C1* exon 1D (β = 1.737, p = .046, 95% CI: 0.034, 3.441), and blunted diurnal rhythmicity (AUCi) was associated with lower DNA % methylation in *FKBP5* intron 7 (β = -0.369, p = .047, 95% CI: -0.733, -0.005). No associations were detected between the maternal diurnal cortisol levels during the second or third trimesters and the epigenetic signatures studied for FKBP5 and NR3C1 in the chorionic villi.

3. Placental gene methylation and birth and infant outcomes

Regarding birth outcomes, in the maternal side of the placenta more *FKBP5* DNA methylation at intron 1 (β = -0.108, p = .041, 95% CI: -0.311, -0.005), and at intron 7 (β = -0.177, p = .004, 95% CI: -0.295, -0.060), was associated with lower gestational age at birth. However, this association was not reported for the chorionic villi. The methylation signatures at intron 5 of FKBP5 and exon 1D of *NR3C1* were not associated with infant gestational age at birth in any of the placental layers. Furthermore, no associations for the studied DNA methylation signatures were observed in either maternal decidua or chorionic villi concerning birth weight.

Finally, no associations were observed between placental methylation signatures and the neurodevelopmental milestones in the NBAS

domains, nor were there associations with newborn cortisol levels during the NBAS procedure (refer to Table 3 for NBAS scale dimensions, basal cortisol, AUCg, and AUCi values).

Discussion

This pilot study investigated how maternal stress and HPA axis activity during pregnancy impact placental epigenetic markers (*FKBP5*, *NR3C1*, *HSD11B2*), exploring their influence on fetal and infant outcomes, including gestational age, birth weight, newborn neurobehavior at 7 weeks, and cortisol reactivity. To the best of our knowledge, this is the first study to analyze and compare epigenetic signatures in two distinctly layered placental samples of primiparous women exposed and non-exposed to stress during pregnancy.

Our results suggest a significant association between elevated maternal diurnal cortisol levels in the first trimester of pregnancy and an increased DNA methylation at exon 1_D of placental *NR3C1* gene. Furthermore, a blunted maternal circadian cortisol pattern, also during the first trimester, correlated with lower mean methylation levels at intron 7 of *FKBP5* gene. Interestingly, these findings were only observed for the chorionic villi layer. These results align with existing human studies demonstrating that DNA methylation of *NR3C1* and *FKBP5* genes in placental tissue varies in response to adversity during pregnancy (Capron et al., 2018; Hogg et al., 2013; Monk et al., 2016).

In this regard, Watkeys and colleagues (2018) conducted a literature review and found that heightened DNA methylation in the promoter region of *NR3C1* was associated with decreased expression of the GR in individuals exposed to adverse conditions (Watkeys et al., 2018). In the presence of cortisol, the cytoplasmic GR undergoes translocation to the nucleus; within the nucleus, it interacts with specific regions in various genes, thereby either inducing or inhibiting the transcription of different genes. In this context, the elevated presence of the FKBP5 cochaperone can reduce cortisol binding to the GR, resulting in diminished cellular cortisol regulation. Interestingly, the GR exerts long-range regulation of FKBP5 through distal intronic and intergenic sites identified in the promoter region of *FKBP5* gene (Paakinaho et al., 2010). Thus, decreased methylation of the *FKBP5* gene may impede the binding interaction with the GR, ultimately leading to alterations in the expression of the FKBP5 cochaperone. Consequently, changes in the methylation of both genes in chorionic villi cells could disrupt their interaction and compromise the placenta's ability to adequately respond to maternal stress signals mediated by cortisol. Overall, this could lead to heightened and prolonged exposure of the developing fetus to an excess of maternal cortisol.

It is noteworthy that previous studies have focused solely on maternal psychological stress conditions and often lack biological measures, such as cortisol. Monk et al. (2016) stands as an exception incorporating cortisol measures during mid-late pregnancy. However, they did not report any associations between cortisol and the methylation of *FKBP5* and *NR3C1* genes (Monk et al., 2016). Our results could point towards a possible distinctive role of placental epigenetic mechanisms during early pregnancy in response to stress. In early gestation, the placenta performs essential functions, including invasion into the maternal endometrium, remodeling maternal vasculature, and secreting hormones crucial for maintaining pregnancy (Nelissen et al., 2011). For example, early hypoxic conditions could foster trophoblast cell proliferation within the chorionic villi (Schroeder et al., 2013). Following the 12^{th} week of gestation, villi's trophoblast growth decelerates, and vascularization becomes favored in the other placental layers. As pregnancy progresses into later stages, the placenta reaches full maturation and may become less sensitive to environmental changes due to the existence of new compensatory mechanisms in its physiology, as noncoding RNAs (Apicella et al., 2019). This could explain why in our study the impacts of maternal stress are primarily observed during the first trimester and, specifically, within the chorionic villi.

In our study, we did not identify direct associations between the maternal depressive state and the methylation profile of the genes of interest. This could be attributed to our study population design consisting of healthy pregnant women without any severe medical complications, including psychiatric disorders. This suggests that more severe pathologies might be necessary to induce relevant changes in placental gene methylation. Notably, a study conducted by Hogg et al. (2013) observed an increase in placental *NR3C1* methylation in women at high risk of preeclampsia (Hogg et al., 2013). On the other hand, some studies have linked elevated levels of maternal perceived stress with *FKBP5* and *NR3C1*

methylation signatures, although they did no observe significant associations with maternal depressive symptoms (Capron et al., 2018; Monk et al., 2016). It is plausible that perceived stress induces alterations in the biological mechanisms related to the regulation of cellular cortisol response more directly than depressive symptoms themselves. Furthermore, it is essential to consider that several of these studies collect retrospective psychological and psychiatric information, providing insights only into the end of pregnancy. In this regard, women who have experienced complications during delivery may exhibit increased stress only in the postpartum period, and the variations in the methylation profile in the placenta could be attributed to these delivery complications and not to prenatal stress. Ultimately, while depressive symptoms may impact the placenta, it is conceivable that they do so through the methylation of other genes, potentially associated with inflammatory pathways. In fact, in a study performed by Czamara et al., (2021) observed that placental demethylation of FKBP5 was associated with increased expression of genes upregulated in preeclamsia and linked to inflammatory and immune response pathways (Czamara et al., 2021). Thus, exploring various dimensions of maternal stress is imperative to comprehend how distinct stressors might manifest as epigenetic modifications in the placenta.

Regarding the analyses in the placental maternal decidua, we detected a robust association between the level of methylation of intron 7 of *FKBP5* and delivery anticipation. Additionally, although with less statistical robustness, association with the methylation of intron 1 of *FKBP5* was also noted. Intron 7 has been shown to interact with the transcriptional start site of *FKBP5* using chromatin confirmation capture, and methylation alters FKBP5 induction (Klengel et al., 2013). Furthermore, intron 1 contains a glucocorticoid responsive element, and its methylation could similarly alter FKBP5 induction (Czamara et al., 2021). The highest levels of methylation of *FKBP5* could decrease FKBP5 expression, resulting in increased cortisol activation of GR targets within the placenta. It should be noted that cortisol assumes an essential role in the orchestration of parturition. For example, cortisol stimulates the synthesis of prostaglandins, which play a key role in promoting uterine contractions (Mitchell, 1994). Thus, the absence of FKBP5 in the cytoplasm due to increase *FKBP5* methylation may activate cortisol actions via the GR, leading to the anticipation of delivery. The fact that we have only observed these methylation signatures in the maternal decidua may be because it is the placental layer closest to the uterus and may play a more significant role in transmitting the onset of labor. However, it is crucial to remember that, even if labor occurred earlier, all the children in the study were born at full term.

In our pilot study, we did not observe any discernible impact of maternal stress or other maternal variables on *HSD11B2* methylation signatures. This result was somewhat unexpected, considering the gene's well-established nature and its regulatory mechanism described by other researchers in clear association with stress. For instance, existing literature associates maternal anxiety and perceived stress during pregnancy with elevated placental *HSD11B2* DNA methylation in the promoter region (Conradt et al., 2013; Monk et al., 2016). However, as discussed by Monk et al. (2016), this gene's promoter region tends to exhibit minimal methylation in their studies (Monk et al., 2016). In this respect, our findings align consistently with the cohorts explored by Monk and colleagues (2016). The activity of 11βHSD2 typically increases during pregnancy, and the hypomethylation of its promoter correlate with an activation of this enzyme (Green et al., 2015; Jahnke et al., 2021). This implies that this gene, in hypomethylated conditions, could effectively buffer cortisol levels throughout pregnancy. The hypomethylation profile of *HSD11B2* could also suggest that, in our sample, the cortisol levels received by infants were potentially at optimal levels, particularly in the later stages of pregnancy. In fact, elevated cortisol levels at the end of pregnancy are deemed necessary and have been correlated with improved cognitive task performance, whereas higher cortisol levels at the beginning of pregnancy are associated with diminished cognitive performance (46). Nonetheless, the low methylation we observed for the introns analyzed in this gene, both in the villi and the placenta, hinders further analytical scrutiny.

Our pilot study has strengths and limitations that should be noted. The sample size is the most significant constraint. However, as described in the methods section, the subsample was selected to represent a case/control scenario from a larger pool of women from the general population, taking into account the presence of elevated or low stress-related

conditions. This approach allowed our sample to be representative from our larger cohort. Furthermore, this selection minimized missing data, a crucial consideration, especially in longitudinal designs.

Moreover, the fact that this sample comprises healthy pregnant women from the general population implies the absence of concurrent clinical conditions, such as hypertension, diabetes, eclampsia, or psychiatric disorders. The examination of depressive traits or symptoms, more prevalent than a categorical diagnosis among pregnant women, significantly contributes to a nuanced understanding of this profile within the general population and its associated placental epigenetic signatures. This consideration may elucidate why we did not observe differences in *HSD11B2* gene methylation due to maternal distress. Despite that, the observation of subtle modifications in the methylation profile of genes related to the functioning of the GR, such as *NR3C1* and *FKBP5*, could demonstrate their higher sensitivity to subtle changes in cortisol associated with moderate stress (33, 47). This heightened sensitivity is attributed to the intrinsic design of these genes to capture stress. Furthermore, the specificity of these associations, observed primarily in the first trimester and within the chorionic villi, accentuates the critical importance of considering temporal dynamics in evaluating the interplay between maternal stress and placental structure.

Finally, it should be mentioned that we employed a novel technique for detecting methylation signatures, namely target bisulfite sequencing. Further refinement and validation of this methodology are warranted to ensure the accuracy and reliability of the results obtained.

Conclusions

The downregulation of the HPA axis during the first trimester of pregnancy, associated with moderate stress of the mother, seems to be linked with subtle epigenetic changes in the GR gene, *NR3C1*, and the *FKBP5* gene in the chorionic villi. Furthermore, on the maternal side of the placenta, methylation of intron 1, but especially intron 7 of *FKBP5*, is associated with anticipated birth, highlighting the significant role of this layer in signaling the initiation of labor. In our study we did not observe *HSD11B2* methylation for neither of both placental sides. Further analyses involving larger samples of mothers from the general population exposed to moderate stress and studies focusing on pregnancies with very high risk, not considered here, may contribute to clarifying a potential dose-effect relationship between maternal psychological stress and the magnitude of observed epigenetic changes in stress-regulating genes, for each of the placental layers.

Methods

Study population

Participants were 45 healthy primiparous pregnant women representative from the general Spanish population. Participants in this study were a subset of a larger study cohort (*Intramural Maternal Epi-Project*, n = 150) recruited from May 2016 to December 2020. Participants were recruited from the maternity units of two public Spanish general hospitals (Hospital Clinic of Barcelona and Hospital Universitario Central de Asturias) before undergoing their first pregnancy ultrasound at week 13th. Inclusion criteria were: (1) less than thirteen weeks of pregnancy, (2) age between 18-40 years, (3) first pregnancy, (4) no history of physical, neurological, or mental diseases, (5) singleton pregnancy. All enrolled participants provided written, informed consent and all procedures were approved by the medical ethical committee of the local hospitals and were conducted in full compliance with Helsinki declaration.

The subsample for this pilot study was selected using a case/control design, including 23 women (51%) exposed to psychosocial stress conditions during pregnancy (with elevated depressive symptoms in at least two trimesters and/or a history of violence before pregnancy) and 22 control women (49%) who were not exposed. These groups were paired, when possible, 2 by 2 based on mothers' age and other controllable sociodemographic information.

The protocol of the project is summarized in Figure 1.

Study protocol

Pregnancy was monitored through the three ultrasounds offered by the Spanish Public Healthcare system: between 11th-13th weeks (first trimester), between 20th-22nd weeks (second trimester) and between 32nd-34th weeks (third trimester), during which the assessments were conducted. At the end of each interview, participants were instructed to collect four salivary samples at home. At birth, placenta samples were collected and stored, and perinatal

outcomes (gestational age at birth and birth weight) of 45 infants were recorded. Infant neurodevelopment was assessed by a certified medical practitioner at 6-8 weeks using the NBAS neurobehavioral scale. At the beginning and the end of the evaluation protocol, two infant salivary samples were collected.

Maternal characteristics

Maternal Body Mass Index (BMI) was calculated using self-reported measures of weight and height at first trimester of pregnancy.

Maternal depressive symptoms. Participants completed the Edinburgh (Postnatal) Depression Scale E(P)DS (48) in each trimester of pregnancy, which is a screening scale for depressive symptoms during pregnancy and the postpartum period (49). This hetero-administered questionnaire consists of 10 questions, measured on a Likert scale from 0 to 3, regarding women's emotions on the previous 7 days. The total score ranges between 0 (not depressed) and 30 (severely depressed). For a better characterization of our sample, we stablished a threshold for depression risk according with Bergink et al. (2011) (50): low depression risk includes EPDS scores lower than 11 for 1st and lower than 10 for 2nd and 3rd trimester of gestation while high depression risk included EPDS scores equal or higher to 11 for 1st and equal or higher than 10 for 2nd and 3rd trimester of gestation.

Maternal saliva samples. Maternal saliva samples were collected with specially designed test-tubes (Salivette®, Sarstedt, Germany) during a non-stress ordinary day each trimester of pregnancy. Moreover, to avoid possible biases, clear written instructions for salivary self-collection were given (Section 1.1., Supplementary material). Participants were instructed to collect a total of four samples in the following order: immediately after awakening (B1), 30 min after awakening (B2), before lunch (B3), and before going to bed (B4) and stored them in their fridge freezers (4ºC). In the next appointment, samples were carried to the lab and stored at -25ºC until analysis.

Infant Characteristics

Birth outcomes. Gestational age at birth, birth weight and sex of the infant were determined from the medical record.

Infant neurodevelopment. Infant neurodevelopment was assessed using the Neonatal Behavioral Assessment Scale (NBAS) (51). The NBAS is a 53-items scale which assesses the capacity of the newborn to interact and respond to the environment, according to six domains (habituation, social-interactive responses and capabilities, motor system, state organization and regulation, autonomic system). Due to specific infant states required for some items (e.g., the habituation package should be administered preferably when the infant is asleep), this study focused on exploring social-interactive responses and capabilities, motor system, state organization and regulation, and the autonomic system.

Infant Saliva Samples. At 6-8 weeks, before and after administration of the NBAS, infant saliva samples were collected with two SalivaBio's Infant's Swab (SIS) tubes. Samples were stored at -25ºC until analysis.

Salivary Cortisol Measurement

Maternal and infant salivary cortisol levels were determined using a highly sensitive enzyme-linked immunosorbent assay (ELISA) (commercial kit Salimetrics, LLC, State College, PA). Samples were tested in duplicate, and the mean was calculated (µg/dL). The lower limit of sensitivity was 0.007µg/dL. Any mean concentration with a coefficient of variation (%CV) higher than 15 was determined in duplicates for a second time to be included in downstream analysis. 47 maternal samples out of 508 (9.25%) and 8 infant samples out of 72 (11.11%) still had %CV > 15% after performing duplicates. These 55 samples were included in downstream analysis since 36 of the 47 maternal samples (76.60%) corresponded to the evening and night measures. Lower cortisol levels in the evening and night are commonly expected, so they will not be indicative of real differences among cortisol concentrations between duplicates.

Placental sample collection

Placenta samples were collected within fifteen minutes of placental delivery. Placentas were oriented with the fetal surface facing up, divided into four quadrants, and a piece of tissue was excised from each quadrant. The extracted tissue was required to maintain a minimum distance of 1.5 cm from both the center and edge of the placental disc. Samples needed to be representative of the whole placenta, excluding areas with thrombosis, infarcts, or other anomalies. The excised tissue pieces were transferred to a

container with cold, fresh, sterile saline solution and carefully rinsed to remove blood. Subsequently, samples were sectioned into three layers: the chorionic plate (fetal side), the chorionic villi, and the decidua (maternal side). Initially, all samples were stored at 4 ºC and later at -80 ºC.

Given that *HSD11B2* is predominantly expressed in the syncytiotrophoblast cells of the chorionic villi and in the epithelial cells of maternal decidua (Zhu et al., 2019), this study focused on exploring these two layers exclusively.

Placental DNA extraction

DNA was extracted from the chorionic villi and the from the maternal side samples and purified using the QIAGEN DNeasy Blood & Tissue kit. Thirty mg of placental tissue were used. Placental tissue was homogenized using a disruptor. Samples were then lysed using proteinase K and RNase. Buffering conditions were adjusted to provide optimal DNA-binding conditions and the lysate was loaded onto the DNeasy Mini spin column or the DNeasy 96 plate. During centrifugation, DNA was selectively bound to the DNeasy membrane as contaminants passed through. Remaining contaminants and enzyme inhibitors were removed in two efficient wash steps and DNA was eluted in buffer.

DNA methylation: Targeted bisulfite sequencing (TBS)

This method was developed by the Department of Translational Research in Psychiatry at the Max Planck Institute where these epigenetic analyses were conducted (37, 52).

Amplicon selection and amplification by PCR: We optimized the amplifications of 11 DNA regions, 5 for *FKBP5*, 3 for *HSD11B2* and 3 for *NR3C1*, covering 19, 116 and 80 CpGs respectively (primer sequences can be found in the supplementary material). In order to reduce cost and maximize the number of samples per sequencing run, triplicate bisulfite treatments were performed for each sample and then pooled to run one PCR amplification per amplicon (1). Overall, 200 ng to 500 ng of DNA was used per sample and bisulfite treated using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA). From this, 20 ng of bisulfite converted DNA was then used for each PCR amplification employing Takara EpiTaq HS Polymerase (Clontech, Saint-Germain-en-Laye, France) and 49 amplification cycles. PCR amplicons were then quantified with the Agilent 2200 TapeStation (Agilent Technologies, Waldbronn, Germany) and pooled in equimolar quantities for each sample. AMPure XP beads (Beckman Coulter, Krefeld, Germany) were used for a double size selection (200-500 bp) to remove primer dimers and high molecular DNA fragments.

Sequencing: Libraries were generated using the TruSeq DNA PCR-Free HT Library Prep Kit (Illumina, San Diego, CA) according to the manufacturer's instructions. Each library was quantified with the Qubit® 1.0 (Thermo Fisher Scientific Inc., Schwerte, Germany), normalized to 4 nM and pooled. Library concentration and fragment sizes were checked via Agilent's 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and quantitative PCR using the Kapa HIFI Library quantification kit (Kapa Biosystems, Wilmington, MA). Paired-end sequencing was performed on an Illumina MiSeq Instrument (Illumina, San Diego, CA) with their MiSeq Reagent Kit v3 (2 x 300-cycles) with the addition of 30% of PhiX Library.

Sequencing data processing: The quality of the sequencing reads was checked with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and Illumina adapter sequences were removed using Cutadapt (Marcel, 2011). Bismark (https://www.bioinformatics.babraham.ac.uk/projects/bismark/) was used for the alignment to a restricted reference limited to our PCR targets. In order to stitch paired-end reads, an in-house Perl script has been developed to remove the low-quality ends of the paired-end reads if they overlapped. The methylation levels for all CpGs, CHGs and CHHs were quantified using the R package methylKit 1.8.1 (3). The resulting DNA methylation calls were submitted to a 3-step quality control. First, PCR artifacts introducing false CpGs of low coverage at 0 or 100% methylation level were removed. Second, CHH methylation levels were analysed, and samples with insufficient bisulfite conversion rate (< 95%) were excluded. Finally, CpG sites with a coverage lower than 1,000 reads were removed. We excluded all CpGs with a call rate below 95% resulting in a final dataset of 19 CpG-sites for *FKBP5*, 91 CpG-sites for HSD11B2 and 36 CpG-sites for NR3C1. A list of all amplicons that passed quality control is given in Supplementary Material Table X and Additional File X. Four amplicons in PCRX and PCRY (FKBP5), PCRZ (HSD11B2) and PCRA (NR3C1) targeted duplicated

CpG-sites (PCR_1 at bp 127 equals PCR 2 at bp 29, PCR1 at bp 152 equals PCR 2 at bp 54 and PCR 1 at bp 205 equals PCR 2 at bp 107). All these amplicons passed quality control criteria and presented with association with the same effect direction in the statistical analyses. To remove redundancies, which can lead to inflated p-values, we used the mean methylation of the repeated amplicons in the final statistical analysis. Only CpG sites with available DNA methylation values in all subjects were included in downstream analysis. One maternal decidua sample was filtered out due to the high number of missing values (17.47%). Due to the overall hypomethylation of all *HSD11B2* gene regions (see Table 2 for details), CpG sites located in this gene were further excluded from analysis.

After QC, 19 CpG sites at the FKBP5 gene and 35 CpG sites at the NR3C1 genes as measured in 45 chorionic villi and 44 maternal decidua samples were included in the final analysis. Methylation levels across each of the 4 *FKBP5* intron 1 CpG sites, the 8 *FKBP5* intron 5 CPG sites, the 7 *FKBP5* intron 7 CPG sites and the 35 *NR3C1* exon 1D CpG sites were averaged to obtain an overall measure of methylation for each gene segment (see Table 2 for details).

Statistical analysis

Analyses were conducted using R Statistical Software (v4.2.1; R Core Team 2021) and SPSS 27.0 (IBM, Chicago, Illinois, USA) for Windows.

Maternal depressive symptoms were normally distributed. Salivary cortisol concentrations were not normally distributed and were log10 transformed to approach the normality. To index maternal cortisol diurnal activity during pregnancy, cortisol area under the curve with respect to the ground (AUCg) and to the increase (AUCi) were calculated for each trimester using the four log10 transformed cortisol concentrations from the wake-up time to bedtime. To index infant cortisol reactivity to the NBAS test, AUCg and AUCi were also calculated using the two log10 transformed cortisol concentrations collected before and after the test. AUCg indicates the mean cortisol levels over the course measurement (e.g., across the day or during the Brazelton test), while AUCi indicates oscillatory changes in cortisol levels during the measurement.

First, linear regression analyses with robust standard errors were used to assess the relationship between methylation in placental maternal side and chorionic villi of the four studied gene segments. Then, linear regression analyses with robust standard errors were also used to test the association between *FKBP5* intron 1, intron5 and intron 7 and *NR3C1* exon 1D overall methylation and i) maternal depressive symptoms, ii) diurnal cortisol levels (AUCg, AUCi) in each trimester of pregnancy, iii) infant gestational age and weight at birth, iv) infant cortisol during NBAS test (pre-test cortisol levels, AUCg, AUCi), and v) infant neurodevelopment (state organization, state regulation, social interactive, motor system, autonomous system). Each analysis was assessed for both the chorionic villi and the maternal side of the placenta separately. In all analyses, maternal age, maternal BMI and sex of the baby were included as covariates. Additionally, analyses that included infant variables collected during NBAS (neurobehavioral dimensions and cortisol) were also adjusted for the sum of infant gestational age at birth and infant age at NBAS in weeks. Linear regressions analyses for each individual CpG site assessed are depicted in the supplementary material.

All tests were two-tailed with significance defined as p-value <0.05.

Abbreviations

11βHSD2	11β-hydroxysteroid dehydrogenase type 2
AUCg	Area under the curve with respect to the ground
AUCi	Area under the curve with respect to the increase
BMI	Maternal Body Mass Index
DOHAD	Developmental Origin of Health and Disease
ELISA	Enzyme-linked immunosorbent assay
EPDS	Edinburgh Postnatal Depression Scale
GR	Glucocorticoid receptor
FKBP5	FK506 binding protein gene
HPA axis	Hypothalamic-Pituitary-Adrenal axis
NBAS	Neonatal Behavioral Assessment Scale
NR3C1	Glucocorticoid receptor gene
TBS	Targeted bisulfite sequencing

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Authors contribution

Conceptualization, A.C.-Q., H.P.-G., E.B., L.F; Methodology, A.C.-Q, E.E., N.S.M.-G., F.C., M.P.G.-P., L.F.; software, A.C.-Q.,

S.R.; Validation, A.C-Q., H.P.-G., S.S., J.L.M.-G.; formal analysis, A.C.-Q., H.P.-G. and S.R.; Investigation, A.C.-Q-, H.P.-G., E.E., S.S., M.R.-H., J.L.M.-G., F.C., M.P.G.-P., E.B., L.F. ; Resources, A.C.-Q-, H.P.-G., E.E., M.R.-H., J.L.M.-G., F.C., M.P.G.-P., E.B. and L.F.; Data curation, A.C.-Q., N.S.M-G., J.L.M.-G.; Writing—original draft preparation, A.C.-Q. and H.P.-G.; Writing—review and editing, A.C.-Q., H.P.-G., N.S.M-G., and L.F.; Visualization, A.C.-Q ., H.P.-G., N.S.M-G., and L.F.; Supervision, A.C.-Q ., H.P.-G., E.E., N.S.M.-G., S.R., S.S., M.R.-H., J.L.M.-G., F.C., M.P.G.-P., E.B. and L.F.; Project Administration, A.C.Q., E.E., F.C., M.P.G.-P., E.B. and L.F.; Funding acquisition, A.C.-Q., E.E., M.R.-H., F.C., M.P.G.-P., E.B. and L.F.

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Availability of data and material

All data are available for use by qualifed researchers following a standardised application procedure

Declarations

Ethics approval and consent to participate

The Intramural Study was approved by the Ethics Committee of the University of Barcelona, of the Hospital Clinic de Barcelona and the Hospital Universitario Central de Asturias and was conducted in full compliance with Helsinki declaration. At the beginning of the study, participants signed a written informed consent form.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to the sensitivity of the data provided by the included subjects and their consent but are available from the Study Boards on reasonable request.

Competing interest

The authors declare that they have no competing interests.

Author's information

Not available

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Tables

Table 1. Sociodemographic characteristics of the recruited pregnant women and their infants in the pilot study.

Variables	Total sample (n=45)	
Maternal age (years) (M, S.D., range)	32.47 (4.47) [21-40]	
Country of birth (n, %)	Spain	35 (77.8)
	Latin American	10 (22.2)
Level of education [a] (n, %)	Low	6 (13)
	Moderate	9 (20)
	High	30 (67)
1st trimester BMI (kg/m²) (M, S.D., range)	23.00 (4.24) [17.29-39.02]	
Depressive symptoms EPDS 1st Trimester [b, c] (M, S.D., range)	7.38 (4.56) [0-19]	
	Low Risk (*n, %*)	32 (78)
	High Risk (*n, %*)	13 (22)
Depressive symptoms EPDS 2nd Trimester [b, c] (M, S.D., range)	6.98 (5.08) [0-20]	
	Low Risk (*n, %*)	31 (74)
	High Risk (*n, %*)	11 (26)
Depressive symptoms EPDS 3rd Trimester [b, c] (M, S.D., range)	7.79 (5.35) [0-19]	
	Low Risk (*n, %*)	28 (65)
	High Risk (*n, %*)	15 (35)
Diurnal cortisol levels 1st trimester (M, S.D., range)	AUCg[d]	-12.44 (2.62) [-17.19 - -7.73]
	AUCi[e]	-4.83 (2.73) [-10.74 - -1.05]
Diurnal cortisol levels 2nd trimester (M, S.D., range)	AUCg[d]	-10.25 (3.09) [-17.90 - -3.11]
	AUCi[e]	-3.99 (4.11) [-9.61 – 10.33]
Diurnal cortisol levels 3rd trimester (M, S.D., range)	AUCg[d]	-10.14 (3.14) [-21.73 - -5.20]
	AUCi[e]	-2.30 (3.81) [-8.67 - -7.20]
Infant birth weight (kg)(M, S.D., range)	3.36 (0.44) [2.16-4.13]	
Gestational Age at delivery (weeks) (M, S.D., range)	39.85 (1.17) [36-42]	
Infant Sex	Male (*n, %*)	13 (28.9%)
	Female (*n, %*)	32 (71.1%)
Log Basal cortisol (pre-NBAS) (µg/dL) (M, S.D., range)	-0.93 (0.48) [-2.44 - -0.23]	
Infant cortisol levels across the NBAS test	AUCg[d] (M, S.D., range)	-0.4621 (0.64) [-3.52 - -0.05]
	AUCi[e] (M, S.D., range)	0.0856 (0.20) [-0.09 – 0.91]

[a] Low level of education includes no education, lower general secondary education and intermediate vocational education. Moderate level of education includes higher general secondary education, pre-university education and higher vocational education, High level of education includes university (degree, master or doctorate)

[b] n Edinburg Postnatal Depression Scale (EPDS): 1st trimester = 45, 2nd trimester = 42, 3rd trimester = 43

[c] Low depression risk includes EPDS scores lower than 11 for 1st and lower than 10 for 2nd and 3rd trimester of gestation while high depression risk included EPDS scores equal or higher to 11 for 1st and equal or higher than 10 for 2nd and 3rd trimester of gestation.

[d] Area Under the Curve with respect to the ground AUCg: indicates mean cortisol levels over the course of the measurement.

[e] Area Under the Curve with respect to the increase, AUCi: indicates mean cortisol levels over the course of the measurement.

Table 2. Average DNA methylation of the regions explored in the genes of interest, *FKBP5*, *NR3C1*, and *HSD11B2*, for the two placental layers: maternal side and chorionic villi

Variables	**Total sample** M, (S.D.) [range]	
% methylation, average all *FKBP5* intron 1 CpG sites	Maternal side	71.28 (3.38) [65.85-80.97]
	Chorionic Villi	72.02 (3.63) [64.23-80.38]
% methylation, average all *FKBP5* intron 5 CpG sites	Maternal side	42.29 (1.89) [38.31-46.37]
	Chorionic Villi	42.23 (1.55) [39.59-45.39]
% methylation, average all *FKBP5* intron 7 CpG sites	Maternal side	82.01 (2.77) [75.90-88.17]
	Chorionic Villi	82.49 (2.61) [77.67-87.81]
% methylation, average all *NR3C1* exon 1D CpG sites	Maternal side	14.04 (3.38) [3.10-57.58]
	Chorionic Villi	14.07 (13.38) [2.98-56.17]
% methylation, average all *HSD11B2* island 1 CpG sites	Maternal side	2.00 (0.64) [0.97-3.33]
	Chorionic Villi	2.11 (0.65) [0.81-4.21]
% methylation, average all *HSD11B2* island 2 CpG sites	Maternal side	0.64 (0.10) [0.47-0.94]
	Chorionic Villi	0.63 (0.10) [0.42-0.91]

Table 3. Adjusted regression analyses for variables of interest in conceptual model

	Exposure	Outcome	Chorionic Villi			Maternal Side		
	Maternal stress[a]		**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Mother	1st trimester	Depressive symptoms EPDS	FKBP5 intron 1	0.190	-0.079, 0.459	.162	0.132	-0.113, 0.377	.283
			FKBP5 intron 5	0.011	-0.103, 0.124	.850	-0.021	-0.162, 0.121	.764
			FKBP5 intron 7	-0.037	-0.251, 0.177	.729	0.102	-0.127, 0.330	.375
			NR3C1 exon 1D	0.438	-0.548, 1.424	.375	0.486	-0.524, 1.520	.348
	2nd trimester	Depressive symptoms EPDS	FKBP5 intron 1	-0.025	-0.270, 0.220	.839	-0.067	-0.291, 0.157	.547
			FKBP5 intron 5	0.014	-0.092, 0.121	.789	0.011	-0.122, 0.144	.871
			FKBP5 intron 7	-0.008	-0.186, 0.170	.925	0.107	-0.087, 0.300	.272
			NR3C1 exon 1D	0.424	-0.517, 1.366	.913	0.298	-0.714, 1.309	.554
	3rd trimester	Depressive symptoms EPDS	FKBP5 intron 1	-0.118	-0.362, 0.126	.333	-0.086	-0.311, 0.139	.441
			FKBP5 intron 5	0.025	-0.076, 0.125	.623	0.009	-0.118, 0.135	.892
			FKBP5 intron 7	-0.140	-0.312, 0.032	.108	-0.051	-0.246, .144	.598
			NR3C1 exon 1D	0.654	-0.177, 1.486	.120	-0.020	-0.246, .144	.966
	Diurnal cortisol levels (circadian rhythm)								
	1st trimester	AUCg	FKBP5 intron 1	-0.378	-0.876, 0.119	.131	-0.085	-0.557, 0.387	.715
			FKBP5 intron 5	0.033	-0.180, 0.246	.754	-0.124	-0.383, 0.134	.333
			FKBP5 intron 7	0.092	-0.271, 0.454	.609	0.038	-0.304, 0.381	.820
			NR3C1 exon 1D	**1.737**	**0.034, 3.441**	**.046***	1.243	-0.792, 3.279	.222
		AUCi	FKBP5 intron 1	-0.345	-0.865, 0.176	.186	-0.336	-0.810, 0.137	.157
			FKBP5 intron 5	-0.052	-0.272, 0.168	.632	-0.099	-0.369, 0.171	.460
			FKBP5 intron 7	**-0.369**	**-0.733, -0.005**	**.047***	-0.325	-0.671, 0.021	.064
			NR3C1 exon 1D	0.174	-2.858, 1.442	.852	-0.708	-0.369, 0.171	.506
	2nd trimester	AUCg	FKBP5 intron 1	-0.307	-0.669, 0.055	.094	0.002	-0.349, 0.353	.992
			FKBP5 intron 5	0.067	-0.093, 0.228	.401	0.080	-0.121, 0.281	.427
			FKBP5 intron 7	0.069	-0.186, 0.325	.584	-0.021	-0.300, 0.258	.879
			NR3C1 exon 1D	0.524	-0.859, 1.908	.447	0.214	-1.294, 1.723	.775
		AUCi	FKBP5 intron 1	-0.095	-0.394, 0.204	.524	-0.001	-0.278, 0.276	.994
			FKBP5 intron 5	0.050	-0.078, 0.178	.434	.0.30	-0.129, 0.190	.702
			FKBP5 intron 7	0.008	-0.196, 0.213	.935	0.050	-0.169, 0.269	.646
			NR3C1 exon 1D	-0.451	-1.553, 0.652	.413	-0.362	-1.546, 0.822	.539

	3rd trimester	AUCg	FKBP5 intron 1	-0.208	-0.596, 0.180	.285	-0.093	-0.451, 0.265	.603
			FKBP5 intron 5	0.001	-0.157, 0.159	.992	0.004	-0.194, 0.202	.967
			FKBP5 intron 7	0.027	-0.245, 0.299	.843	0.092	-0.200, 0.385	.525
			NR3C1 exon 1D	-0.827	-2.167, 0.512	.219	-0.442	-1.896, 1.012	.541
		AUCi	FKBP5 intron 1	-0.049	-0.395, 0.296	.774	-0.002	-0.317, 0.314	.991
			FKBP5 intron 5	-0.009	-0.148, 0.130	.898	-0.073	-0.245, 0.100	.399
			FKBP5 intron 7	0.178	-0.051, 0.407	.124	0.099	-0.154, 0.352	.431
			NR3C1 exon 1D	0.314	-0.883, 1.510	.598	-0.176	-1.459, 1.107	.782

Infant	**Birth Outcomes**								
	0 weeks	**FKBP5 intron 1**	Gestational Age	0.028	-0.131, 0.074	.579	**-0.108**	**-0.211, -0.005**	**.041***
		FKBP5 intron 5		-0.012	-0.262, 0.238	.925	-0.049	-0.240, 0.142	.608
		FKBP5 intron 7		-0.029	-0.177, 0.120	.698	**-0.177**	**-0.295, -0.060**	**.004****
		NR3C1 exon 1D		0.023	-0.004, 0.05	.099	0.015	-0.010, 0.04	.242
		FKBP5 intron 1	Birth Weigh	0.005	-0.036, 0.026	.740	0.015	-0.048, 0.018	.351
		FKBP5 intron 5		0.015	-0.060, 0.091	.680	0.010	-0.048, 0.069	.720
		FKBP5 intron 7		0.001	-0.044, 0.046	.979	-0.029	-0.069, 0.010	.139
		NR3C1 exon 1D		0.005	-0.003, 0.013	.231	0.002	-0.005, 0.011	.485
	Cortisol levels (during NBAS Scale)								
	8 weeks	FKBP5 intron 1	Basal Cortisol (Pre)	0.005	-0.049, 0.059	.844	-0.014	-0.072, 0.043	.616
		FKBP5 intron 5		0.084	-0.021, 0.189	.111	0.015	-0.075, 0.104	.741
		FKBP5 intron 7		0.028	-0.040, 0.096	.410	-0.007	-0.077, 0.063	.835
		NR3C1 exon 1D		0.007	-0.005, 0.020	.251	0.000	-0.016, 0.016	.998
		FKBP5 intron 1	Cortisol Secretion (AUCg)	-0.011	-0.073, 0.050	0.709	-0.032	-0.105, 0.040	.365
		FKBP5 intron 5		0.052	-0.093, 0.196	.470	0.025	-0.095, 0.144	.675
		FKBP5 intron 7		0.009	-0.083, 0.101	.842	0.015	-0.078, 0.108	.741
		NR3C1 exon 1D		0.009	-0.008, 0.026	.289	0.005	-0.015, 0.026	.599
		FKBP5 intron 1	Cortisol Reactivity (AUCi)	0.003	-0.017, 0.023	.766	0.009	-0.015, 0.033	.461
		FKBP5 intron 5		-0.008	-0.056, 0.040	.733	0.005	-0.034, 0.044	.798
		FKBP5 intron 7		0.005	-0.025, 0.035	.747	0.002	-0.029, 0.032	.900
		NR3C1 exon 1D		-0.002	-0.007, 0.004	.583	0.000	-0.006, 0.007	.896

		Neurodevelopmental milestones (NBAS Scale dimensions)							
Infant	**8 weeks**	FKBP5 intron 1	State Organization	-0.001	-0.340, 0.302	.993	0.008	-0.339, 0.354	.965
		FKBP5 intron 5		0.260	-0.447, 0.967	.460	0.331	-0.221, 0.883	.231
		FKBP5 intron 7		0.173	-0.254, 0.600	.417	0.205	-0.217, 0.627	.330
		NR3C1 exon 1D		0.065	-0.015, 0.145	.108	-0.002	-0.081, 0.078	.966
		FKBP5 intron 1	State Regulation	-0.078	-0.793, 0.636	.825	-0.062	-0.670, 0.547	.838
		FKBP5 intron 5		0.281	-1.339, 1.962	.736	-0.090	-1.444, 1.265	.894
		FKBP5 intron 7		0.069	-0.955, 1.092	.892	0.220	-0.812, 1.252	.667
		NR3C1 exon 1D		0.121	-0.161, 0.219	.209	0.029	-0.015, 0.145	.760
		FKBP5 intron 1	Social Interactive	0.234	-0.901, 1.369	.678	-0.248	-1.565, 1.069	.704
		FKBP5 intron 5		0.843	-1.820, 3.506	.525	0.323	-1.823, 2.469	.762
		FKBP5 intron 7		0.506	-1.111, 2.123	.529	0.231	-1.406, 1.867	.776
		NR3C1 exon 1D		0.279	-0.019, 0.576	.065	0.109	-0.191, 0.409	.466
		FKBP5 intron 1	Motor System	0.051	-0.318, 0.420	.781	-0.015	-0.438, 0.408	.944
		FKBP5 intron 5		-0.132	-1.000, 0.737	.760	0.166	-0.521, 0.852	.627
		FKBP5 intron 7		0.094	-0.433, 0.621	.719	-0.033	-0.558, 0.492	.900
		NR3C1 exon 1D		-0.002	-0.104, 0.099	.965	0.045	-0.051, 0.140	.349
		FKBP5 intron 1	Autonomous System	-0.067	-0.342, 0.208	.625	0.076	-0.242, 0.395	.629
		FKBP5 intron 5		0.391	-0.245, 1.027	.220	0.291	-0.220, 0.802	.256
		FKBP5 intron 7		0.094	-0.300, 0.487	.632	-0.059	-0.455, 0.338	.766
		NR3C1 exon 1D		0.007	-0.069, 0.082	.859	0.010	-0.064, 0.083	.791

[a] n Edinburg Postnatal Depression Scale (EPDS): 1st trimester = 45, 2nd trimester = 42, 3rd trimester = 43

p values: *p ≤ 0.05, **p ≤ 0.01

Figures

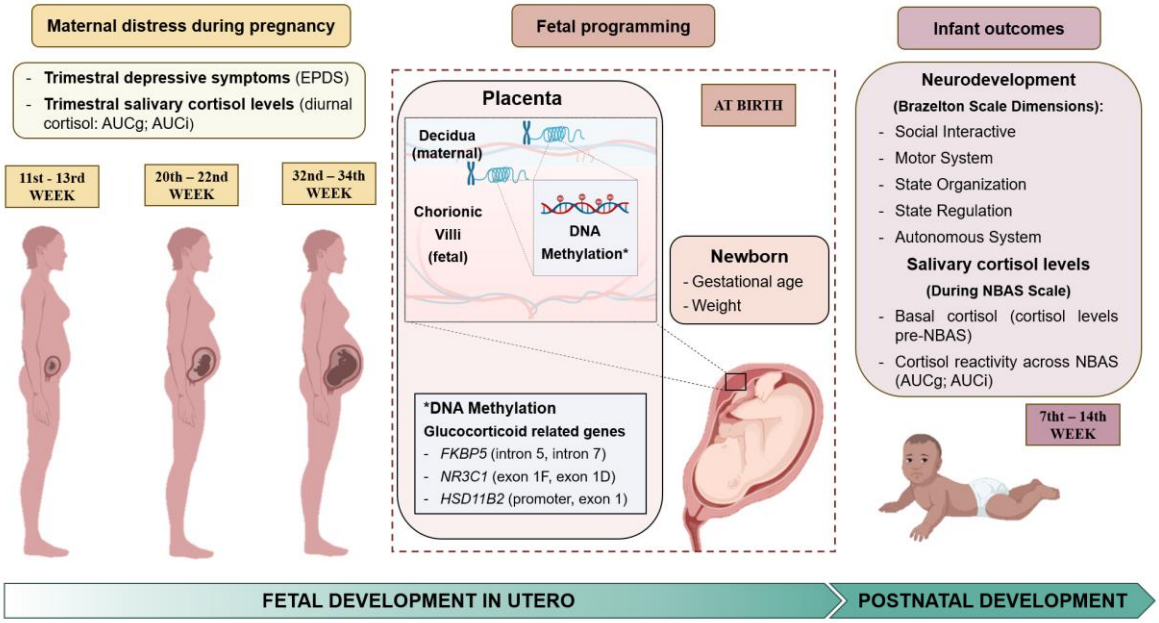

Figure 1. Summary of the study protocol. EPDS, Edinburgh Prenatal Depression Scale; AUCg, Area Under the Curve with respect to the ground; AUCi, Area Under the Curve with respect to the increase. Image created with BioRender.com.

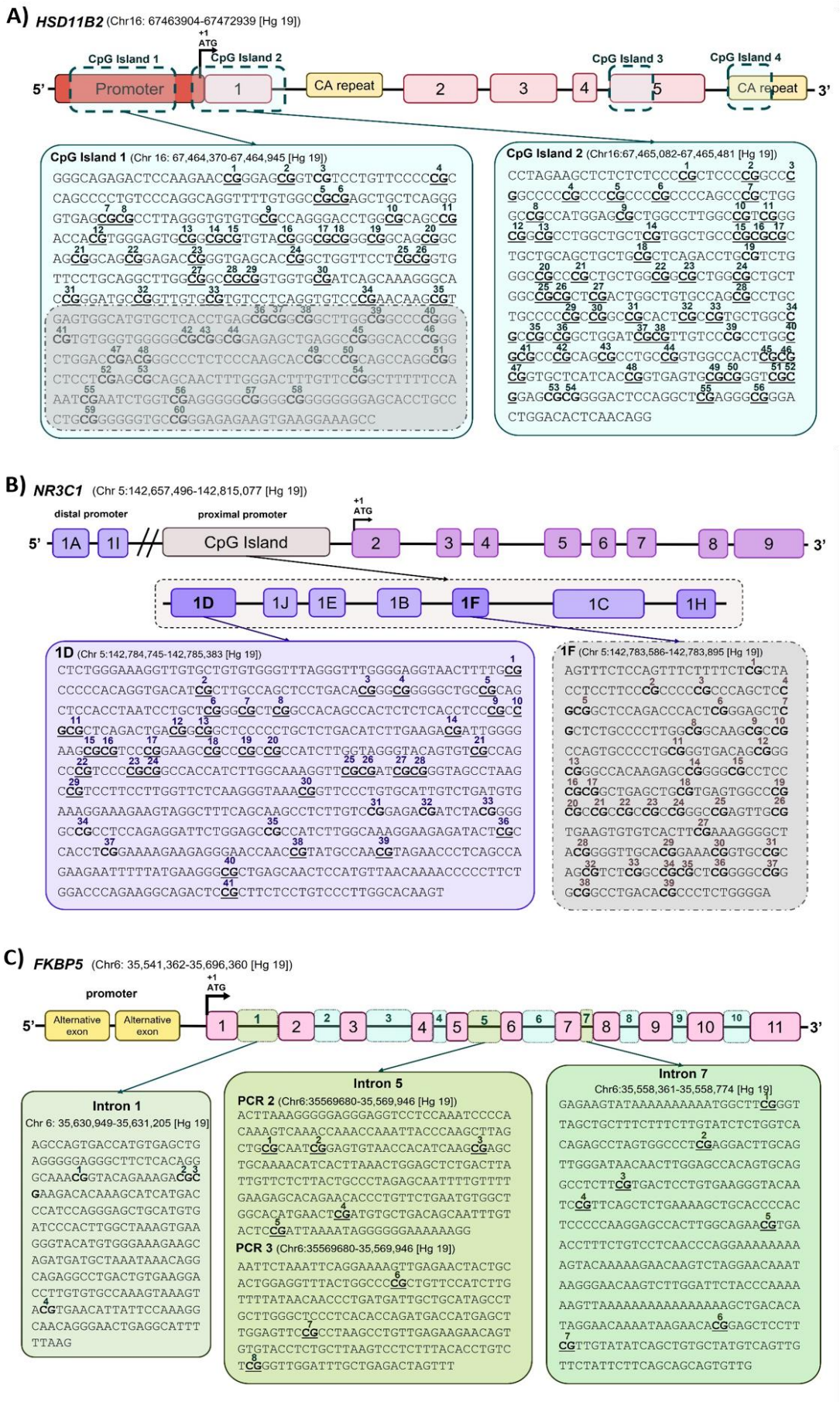
A) HSD11B2 (Chr16: 67463904-67472939 [Hg 19])
+1 ATG
CpG Island 1
CpG Island 2
CpG Island 3
CpG Island 4
5'
Promoter
1
CA repeat
2
3
4
5
CA repeat
3'
CpG Island 1 (Chr 16: 67,464,370-67,464,945 [Hg 19])
CpG Island 2 (Chr16:67,465,082-67,465,481 [Hg 19])
B) NR3C1 (Chr 5:142,657,496-142,815,077 [Hg 19])
distal promoter
proximal promoter
+1 ATG
5'
1A
1I
CpG Island
2
3
4
5
6
7
8
9
3'
1D
1J
1E
1B
1F
1C
1H
1D (Chr 5:142,784,745-142,785,383 [Hg 19])
1F (Chr 5:142,783,586-142,783,895 [Hg 19])
C) FKBP5 (Chr6: 35,541,362-35,696,360 [Hg 19])
promoter
+1 ATG
5'
Alternative exon
Alternative exon
1
2
3
4
5
6
7
8
9
10
11
3'
Intron 1
Chr 6: 35,630,949-35,631,205 [Hg 19]
Intron 5
PCR 2 (Chr6:35569680-35,569,946 [Hg 19])
PCR 3 (Chr6:35569680-35,569,946 [Hg 19])
Intron 7
Chr6:35,558,361-35,558,774 [Hg 19]

Figure 2. Sequencing strategy. Gene structure of the three genes included in this study in humans. Solid black line boxes with a number represent the different exons and the 5'–3' orientation goes from left to right. A) *HSD11B2* gene is located in the chromosome 16q22. Solid black line boxes with a number and colored in pink represent the different exons. The *HSD11B2* gene contains five exons (pink), two tandems of repeats of cytosine-adenine (yellow) and four GC islands (blue). 35 sites for island 1 and 56 CpGs for island 2 were analyzed. 25 CpG sites of island 1 did not pass the amplification and were not included in the analyses (gray) B) *NR3C1* gene is located in the chromosome 5. The *NR3C1* gene is composed for multiple exons: two distal in the promoter region (1_A and 1_I), seven in the proximal promoter (1_D, 1_J, 1_E, 1_B, 1_F, 1_C, 1_H) (violet), and nine common exons (lavender). 41 CpG sites for exon 1_D were included in the analyses. 39 CpG sites for exon 1_F did not pass the amplification and were not included in the analyses (gray). C) *FKBP5* gene is located in the chromosome 6. The FKBP5 gene is composed for two alternative exons in the proximal promoter region (yellow), eleven common exons (pink) and ten introns (blue), which usually contain CpG sites. 4CGs sites for intron 1, 8CGs sites for intron 5 and 7 CGs sites for intron 7 were included in the analyses.

UNIVERSITAT DE BARCELONA

Dr. Lourdes Fañanás Saura
Section of Zoology and Biological Anthropology
Dept. Evolutionary Biology, Ecology and Environmental Sciences
Faculty of Biologia, Universitat de Barcelona

Supervisor's report on the contribution of the PhD applicant to the article.

Prof. Dr. Lourdes Fañanás Saura, full professor at the Department of Evolutionary Biology, Ecology and Environmental Sciences of the Faculty of Biology (Universitat de Barcelona) and supervisor of the present doctoral thesis by Águeda Castro Quintas, hereby certifies that the participation of the PhD applicant in the article "*Distress during pregnancy and placental epigenetic patterns at glucocorticoid related genes: a pilot study in Spanish primiparous women*" included the following tasks:

- Participation in the conception and design of the study
- Recruitment and evaluation of the included participants
- Coordination of the project
- Statistical analyses and data interpretation
- Writing of the first manuscript draft
- Critical revision of the article for intellectual content

Signed by Prof. Lourdes Fañanás

Barcelona, 5th December 2023

3.3. Exploring the impact of COVID-19 on newborn neurodevelopment: a pilot study

Rosa Ayesa-Arriola*, **Águeda Castro-Quintas***, Victor Ortiz-Garcia de la Foz, Margarita Miguel-Corredera, Nerea San Martin-Gonzalez, Nancy Murillo-Garcia, Karl Neergaard, Lourdes Fañanás, Isabel de las Cuevas-Terán

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Exploring the impact of COVID-19 on newborn neurodevelopment: a pilot study

Rosa Ayesa-Arriola[1,3,4,6]✉, Águeda Castro Quintas[2,3,6], Víctor Ortiz-García de la Foz[3,4], Margarita Miguel Corredera[4], Nerea San Martín González[2], Nancy Murillo-García[1,4], Karl Neergaard[4], Lourdes Fañanás Saura[2,3] & Isabel de las Cuevas-Terán[1,5]

The COVID-19 pandemic can seize the opportunity to explore the hypothesis of prenatal exposure to viral infections increases the risk for neurodevelopmental disorders. Advancing our knowledge in this regard would improve primary prevention of mental disorders in children. For this pilot study, six-week-old infants born to mothers exposed (n = 21) or unexposed (n = 21) to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) were assessed in Santander-Cantabria (Spain) using the Neonatal Behavioral Assessment Scale (NBAS). Groups comparisons were performed to explore the effects that infection and timing of exposure (in terms of the three trimesters of pregnancy). The infants' competencies and performances on the NBAS were generally similar in the exposed and unexposed to SARS-CoV-2 groups. The most significant difference found was a less optimally response to cuddliness (item on the state regulation domain) particularly in infants born to mothers exposed in the third trimester of pregnancy, and in pull-to-sit (item on the motor system domain). Although our interpretations must be careful, these preliminary results highlight the possible association between prenatal SARS-CoV-2 exposure and poorer development in motor skills and infant interactive behavior. Further longitudinal studies are needed to explore these relationships and disentangle the biological mechanisms implicated.

Prenatal exposure to viral infection has been related to later development of neuropsychiatric disorders[1]. This has been observed after historical pandemics, such as the 1918–1919 Spanish influenza[2]. However, studies addressing the association between prenatal viral infections and mental disorders, such as the viral hypothesis of schizophrenia[3], have not been able to confirm a causal relationship. In terms of the pandemic of the 2019 Coronavirus Disease (COVID-19), the mechanisms related to the maternal immune response have been proposed to interfere with fetal brain development[4]. The current conditions of the COVID-19 pandemic, including the psychiatric effects, such as worry and anxiety in expectant mothers[5], and neurological sequelae, such as headache and difficulties with concentration[6], provide a unique opportunity to determine the associations between fetal exposure to maternal SARS-CoV-2 and postnatal neurodevelopment[4,7].

Several studies addressing the implications of COVID-19 disease during pregnancy have focused on the effects of the virus on obstetric and delivery complications[8,9]. A recent review indicates that there may be a possible relationship between prenatal SARS-CoV-2 infection and pre-term birth, intrauterine growth restriction and low birth weight[10]. Considering that these neonatal outcomes have been previously associated with a higher vulnerability for later neurodevelopmental disorders[11,12], children born from SARS-CoV-2 infected mothers could be considered at higher risk along lifetime.

Prenatal infections have been associated with the appearance of unspecific early risk markers, reflected in cognitive, emotional and behavioural deviations in the development of the newborn[13]. Upper respiratory infections during early and middle pregnancy seem to be associated with greater behavioural issues among later offspring[14]. Meanwhile, previous birth cohort studies have identified relationships between maternal immune activation in the third trimester of pregnancy and deviations in the expected behavioural and cognitive scores at 14 months[15].

[1]University of Cantabria, Santander, Spain. [2]Department of Evolutionary Biology, Ecology and Environmental Sciences (BEECA), Faculty of Biology, University of Barcelona, Institute of Biomedicine of the University of Barcelona (IBUB), Barcelona, Spain. [3]Centro Investigación Biomédica en Red de Salud Mental (CIBERSAM), Madrid, Spain. [4]Mental Illnesses Research Unit, Marqués de Valdecilla Research Institute, IDIVAL, Santander, Spain. [5]Neonatal Unit, Pediatric Service, University Hospital Marqués de Valdecilla, Santander, Spain. [6]These authors contributed equally: Rosa Ayesa-Arriola and Águeda Castro Quintas. ✉email: rayesa@humv.es

The evaluation of neonatal behavior has an enormous value for predicting later developmental trajectories[16]. It has been well established that, in the first weeks of life, newborns' brains are prepared to actively react to the environment, but also to regulate their inner mental states[17]. Different studies indicate that neonates develop some "rudimentary" abilities for regulating their emotions and their social interactions by integrating sensory, motor and kinetic information[18–20]. The Neonatal Behavioral Assessment Scale (NBAS)[21] evaluates innate characteristics and behavioral responses in the offspring, with the focus on the interaction with environment and coping strategies.

In this preliminary analysis of a larger longitudinal study conducted in Santander-Cantabria (Spain), we sought to explore the consequences of prenatal exposure to SARS-CoV-2 in early neurodevelopmental outcomes of post-birth offspring. The NBAS scale was used to compare the performance of six-week-old infants that were both exposed (clinical group) and unexposed (control group) prenatally to SARS-CoV-2.

Materials and methods

Study design and participants. The present pilot study included infants enrolled in COGESTCOV-19 (Cohort of COVID-19 pregnant women and newborns: study of biological and psychological aspects related to neurodevelopment). All mother-infant dyads were enrolled during pregnancy. For each dyad exposed to SARS-CoV-2 infection during pregnancy, 1 unexposed dyad, defined as the absence of maternal SARS-CoV-2 infection during pregnancy and at delivery, was selected and invited to participate based on infant gestational age at birth and date of birth within a 4-week window. Study procedures approved by the Marqués de Valdecilla University Hospital Review Board (internal code 2020.190). This research was performed in accordance with relevant guidelines/regulations, including the Declaration of Helsinki, and all participants signed an informed consent prior to their inclusion in the study. This pilot study included data from 42 mothers and infants (n = 21 exposed, n = 21 unexposed). Primiparous and multiparous women were included.

Interviews with pregnant women. Participants in COGESTCOV-19 were reached through midwives and obstetricians who voluntary agreed to inform pregnant women about the research project. In addition, the information was published on social media platforms (Facebook, Instagram and Twitter). In the first visit, prior to both blood test and interview, we obtained written informed consent. Participants were informed that their data and those of their offspring would be kept confidential because all identifiers would be removed from the data to enable anonymity.

For data collection, we used semi-structured interviews to allow for a detailed exploration of the participants' situations and experiences. During the prenatal interview, mothers were asked for their sociodemographic data and their medical and psychological status. During the postnatal interview, information was obtained regarding their delivery and their infant's neonatal outcomes. We developed the interview by critically reviewing the literature. We used open ended questions to avoid limiting discussion through prior categorization or by structuring interviews around the researcher's ideas and assumptions The interview was administrated by expert psychologists.

All of the women in the exposed group showed positive results for SARS-CoV-2 polymerase chain reaction tests. In addition, in terms of the study protocol, blood serological tests were performed in order to confirm the absence of antibodies for SARS-CoV-2 in the control group. Infection severity was categorized as previously described by Gandhi et al.[22], attending that patients with mild disease typically recover at home, while patients with moderate or severe SARS-CoV-2 infection are usually hospitalized for observation and supportive care.

Neurodevelopmental assessment in infants. Infants in this pilot study, born between December 2020 and November 2021, were recruited for COGESTCOV-19 as part of a separate protocol to examine the association between prenatal SARS-CoV-2 infection exposure and newborn 6-weeks neurodevelopment.

Infants' neurobehavioral functioning was evaluated using the Neonatal Behavioral Assessment Scale (NBAS)[21] at six-weeks old. The NBAS is an scale that assesses the capacity of the newborn to interact and respond to the environment, according to six subdomains: habituation, orientation, motor system, state organization and regulation, autonomic stability and reflexes.

This examination was conducted in a particular sequence according to the authors' instructions, midway between feedings in a quiet and semi-darkened room with a temperature of 22–27 °C. The NBAS was scored immediately after being performed by a trained, certified and independent reliable examiner. Because the administration of some items required the infant to be in specific states to be administered (e.g., the habituation package should be administered preferably in state 1 (deep sleep) or 2 (light sleep), and the orientation package must be administered while the infant is in state 4 (alert state), we anticipated that some items could not be completed. The test was administrated by a neonatologist and pediatrics expert certified to administrate the NBAS.

Statistical analysis. Chi-Square and t-tests were used to compare mothers and infant's information on the prenatally exposed to SARS-CoV-2 (clinical group) and unexposed (control group). Analysis of covariance (ANCOVAs) with mother age, gestational age, age at NBAS assessment and infants sex as covariates were performed to compare clinical and control groups as well as clinical groups attending trimester of pregnancy in the moment of the infection. Post hoc analyses were performed on all main effects (Bonferroni corrected $p < 0.05$). R version 4.1.2 (2021-11-01) was used for statistical analysis. All statistical tests were two-tailed, and significance was set at the 0.05 level.

Results

This pilot study focused on comparing two groups (prenatally exposed or unexposed to SARS-CoV-2) of 21 infants each, aged 6 weeks, matched 2 by 2 according to mothers age and other controllable sociodemographic information (Table 1). The two groups did not differ with respect to the children's characteristics, such as gestational age, length and birth weight. Noticeably, mothers did differ on educational level ($p < 0.001$), with lower scores on exposed compared to unexposed.

The means of both groups were calculated and compared for the 35 items of the NBAS linked to habituation, social interactive, motor system, state organization, state regulation, and autonomic and autonomic nervous system clusters. The infants' competencies and performances were generally similar in the clinical group and in the control group (Table 2). Statistically significant differences were found for the following items: cuddliness ($F = 7.750$; $p = 0.009$) (the ability to "let themselves go" in the arms of an adult and relax in a comforting position), which revealed that the unexposed group had significantly higher scores than those in the exposed group; and pull-to-sit ($F = 3.678$; $p = 0.063$) (hold the head in line with the trunk during the pull-to-sit maneuver) pointing towards higher scores for the unexposed group. Those items respectively belong to state regulation and motor system NBAS domains.

A multiple comparisons analysis was conducted to identify whether the timing of exposure, consisting of the first, second and third trimester of pregnancy, had an effect on neurodevelopment. Results showed that infants exposed to infection on the second trimester of pregnancy presented significantly worse habituation on the response to bell ($F = 5.737$; $p = 0.018$) (response to a small bell that reflects infants' aptitude for getting used to external stimulation). In addition, those infants exposed to infection on the third trimester of pregnancy presented a poorer response to cuddliness ($F = 3.027$; $p = 0.043$) (Table 3).

Discussion

Although the results of this pilot study are preliminary and should be interpreted with caution, they are very interesting and deserve attention. The observation that the performance in the item cuddliness has a lower score in infants whose mothers were exposed to SARS-CoV-2, and more specifically in those exposed during the third trimester of pregnancy, is particularly interesting. From birth, newborns recognize and create structures of social interaction with others that are critical for optimal brain development[23,24]. Social interactions, especially with their caregivers, are based on face-to-face exchanges and close physical contact that lead to attachment[25]. Previous studies have related a less optimal response to cuddliness in infants of depressed mothers, suggesting that those infants were more aroused[26]. It is interesting to note that none of the infants whose mothers were exposed to SARS-CoV-2 in the first trimester of pregnancy completed the item response to bell. This item requires that

	Cases		Controls				
	N = 21		N = 21				
	Mean	SD	Mean	SD	Statistic	Value	*p*
Mother age, y	34.7	3.8	35.1	3.6	t	− 0.378	0.708
Gestational age, w	39.9	1.7	40.0	1.0	W	255.0	0.392
Mother education, y	13.4	4.6	19.6	3.0	t	− 5.136	< 0.001
Weight	3257.9	541.7	3456.9	349.2	t	− 1.415	0.165
Length, cm	49.9	2.1	50.5	2.3	t	− 0.903	0.372
Age at Brazelton assessment, w	6.4	1.5	6.2	0.4	W	208.5	0.771
	N	%	N	%			
Sex (Boy)	12	57.1	10	47.6	X^2	0.382	0.537
Delivery hospitalization (Scheduled)	5	27.8	4	21.1	Fisher	0.227	0.714
Birth					Fisher	0.734	0.759
Natural	11	55.0	8	42.1			
Induction	6	30.0	8	42.1			
C-section	3	15.0	3	15.8			
Primiparous	14	66.7	14	66.7	X^2	0.000	1.000
Vaginal delivery	20	95.2	19	90.5	Fisher	0.359	1.000
Full term	19	95.0	19	100.0	Fisher	0.975	1.000
Infection term							
1st	3	14.3					
2nd	8	38.1					
3rd	10	47.6					
Infection severity							
Mild	20	95.2					
Moderate	1	4.8					

Table 1. Pilot sample characteristics.

	Cases		Controls				
	N = 21		N = 21				
	Mean	SE	Mean	SE	Statistic	Value	p
Habituation							
1—Response decrement to light	5.8	1.0	6.0	0.9	F	0.042	0.841
2—Response decrement to rattle	3.8	0.9	4.9	0.9	F	0.722	0.407
3—Response decrement to bell	5.2	1.2	5.0	1.0	F	0.023	0.881
4—Response decrement to tactile stimulation of the foot	4.8	1.5	5.7	1.5	F	0.135	0.732
Orientation							
5—Animate visual orientation	4.7	0.6	5.4	0.6	F	0.681	0.415
6—Animate visual and auditory orientation	5.7	0.6	6.6	0.6	F	0.991	0.327
7—Inanimate visual orientation	3.5	0.7	3.5	0.7	F	0.005	0.946
8—Inanimate visual and auditory orientation	5.1	0.6	4.2	0.6	F	1.050	0.314
9—Animate auditory orientation	6.4	0.6	5.7	0.6	F	0.593	0.448
10—Inanimate auditory orientation	6.3	0.5	5.3	0.5	F	2.260	0.143
11—Alertness	5.6	0.6	5.2	0.5	F	0.272	0.606
Motor System							
12—General tone	8.7	0.3	9.2	0.3	F	1.445	0.237
13—Motor maturity	6.2	0.6	6.3	0.6	F	0.038	0.846
14—Pull-to-sit	4.8	0.4	6.1	0.4	F	3.678	0.063
15—Defensive movement	7.1	0.5	6.6	0.5	F	0.515	0.478
16—Activity	7.9	0.6	7.0	0.7	F	0.955	0.335
State Organization							
17—Peak of excitement	5.6	0.6	4.5	0.6	F	1.963	0.170
18—Rapidity of build-up	6.1	0.6	5.9	0.6	F	0.072	0.790
19—Irritability	4.1	0.5	4.8	0.5	F	0.662	0.421
20—Lability of states	5.8	0.6	4.8	0.6	F	1.608	0.213
State Regulation							
21—Cuddliness	4.4	0.3	5.6	0.3	F	7.750	0.009
22—Consolabity	5.1	0.7	4.9	0.6	F	0.025	0.874
23—Self-quieting	5.1	0.6	4.4	0.6	F	0.609	0.441
24—Hand-to-mouth	4.1	0.7	3.8	0.7	F	0.088	0.768
Autonomic Stability							
25—Tremolousness	8.1	0.6	8.3	0.6	F	0.075	0.785
26—Startles	7.0	0.5	6.7	0.5	F	0.255	0.616
27—Lability of skin	6.9	0.6	7.2	0.6	F	0.152	0.698
Smiles							
28—Smiles	2.1	0.4	2.0	0.4	F	0.124	0.727
Supplementary Items							
29—Quality of alertness	5.2	0.5	5.8	0.5	F	0.827	0.369
30—Cost of attention	5.5	0.4	5.0	0.4	F	0.665	0.420
31—Examiner facilitation	5.4	0.4	4.9	0.4	F	0.935	0.340
32—General irritability	5.8	0.5	5.4	0.5	F	0.331	0.569
33—Robustness and endurance	6.6	0.5	5.6	0.5	F	1.581	0.217
34—State regulation	6.9	0.4	7.2	0.4	F	0.553	0.462
35—Examiner's emotional response	7.3	0.5	8.1	0.5	F	1.595	0.215

Table 2. NBAS items comparisons between cases and controls. Using mother age, gestational age, age at NBAS assessment and sex as covariates, and Bonferroni adjusted.

the children be in state 1 (deep sleep) in order to initiate the administration. This could be due to an excess of excitation that is not triggered by the environment but is residual. One possible interpretation of this detail is that the level of arousal of the cases group exposed during the first and second trimester of pregnancy was higher than in those exposed in the third trimester that were similar to controls, impairing a proper state regulation. In addition, it would be possible that postnatal influences, such as maternal behavior linked to a variety of changes related to SARS-CoV-2 exposure and pandemic situation in general, could also contribute and be particularly relevant for this finding.

Those findings points towards that those infants prenatally exposed to SARS-CoV-2 could present difficulties on state regulation, that in accordance with Belot et al.[27] could be partially explained by physiological alterations

	1st Term		2nd Term		3rd Term		Control					
	N = 3		N = 8		N = 10		N = 21					
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Statistic	Value	p	Post-Hoc
Habituation												
1—Response decrement to light	3.5	2.5	3.6	1.7	8.2	1.4	5.9	0.9	F	1.664	0.210	
2—Response decrement to rattle	0.7	2.5	3.8	1.7	4.9	1.4	4.8	0.9	F	0.892	0.467	
3—Response decrement to bell			2.1	1.3	8.4	1.3	4.9	0.8	F	5.737	0.018	2 < 3 *
4—Response decrement to tactile stimulation of the foot			-4.5	5.6	6.7	1.7	6.0	1.3	F	1.531	0.348	
Orientation												
5—Animate visual orientation	3.4	1.5	4.1	1.0	5.7	0.9	5.4	0.6	F	0.881	0.462	
6—Animate visual and auditory orientation	4.7	1.7	5.0	1.1	6.5	1.0	6.5	0.6	F	0.717	0.550	
7—Inanimate visual orientation	4.3	1.9	2.9	1.2	3.7	1.1	3.5	0.7	F	0.148	0.930	
8—Inanimate visual and auditory orientation	4.7	2.1	5.7	1.1	4.8	1.0	4.3	0.6	F	0.485	0.696	
9—Animate auditory orientation	4.0	1.9	5.5	1.0	7.8	1.0	5.7	0.6	F	1.477	0.244	
10—Inanimate auditory orientation	6.7	1.3	6.5	0.8	6.0	0.8	5.3	0.5	F	0.797	0.506	
11—Alertness	5.8	1.6	5.2	1.1	5.8	0.9	5.2	0.6	F	0.161	0.922	
Motor system												
12—General tone	7.5	0.9	9.1	0.5	8.7	0.5	9.2	0.3	F	1.346	0.276	
13—Motor maturity	5.3	1.8	5.7	1.0	6.9	1.0	6.3	0.6	F	0.283	0.837	
14—Pull-to-sit	5.4	1.3	4.0	0.7	5.4	0.7	6.0	0.4	F	2.036	0.127	
15—Defensive movement	7.4	1.3	6.8	0.8	7.1	0.8	6.6	0.5	F	0.218	0.883	
16—Activity	7.6	1.9	8.1	1.1	7.9	1.0	7.0	0.7	F	0.321	0.810	
State organization												
17—Peak of excitement	7.2	1.6	6.7	0.9	4.2	0.9	4.6	0.6	F	2.215	0.104	
18—Rapidity of build-up	7.6	1.7	6.6	1.0	5.1	1.0	5.9	0.6	F	0.609	0.614	
19—Irritability	3.7	1.6	4.6	0.9	3.9	0.9	4.8	0.6	F	0.364	0.780	
20—Lability of states	7.0	1.6	6.7	0.9	4.6	0.9	4.8	0.6	F	1.633	0.200	
State regulation												
21—Cuddliness	5.0	0.9	4.6	0.5	3.9	0.5	5.7	0.3	F	3.027	0.043	3 < 4 *
22—Consolability	6.9	2.1	4.8	1.1	4.7	1.0	5.0	0.6	F	0.276	0.842	
23—Self-quieting	5.3	1.7	6.3	0.9	3.7	1.0	4.4	0.6	F	1.422	0.254	
24—Hand-to-mouth	5.2	2.0	4.7	1.1	3.1	1.1	3.8	0.7	F	0.429	0.734	
Autonomic stability												
25—Tremolousness	7.0	1.7	7.6	0.9	8.9	0.9	8.3	0.6	F	0.481	0.697	
26—Startles	8.3	1.4	6.8	0.8	6.7	0.8	6.7	0.5	F	0.432	0.731	
27—Lability of skin	9.1	1.5	7.8	0.9	5.3	0.8	7.3	0.5	F	2.088	0.120	
Smiles												
28—Smiles	3.5	1.1	1.6	0.6	2.1	0.6	2.0	0.4	F	0.860	0.471	
Supplementary items												
29—Quality of alertness	4.9	1.4	4.9	0.8	5.5	0.8	5.8	0.5	F	0.385	0.764	
30—Cost of attention	6.4	1.1	5.1	0.6	5.5	0.6	5.0	0.4	F	0.576	0.635	
31—Examiner facilitation	5.9	1.1	5.8	0.6	4.9	0.6	4.9	0.4	F	0.678	0.572	
32—General irritability	7.1	1.4	6.3	0.8	4.8	0.8	5.4	0.5	F	0.995	0.407	
33—Robustness and endurance	5.9	1.6	6.7	0.9	6.7	0.9	5.6	0.6	F	0.559	0.645	
34—State regulation	5.9	1.1	7.0	0.6	7.1	0.6	7.2	0.4	F	0.466	0.708	
35—Examiner's emotional response	7.3	1.3	8.0	0.7	6.6	0.7	8.1	0.5	F	1.092	0.366	

Table 3. NBAS items comparisons attending trimester of pregnancy during COVID-19 infection. Using mother age, gestational age, age at NBAS assessment and sex as covariates, and Bonferroni adjusted.

in the nervous system. This higher level of arousal could be associated with greater activation of infants' hypothalamic–pituitary–adrenal (HPA) axis, and relates to stress exposure during critical periods, which in turn increases the risk on psychopathology[28]. Noticeably, Provenzi et al.[29] observed alterations in the regulatory capacity of 3-month-old infants whose mothers were pregnant during the COVID-19 pandemic compared to infants whose mother were pregnant previous to this period. This finding highlights the importance of psychosocial stress for regulating children's HPA axis functioning, especially in the third trimester of pregnancy when this system has already been formed. These results are in line with findings from Shuffrey et al.[30], who observed social and motor developmental alterations in children born from mothers pregnant during COVID-19 pandemic period, but not

in those exposed prenatally to SARS-CoV-2. Biological data, such as cortisol levels obtained from saliva samples in those infants, will be very useful to explore the functioning of their HPA axis.

Another result that worth considering is that of the poorer performance on pull-to-sit item, which is related to head control, in infants whose mothers were exposed to SARS-CoV-2. Vulnerabilities in infants' fine and gross motor skills may have significant consequences for later motor development[31]. Specifically, poor postural control during pull-to-sit has been suggested as a predictor of developmental disruption[32]. Previous studies have linked childhood motor deficits with the risk of developing autism-like disorders[33] and adult schizophrenia spectrum disorders[31]. Lower scores in our cases could be indicating a certain motor immaturity or what have been termed neurological soft signs (NSS). NSS entail minor motor and sensory differences, a common feature in children with neuropsychiatric disorders[17,34], that could be considered a proxy marker for underlying neuropsychological deficits.

This study has several limitations. First, since it is a pilot study, the sample is relatively small (only 21 exposed and 21 unexposed). However, as it is an ongoing project, more participants are being recruited, adding to the current sample in later analyses. It should be noted, however, that sample sizes found in the literature related to the NBAS scale are not much larger than our current sample. This is possibly due to the extensive training required to administer the NBAS scale. Second, none of the cases in the study presented with severe COVID-19 symptoms. This circumstance may either be due to a lack of serious cases in our community at the time, or that the few severe cases that might have occurred suffered too much to be enrolled in a research study of this kind. Third, attending that COVID-19 vaccination safety for pregnant people was not confirmed until august 2021, when most of the women in this pilot study have been already evaluated, information about vaccine status and vaccine attitude will be the considered in coming studies[35]. Finally, pairing was not optimal for years of education since, in line with results reported by Shuffrey et al.[30], significantly differed between exposed and unexposed women. A possible interpretation is that voluntary participation was related to higher education in the unexposed group while negatively related to enroll in the exposed group. In addition, to the already known unequal distribution of the disease, which in already marginalised populations present higher rates and risks[36].

Conclusions

Our findings suggest that prenatal exposure to SARS-CoV-2 infection could be related to alterations in different neurodevelopmental domains, particularly those related to interactive behavior and motor development. The timing of exposure to the infection could play an important role on the severity and type of these deficits. It is essential to continue studying these cohorts of children over time with a particular focus on primary prevention and early intervention.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

R.A.A., L.F.S., and I.C.T. designed the study. R.A.A., A.C.Q., M.M.C., N.M.G., and I.C.T. recruited and assessed the participants. M.M.C. and V.O.F. prepared the database, statistical analysis, and final tables. R.A.A., A.C.Q., N.S.M.G., and I.C.T. wrote the original draft. R.A.A. and K.N. interpreted the results, edited the manuscript, and approved final corrections. R.A.A. and L.F.S. administrated the project. All authors have read and agreed to the published version of the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to R.A.-A.

UNIVERSITAT DE BARCELONA

Dr Lourdes Fañanás Saura
Section of Zoology and Biological Anthropology
Dept. Evolutionary Biology, Ecology and Environmental Sciences
Faculty of Biologia, Universitat de Barcelona

Supervisor's report on the contribution of the PhD applicant to the article.

Prof. Dr. Lourdes Fañanás Saura, full professor at the Department of Evolutionary Biology, Ecology and Environmental Sciences of the Faculty of Biology (Universitat de Barcelona) and supervisor of the present doctoral thesis by Águeda Castro Quintas, hereby certifies that the participation of the PhD applicant in the article "*Exploring the impact of COVID-19 on newborn neurodevelopment: a pilot study*" included the following tasks:

- Participation in the conception and design of the study.
- Participation in the statistical analyses and data interpretation.
- Critical revision of the article for intellectual content.

Signed by Prof. Lourdes Fañanás

Barcelona, 5th December 2023

Section II – Childhood maltreatment, psychosocial stress and responses of the endocrine and immune systems

3.4. Childhood maltreatment disrupts HPA-axis activity under basal and stress conditions in a dose-response relationship in children and adolescents

Laia Marques-Feixa, Helena Palma-Gudiel, Soledad Romero, Iñaki Zorrilla, Hilario Blasco-Fontecilla, Marta Rapado-Castro, **Águeda Castro Quintas**, Jorge Moya-Higueras, Jaume March, Lourdes Fañanás & EPI-Young Stress GROUP.

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Author for correspondence:
Lourdes Fañanás, E-mail: lfananas@ub.edu

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Childhood maltreatment disrupts HPA-axis activity under basal and stress conditions in a dose–response relationship in children and adolescents

Laia Marques-Feixa[1,2], Helena Palma-Gudiel[1,2], Soledad Romero[2,3,4], Jorge Moya-Higueras[2,5], Marta Rapado-Castro[2,6,7], Águeda Castro-Quintas[1,2], Iñaki Zorrilla[2,8], María José Muñoz[9], Maite Ramírez[10], María Mayoral[2,6], Ariadna Mas[3], María José Lobato[11], Hilario Blasco-Fontecilla[2,11], Lourdes Fañanás[1,2] and EPI-Young Stress GROUP*

[1]Department of Evolutionary Biology, Ecology and Environmental Sciences, Faculty of Biology, University of Barcelona, Biomedicine Institute of the University of Barcelona (IBUB), Barcelona, Spain; [2]Network Centre for Biomedical Research in Mental Health (CIBER of Mental Health, CIBERSAM), Spain; [3]Department of Child and Adolescent Psychiatry and Psychology, Institute of Neuroscience, Hospital Clínic de Barcelona, 2017SGR88, Barcelona, Spain; [4]Institut d'Investigacions Biomediques August Pi i Sunyer (IDIBAPS), Barcelona, Spain; [5]Department of Psychology, Faculty of Education, Psychology and Social Work, University of Lleida, Spain; [6]Department of Child and Adolescent Psychiatry, Institute of Psychiatry and Mental Health, Hospital General Universitario Gregorio Marañón, School of Medicine, Universidad Complutense, IiSGM, Madrid, Spain; [7]Department of Psychiatry, Melbourne Neuropsychiatry Centre, The University of Melbourne & Melbourne Health, Victoria, Australia; [8]Department of Psychiatry, Hospital Santiago Apostol, Vitoria-Gasteiz, Spain; [9]Hospital Benito Menni, Adolescent Crisis Unit, Sant Boi de Llobregat, Spain; [10]Galdakao Mental Health Services, Child and Adolescent Mental Health, Galdakao, Spain; [11]Department of Psychiatry, Puerta de Hierro University Hospital-Majadahonda, Autonoma University, ITA Mental Health, Madrid, Spain and [12]Day Hospital for Adolescents, Fundació Orienta, Gavà, Spain

Abstract

Background. This study investigates the impact of childhood maltreatment (CM) on hypothalamic–pituitary–adrenal (HPA)-axis functioning and on anxiety perception. Moreover, the influence of CM severity and frequency was also explored.

Methods. In total, 187 participants aged 7–17 were assessed for CM history using validated questionnaires and *ad hoc* interviews to be classified according to the criteria of the Tool for Assessing the Severity of Situations in which Children are Vulnerable (TASSCV). Psychopathology was ascertained using the K-SADS-PL5. To assess HPA-axis functioning, salivary cortisol samples were collected throughout a normal day and during an acute psychosocial stressor, the Trier Social Stress Test for children (TSST-C). Subjective anxiety was evaluated using STAI/-C.

Results. Youth with a CM history had higher overall diurnal cortisol levels ($p = 0.001$), blunted cortisol response to acute psychosocial stress ($p = 0.002$) and greater perceived anxiety ($p = 0.003$), than those without CM. Specifically, participants exposed to moderate/severe or often/frequent CM showed the greater diurnal cortisol output ($p_{severity} = 0.002$; $p_{frequency} = 0.003$), and blunted cortisol response during the TSST-C ($p_{severity} = 0.006$; $p_{frequency} = 0.008$). Meanwhile, youth with low CM severity/frequency exhibited a similar cortisol response to those without CM. However, perceived anxiety was higher in those exposed to CM ($p < 0.001$), regardless of its severity/frequency.

Conclusions. Disturbances in HPA-axis functioning are already evident early after CM exposure, while psychological and physiological responses to an acute stressor are dissociated in youth exposed to CM. The dose–response relationship described in this paper highlights the need to comprehensively evaluate CM so that vulnerable children can be identified and assigned to proper interventions.

Introduction

Experiences of childhood maltreatment (CM) are one of the main contributors to mental illness (Brown, Harris, & Craig, 2019; Hughes et al., 2017). However, CM is non-specifically associated with psychiatric disorders, i.e. several types of CM can increase vulnerability for a specific disorder in different patients (Vachon, Krueger, Rogosch, & Cicchetti, 2015). CM has been associated with early onset of psychiatric illness, increased symptom severity and comorbidity, and poor clinical outcomes characterized by requiring higher medication

dosages, increased suicidal behavior, and more and longer hospitalizations (Lippard & Nemeroff, 2020). Furthermore, factors such as time of exposure, chronicity, and severity of childhood abuse or neglect play a role in clinical outcomes. Studies indicate a dose–response relationship between multiplicity of exposure, severity or frequency, and risk of mental disorders (Anda et al., 2006).

CM is associated with dysregulation of stress-mediating systems, thereby increasing the risk of mental and physical health problems. Specifically, disruptions in hypothalamic–pituitary–adrenal (HPA)-axis regulation have been studied as a potential mediator of this association (Koss & Gunnar, 2018; Kudielka & Wüst, 2010). The HPA-axis is one of the main stress response systems; cortisol, its final effector, released in direct response to acute stressors, triggers a wide range of actions by regulating gene transcription and epigenetic modifications in several brain areas (Provençal, Arloth, Cattaneo, Anacker, & Cattane, 2019). Furthermore, HPA-axis maintains a diurnal rhythm, with the highest cortisol levels in the morning which decrease progressively during the day until reaching the lowest at midnight. Since the HPA-axis continues to mature during early stages of life, environmental factors such as early-life stress may induce long-lasting changes in its functioning, resulting in the emergence of different disorders (Tarullo & Gunnar, 2006). However, findings regarding alterations in the patterns of cortisol associated with early-life stress have been inconsistent (Fogelman & Canli, 2018).

A recent meta-analysis focusing on CM and diurnal HPA-axis activity in children and adults reported no overall effect on diurnal cortisol slope (Bernard, Frost, Bennett, & Lindhiem, 2017). However, a moderate association was found between CM and blunted awakening cortisol concentrations when considering only sufferers of CM who were referred from child welfare system agencies. In contrast, another recent meta-analysis showed that CM affects HPA-axis reactivity during stressful situations as evidenced by a flattened cortisol pattern during an acute psychosocial stress task in children and adults who faced early-life adversities (Bunea, Szentágotai-t, & Miu, 2017). Interestingly, the effects were more pronounced in studies focused on adults and CM. These findings suggest a pattern of blunted cortisol response during the peak and recovery phases of acute stress, and overall hypocortisolism in individuals exposed to CM. However, some studies report hypercortisolism in subjects exposed to early-life stress, childhood trauma, or insensitive interactions with caregivers (Hunter, Minnis, & Wilson, 2011). Besides, it has been suggested that distinct patterns of cortisol responses may be partially explained by CM severity and frequency (Ouellet-Morin et al., 2019), pubertal stage (King et al., 2017), or sex (Trickett, Gordis, Peckins, & Susman, 2014).

Notably, HPA-axis dysregulation, both hyperactivity and hypoactivity, has been associated with different psychiatric disorders and other disease outcomes (Turner et al., 2020). Although infancy is a sensitive period for HPA-axis regulation, this system remains plastic and it can be recalibrated during specific ontogenic periods, if the environmental conditions improve. In fact, recent studies support puberty as a key recalibration period to trigger shifts in HPA-axis functioning in postinstitutionalized children (DePasquale, Donzella, & Gunnar, 2019).

Thus, the main aim of the current research was to establish the proximal effects of CM on HPA-axis regulation and anxiety perception in children and adolescents, under basal conditions and in response to a psychosocial stressor, as compared with youth without CM. In addition, the differential impact of the severity and frequency of the CM experiences was also analyzed to better dissect the relationship between CM and HPA-axis dysfunction. Finally, anxiety perception was assessed throughout the experimental stress paradigm to verify that all participants underwent a subjective experience of stress (regardless of their CM history); thus, the potential differences in stress perception with regard to CM can be disentangled from actual differences in HPA-axis functioning. Complementarily, anxiety trait was also assessed in relationship with basal diurnal cortisol output. Specifically, we hypothesized that exposure to CM would be associated with blunted HPA-axis functioning and higher anxiety perception. Moreover, more severe and frequent exposure to CM would be associated with greater dysregulation of the HPA-axis following a dose–response relationship.

Methods

The EPI-Young-Stress project is a multi-center study which aims to evaluate HPA-axis functioning, associated epigenetic signatures, and immunological biomarkers involved in the association between CM and youth mental disorders. The research was conducted at the University of Barcelona and six child and adolescent psychiatry departments in Spain: Hospital Benito Menni, Hospital Clínic Barcelona, Hospital Gregorio Marañón, Hospital Puerta de Hierro, Hospital Santiago Apóstol, and Day Hospital Orienta Gavà.

The study was approved by the Ethical Review Board of each participating hospital and university. Families were explicitly informed about the voluntary nature of the study, their rights, and the procedures, risks, and potential benefits involved. Written consent was required from all parents or legal guardians; the children provided written assent after the nature of the procedure had been fully explained.

Participants

A total of 187 children and adolescents aged 7–17 years participated in this study. Children without psychopathology were recruited from advertisements, primary healthcare centers, schools, and other community facilities. Children with current psychopathology were recruited from the above-mentioned hospitals (inpatient clinics, partial hospitalization programs, and outpatient clinics) (see Table 1). Recruitment lasted from April 2016 to March 2020. Exclusion criteria for all participants included diagnosis of autism spectrum disorder, eating disorder with body mass index (BMI)<18.5, intellectual disability (IQ < 70), current drug dependence, non-fluency in Spanish, extreme premature birth (<1500 g at birth), head injury with loss of consciousness, and severe neurological or other pathological conditions likely to affect HPA-axis functioning (such as cancer or autoimmune diseases).

Procedures

Sociodemographic and clinical measures

The interview package included basic demographic information including socioeconomic status (SES) based on the Hollingshead Four-Factor Index of SES (Hollingshead, 1975). Pubertal development was assessed using the Tanner staging questionnaire (Morris & Udry, 1980) and participants were classified as either children (Tanner stages 1–3) or adolescents (Tanner stages 4–5). The Global Family Environment Scale

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Table 1. Sociodemographic and anthropometric data of participants with and without a history of CM

Variable			Total sample ($n = 187$)	Youth without CM ($n = 93$, 50%)	Youth with CM ($n = 94$, 50%)	t/χ^2	p	d/κ
Age (M, S.D.)[a]			13.62 (2.59)	13.20 (2.69)	14.03 (2.44)	−2.204	**0.029***	0.323
Sex[b]	Female (*n*, %)		108 (58%)	48 (52%)	60(64%)	2.860	0.091	0.122
	Male (*n*, %)		79 (42%)	45 (48%)	34 (36%)			
Pubertal stage[b]	Child (Tanner stage 1–3) (*n*, %)		94 (50%)	53 (57%)	41 (44%)	3.344	0.067	0.134
	Adolescent (Tanner stage 4–5) (*n*, %)		93 (50%)	40 (43%)	53 (56%)			
Ethnicity[b]	European (*n*, %)		154 (82%)	87 (93%)	67 (71%)	15.956	**<0.001*****	0.222
	Others[c] (*n*, %)		33 (18%)	6 (7%)	27 (29%)			
Socioeconomic status (SES) (M, S.D.)[a,d]			40.34 (17.93)	47.49 (14.77)	33.12 (18.03)	5.893	**<0.001*****	0.872
CGAS (M, S.D.)[a]			72.07 (21.66)	84.26 (14.37)	59.88 (20.89)	9.270	**<0.001*****	1.359
Current psychiatric diagnosis status[b]	Subjects without current psychiatric diagnosis (*n*, %)		71 (38%)	56 (60%)	15 (16%)	38.879	**<0.001*****	−0.442
	Subjects with current psychiatric diagnosis (*n*, %):		116 (62%)	37 (40%)	79 (84%)			
	Primary psychiatric diagnosis dimensions[b,e]	ADHD	30 (26%)	18 (49%)	12 (15%)	32.235	**<0.001*****	0.119
		Affective disorders	29 (25%)	6 (16%)	23 (29%)			
		Trauma and stress-related disorders	19 (16%)	0 (0%)	19 (24%)			
		Anxiety disorders	15 (13%)	9 (24%)	6 (8%)			
		Behavioral disorders	13 (11%)	1 (3%)	12 (15%)			
		Psychotic disorders	7 (6%)	3 (8%)	4 (5%)			
		Eating disorders	3 (3%)	0 (0%)	3 (4%)			
	Clinical care units of subjects with current psychiatric diagnosis[b,e]	Outpatient	69 (60%)	31 (83%)	38 (48%)	13.458	**0.001****	−0.262
		Inpatient	35 (30%)	5 (14%)	30 (38%)			
		Partial program	12 (10%)	1 (3%)	11 (14%)			
	Psychopharmacological treatment of subjects with current psychiatric diagnosis[b,e]	No (*n*, %)	28 (24%)	9 (24%)	19 (24%)	0.001	0.974	<0.001
		Yes (*n*, %)	88 (76%)	28 (76%)	60 (76%)			
Oral contraceptive use[b,f]	No (*n*, %)		102 (94%)	47 (98%)	55 (92%)	1.985	0.159	0.056
	Yes (*n*, %)		6 (6%)	1 (2%)	5 (8%)			
Corticosteroid medication[b]	No (*n*, %)		184 (98%)	90 (97%)	94 (100%)	3.082	0.079	−0.032
	Yes (*n*, %)		3 (2%)	3 (3%)	0 (0%)			

(*Continued*)

Table 1. (*Continued.*)

Variable		Total sample ($n = 187$)	Youth without CM ($n = 93$, 50%)	Youth with CM ($n = 94$, 50%)	t/χ^2	p	d/κ
Last year global family environmental (GFES) (M, S.D.)[a,g]		78.24 (15.03)	84.53 (9.65)	71.94 (16.76)	6.104	**<0.001*** **	0.920
Illegal drug use[b]	Never	164 (88%)	90 (97%)	74 (79%)	15.242	**0.002** **	0.124
	Less than once a month	10 (5%)	1 (1%)	9 (10%)			
	Once a month or more	7 (4%)	2 (2%)	5 (5%)			
	Daily use	6 (3%)	0 (0%)	6 (6%)			
BMI (M, S.D.)[a,h]		21.45 (5.17)	19.66 (3.75)	23.23 (5.77)	−4.799	**<0.001*** **	0.733
WHR (M, S.D.)[a,h]		0.84 (0.09)	0.84 (0.09)	0.84 (0.09)	0.059	0.953	−0.011

ADHD, attention-deficit/hyperactivity disorder; BMI, body mass index; CGAS, Children's Global Assessment Scale, rating from 1 to 100 with higher ratings indicating better functioning in a wide range of activities; CM, childhood maltreatment (CM group refers to subjects with a confirmed or suspected history of CM); GFES, The Global Family Environment Scale, ranging from 1 to 90, with higher scores indicating a better family environment; SES, socioeconomic status, raw scores range from 8 to 66, with higher scores reflecting higher SES; WHR, waist-to-hip ratio.

[a]Student's *t* test.

[b]χ^2 test.

[c]Other ethnicities included Latin American (66%), Maghrebin (16%), sub-Saharan (9%), and others (9%).

[d]This analysis was conducted with 183 subjects.

[e]This analysis was only conducted with the 116 subjects with a current psychiatric diagnosis.

[f]This analysis was only conducted with the 108 female subjects.

[g]This analysis was conducted with 176 subjects.

[h]This analysis was conducted within 171 subjects.

p values: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. *d* = Cohen's effect size.

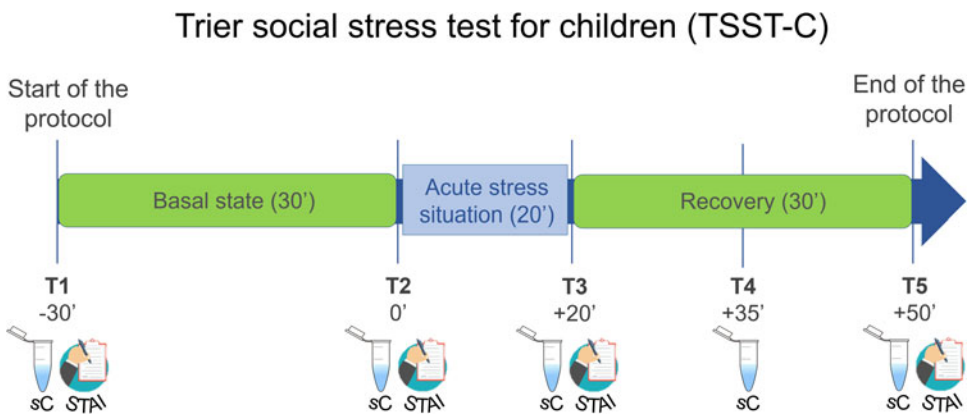

Fig. 1. Summary of the Trier Social Stress Test for children (TSST-C) protocol. sC, salivary cortisol sample; STAI, State/Trait Anxiety Inventory – State.

(GFES) was used to measure the quality of the family environment (Rey et al., 1997). Additionally, ethnicity, BMI and waist-to-hip ratio were recorded.

Both participants and their parents directly recounted the youth's medical history. Psychopathology was assessed using the Spanish version of the Schedule for Affective Disorders and Schizophrenia for School-Age Children Present and Lifetime Version DSM-5 (K-SADS-PL-5) (de la Peña et al., 2018). Information was completed whenever possible using medical records. Final diagnoses were established by consensus, and based on DSM-5 criteria (APA: American Psychiatric Association, 2013), primary psychiatric diagnoses were later classified into dimensions to better characterize the sample (see Table 1). The global level of functioning was measured by the Children's Global Assessment Scale (CGAS) (Shaffer et al., 1983). The use of psychiatric medication was dichotomized as absence/presence, since there were no differences in cortisol levels according to the different drugs (data available upon request). Current illegal drug use was classified into four frequency groups: never, less than once a month, once a month or more, and daily use (Forti et al., 2019).

Childhood maltreatment assessment

All participants and their parents/legal guardians were interviewed separately, face to face, by one trained psychologist or psychiatrist. They were assessed by means of an exhaustive interview focused on the identification of signs of child vulnerability, adverse experiences, and family interactions, based on the criteria of the instrument 'Tool for assessing the severity of situations in which children are vulnerable' (TASSCV), which has been validated by professionals working in child and adolescent care units (see online Supplementary material) (CARM, 2012). Additionally, adolescents older than 12 were assessed for history of CM via the short version of the Childhood Trauma Questionnaire (CTQ-SF) (Bernstein et al., 2003) and the Childhood Experience of Care and Abuse Questionnaire (CECA-Q2) (Kaess et al., 2011). Children under 12 years answered an adapted hetero-administered *ad hoc* questionnaire (see online Supplementary material). Afterwards, the clinicians completed a table summarizing the different forms of CM effected by caregivers or other adults (not by peers), being TASSCV the main measure of CM used in the primary analyses, while the other measures (CTQ-SF, CECA-Q2, *ad hoc* questionnaire, and reports from social services or teachers) were used as an additional source of information for the clinicians. The exhaustive participants' evaluation during the recruitment process allowed for clinicians to enrich their praxis. In addition, after the interviews of this study, a referral system of urgent appointment was implemented for those subjects who requested it, activating the usual protocols that guarantee the children's protection rights. Following the TASSCV criteria, each CM type was coded as either: (i) absent, (ii) suspected (if significant signs of neglect or abuse emerged during the evaluation), or (iii) confirmed (with clear evidence from social services or family). Severity and frequency of different types of CM were rated on a four-point Likert scale according to TASSCV criteria. CM severity was coded according to the characteristics of the experience suffered as low (1), moderate (2), severe (3), or very severe (4); while frequency was coded as whether CM had occurred once (1), sometimes (2), often (3), or frequently (4). Five types of CM were considered in the following analysis: physical neglect, emotional neglect, physical abuse, emotional abuse, and sexual abuse.

HPA-axis functioning

Four saliva samples were collected during a normal day with the aim to assess HPA-axis diurnal functioning (basal condition), specifically, on waking up (B1), 30 min after waking (B2), before lunch (B3), and before bedtime (B4). On a different day, in order to explore HPA-axis reactivity during acute psychosocial stress, the Trier Social Stress Test for children (TSST-C), a validated protocol that reliably induces HPA-axis activation, was applied (Buske-Kirschbaum et al., 1997). Briefly, upon arrival at the lab, the participants waited in a quiet room for 30 min before entering the examination room, where a panel of judges awaited. During the 20 min of the stress situation, the participants had to perform a speaking and an arithmetic task following instructions from the judges while being videotaped. After the stress task, the participants returned to the first room for 30 min (see online Supplementary material for a more detailed description of the procedure). Five saliva samples were collected during this procedure: 30 min before the stressor (T1), immediately before the stressor (T2), immediately after the stressor (T3), 15 min after the stressor (T4), and 30 min after the stressor (T5) (see Fig. 1). All participants were scheduled at 16:00 h to control for diurnal cortisol variability. Previously, further instructions were given to the participants to avoid factors that have been reported to influence cortisol levels (details in the online Supplementary material). Details about collection time of each salivary cortisol sample are available in Table 2.

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Table 2. Cortisol values and anxiety perception according to the presence of CM, CM severity and CM frequency

		Dichotomous CM (mean, s.d.)		Severity of CM (mean, s.d.)		Frequency of CM (mean, s.d.)				
		Youth without CM ($n = 93$)	Youth with CM ($n = 94$)	Youth with low CM ($n = 20$)	Youth with moderate/severe CM ($n = 74$)	Youth once/sometimes exposed to CM ($n = 22$)	Youth often-frequently exposed to CM ($n = 72$)	F (p) dichotomous CM[a]	F (p) severity of CM[a]	F (p) frequency of CM [a]
Diurnal salivary cortisol (µm log-transformed)	B1	−0.66 (0.32)	−0.66 (0.25)	−0.63 (0.24)	−0.67 (0.25)	−0.67 (0.20)	−0.66 (0.26)	1.467 (0.225) B4*	1.214 (0.300) B4*	1.085 (0.375) B4*
	B2	−0.51 (0.31)	−0.52 (0.31)	−0.44 (0.23)	−0.54 (0.33)	−0.40 (0.19)	−0.56 (0.34)			
	B3	−1.09 (0.29)	−1.03 (0.34)	−0.93 (0.34)	−1.05 (0.33)	−0.95 (0.34)	−1.05 (0.34)			
	B4	−1.56 (0.59)	−1.38 (0.45)	−1.46 (0.35)	−1.36 (0.47)	−1.39 (0.38)	−1.38 (0.48)			
	AUCg	−927.77 (262.45)	−831.64 (202.25)	−799.16 (221.60)	−839.42 (198.39)	−765.10 (194.54)	−851.22 (201.84)	**12.244 (0.001**)▲**	**6.349 (0.002**)▲**	**6.068 (0.003**)▲**
	AUCi	−374.70 (326.25)	−291.13 (246.56)	−297.62 (261.08)	−289.58 (244.88)	−234.17 (229.26)	−307.89 (250.57)	3.040 (0.083)	1.716 (0.184)	1.276 (0.282)
Salivary cortisol during TSST-C (µm log-transformed)	T1	−0.97 (0.31)	−0.97 (0.26)	−0.99 (0.30)	−0.96 (0.25)	−0.97 (0.26)	−0.97 (0.26)	**4.530 (0.002**) T3*, T4**, T5***	**2.773 (0.006**) T3*, T4**, T5***	**2.665 (0.008**) T3*, T4**, T5***
	T2	−1.02 (0.26)	−1.01 (0.27)	−1.01 (0.24)	−1.00 (0.28)	−1.01 (0.23)	−1.00 (0.29)			
	T3	−0.89 (0.31)	−0.98 (0.27)	−0.93 (0.25)	−0.99 (0.29)	−0.95 (0.26)	−0.99 (0.29)			
	T4	−0.89 (0.35)	−1.03 (0.32)	−0.99 (0.33)	−1.04 (0.32)	−1.01 (0.30)	−1.03 (0.32)			
	T5	−0.98 (0.35)	−1.07 (0.31)	−1.09 (0.28)	−1.07 (0.32)	−1.07 (0.26)	−1.07 (0.32)			
	AUCg	−75.22 (21.74)	−78.67 (18.75)	−80.52 (22.87)	−78.26 (17.84)	−80.90 (18.94)	−78.02 (18.77)	0.091 (0.763)	0.057 (0.945)	0.074 (0.929)
	AUCi	0.56 (15.55)	−2.79 (15.02)	24 (17.24)	−3.47 (145.3)	−2.19 (16.51)	−2.97 (14.69)	**4.779 (0.030*)**	**3.921 (0.022*)**	**3.194 (0.044*)**
Anxiety trait: STAI-Trait (PC)		36.98 (28.40)	65.39 (32.74)	60.06 (31.50)	66.69 (33.12)	56.10 (34.14)	68.16 (32.04)	**9.129 (0.003**)▲**	**5.109 (0.007**)**	**4.102 (0.019*)**
Perceived anxiety during TSST-C: STAI-State (PC)	T1	25.68 (27.90)	45.34 (33.88)	46.59 (33.13)	45.05 (34.25)	32.05 (28.59)	49.27 (34.50)	1.742 (0.160)	1.670 (0.131)	1.240 (0.287)
	T2	25.20 (27.40)	40.40 (33.04)	32.07 (27.83)	42.07 (33.92)	30.05 (29.99)	43.64 (33.50)			
	T3	43.68 (32.53)	66.58 (32.18)	56.76 (36.70)	68.80 (30.90)	61.62 (32.16)	68.04 (32.27)			
	T5	21.12 (27.42)	42.49 (35.08)	29.41 (34.09)	45.50 (34.84)	27.14 (30.21)	47.10 (35.33)			

AUCg, area under the curve with respect to ground (indicating the total cortisol output); AUCi, area under the curve with respect to increase (reflecting cortisol changes over time); CM, childhood maltreatment (CM group refers to the subjects with a confirmed or suspected history of CM based on TASSCV criteria); STAI-State (PC), percentile scores of state anxiety inventory scale (for adolescents 16–17 years old) and state anxiety inventory for children scale (for participants under 15); STAI-Trait (PC), percentile scores of anxiety trait inventory scale (for adolescents 16–17 years old) and anxiety trait inventory for children scale (for participants under 15); TSST-C, Trier Social Stress Test for children.

Diurnal salivary cortisol was measured at: B1, immediately after awakening; B2, 30 min after waking; B3, before lunch; B4, before bedtime. Mean time for saliva sample collection: 08:52 ± 1:27 (6:00–12:00) (B1); 09:24 ± 1:26 (6:30–12:59) (B2); 14:19 ± 0:53 (12:15–16:40) (B3); and 22:37 ± 01:16 [20:00–2:50(+1day)] (B4). Saliva samples for cortisol measurement during TSST-C were collected at: T1, 30 min before stressor; T2, immediately before stressor; T3, immediately after stressor; T4, 15 min after stressor; T5, 30 min after stressor. Mean time for saliva sample collection during the TSST-C procedure: 16:04 ± 0:11 (15:13–17:15) (T1); 16:33 ± 0:12 (15:42–17:45) (T2); 16:53 ± 0:13 (15:59–18:00) (T3); 17:08 ± 0:13 (16:08-18:16) (T4); and 17:23 ± 0:13 (16:30–18:30) (T5).

Dichotomous CM refers to the analysis comparing youth without CM with youth exposed to any type of CM. Severity of CM refers to the analysis comparing youth without CM, youth exposed to low CM, and youth exposed to moderate/severe CM. Frequency of CM refers to the analysis comparing youth without CM, youth exposed to CM once/sometimes, and youth exposed to CM often/frequently.

[a]Mixed-effects model (for single measurements) and ANOVA (for AUCg and AUCi). The analyses include the following covariates: clinical status, sex, pubertal stage, psychopharmacological treatment, illegal drugs use, oral contraceptive use, corticosteroid medication, ethnicity, SES, and BMI [additionally adjusting by the time of the first cortisol sample collection (B1) for diurnal analysis]. Values in superscript $^{(B4,\ T3,\ T4,\ T5)}$ indicate the samples with a significant difference in the simple effects test in the context of mixed-effect model.

p values: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. ▲$p \leqslant 0.006$ [as the Bonferroni-corrected level of significance for multiple testing (0.05/9 = 0.006)].

Saliva samples were collected using Salivette® tubes (Sarstedt, Inc., Newton, NC, USA) for diurnal cortisol assessment and with Saliva Bio Oral Swabs (SOS) (Salimetrics, LLC, State College, PA, USA) for TSST-C cortisol reactivity. The subjects were asked to chew a swab for 1 min and then transfer it directly from their mouth to the tube. They were instructed to store their saliva samples for diurnal cortisol assessment in a freezer until they could be delivered to the research center, where samples were stored at −20 °C. The saliva samples collected during the TSST-C were directly stored at the research center. Details of salivary cortisol determination procedures are explained in the online Supplementary material.

Anxiety trait and anxiety perception during acute stress

The subscale trait of the State-Trait Anxiety Inventory (STAI) was used to evaluate general proneness to anxious behavior (STAI-Trait for children, for subjects 15 years old and under; STAI-Trait, for adolescents 16–17 years old) (Spielberger, 1973). During the TSST-C, the perceived emotional arousal was assessed via the STAI-State for children scale (for children 15 and under) and the STAI-State subscale (for adolescents 16–17 years old) (Spielberger, 1973). Participants answered the STAI-State questionnaire: 30 min before the stressor (T1), immediately before the stressor (T2), immediately after the stressor (T3), and 30 min after the stressor (T5) (see Fig. 1).

Statistical analysis

All statistical analyses were performed using SPSS 26 for Windows (IBM, Chicago, Illinois, USA). Descriptive statistics were analyzed by Student's t test for continuous variables and a χ^2 test for categorical variables. Cortisol data were log-transformed to reduce skewness. The presence of any type of suspected or confirmed history of CM was included in downstream analysis as a dichotomic variable. The effects of both (i) CM severity (classified as: absent, low, or moderate/severe) and (ii) the frequency of CM (classified as: never, once/sometimes, or often/frequently) were also tested through independent analyses. Sensitivity analysis was conducted to explore the effects of CM when considering only subjects with a confirmed history of CM (with clear evidence from social service reports or family), aggregating those with suspected history of CM together with those without CM (see online Supplementary material).

To examine the effect of CM in diurnal cortisol slopes and changes in cortisol and anxiety perception across the TSST-C, mixed-effects models with a random effect of intercept and a random slope of time, to account for within-subject correlations, were used. Interaction with time was considered the main effect of interest of the model. Time factor had four categories (time-points) for diurnal cortisol and anxiety perception during TSST-C, and five categories for cortisol during TSST-C. In addition, simple effects tests were performed to evaluate the specific time point interaction between groups. Additionally, the overall cortisol secretion during a normal day and throughout the experimental protocol was summarized applying: (i) the area under the curve with respect to ground (AUCg) to explore the total hormonal output, and (ii) the area under the curve with respect to increase (AUCi) to reflect hormonal changes over time (Pruessner, Kirschbaum, Meinlschmidt, & Hellhammer, 2003). Differences in AUCg, AUCi, and STAI-Trait scores between CM groups were tested by ANOVA. All the analyses were adjusted for the following covariates, as previously described to influence cortisol output during the TSST (Allen, Kennedy, Cryan, Dinan, & Clarke, 2014; De Punder, Heim, & Entringer, 2019; Lê-scherban et al., 2018; Marceau & Abel, 2018): clinical status, sex, pubertal stage, psychopharmacological treatment, illegal drugs use, oral contraceptive use, corticosteroid medication, ethnicity, SES, and BMI. In the diurnal cortisol analyses, the time of first cortisol sample (B1) collection was also included as a covariate. Specifically, in the ANOVA analysis, in order to study the direct effect of clinical status, sex, and pubertal stage on cortisol and anxiety, as well as their potential interactions with CM, these variables were included as inter-subject factors. To correct for the testing of three different CM variables (presence/absence of CM, CM severity, and CM frequency) and three different cortisol summary measures (mixed model, AUCg, and AUCi), in Table 2, a Bonferroni correction was applied by dividing the original α level ($p < 0.05$) by 9 (3×3), and obtained a new significance level of $p < 0.006$. Spearman's non-parametric correlation was calculated separately in participants without CM and those with a history of CM, to explore the relationship between anxiety perception and salivary cortisol during basal conditions and during the TSST-C.

Results

Attrition and descriptive analysis

Nine subjects had no information available on diurnal cortisol levels, so they were not included in the diurnal cortisol analysis. Three participants had no information available on cortisol and anxiety perception during the TSST-C, so they were not included in the corresponding analysis. Sixteen subjects were excluded from the analysis due to missing information on covariates such as BMI or SES. All the excluded participants due to missing BMI or SES values were diagnosed with a current psychiatric disorder. There were no significant differences in either sociodemographic factors or cortisol values when comparing the participants excluded and subjects with psychiatric diagnostic included in the analysis; however, the excluded participants exhibited significantly higher CGAS than those included ($t = 2.360$, $p = 0.020$).

A brief summary of the sociodemographic and anthropometric variables, by CM history, is provided in Table 1. Significant group differences according to CM exposure were observed with regard to age, ethnicity, SES, illegal drug use, CGAS, GFES, BMI, current psychiatric disorder, and type of clinical care unit. Mean cortisol values by CM group measures at each diurnal and TSST-C time-point, AUCg and AUCi values, and STAI-Trait and STAI-State scores are summarized in Table 1.

Childhood maltreatment and diurnal salivary cortisol

As expected, cortisol levels fluctuated significantly throughout the day, following a circadian rhythm ($F = 218.307$, $p < 0.001$). No global interaction between time and CM was detected ($F = 1.467$, $p = 0.225$), reflecting a similar cortisol diurnal trajectory in both groups (see Table 2), also evidenced by AUCi levels, $F_{(1,160)} = 3.040$, $p = 0.083$, $\eta_p^2 = 0.021$. However, the simple effects analysis in the context of mixed-effect model revealed a significant time point-specific interaction at B4 (before bedtime) between CM groups ($F = 4.678$, $p = 0.032$). Although cortisol levels consistently decreased from lunchtime to bedtime in both groups, this was less pronounced in the CM group, leading to a higher total hormonal output over the whole day, as evidenced

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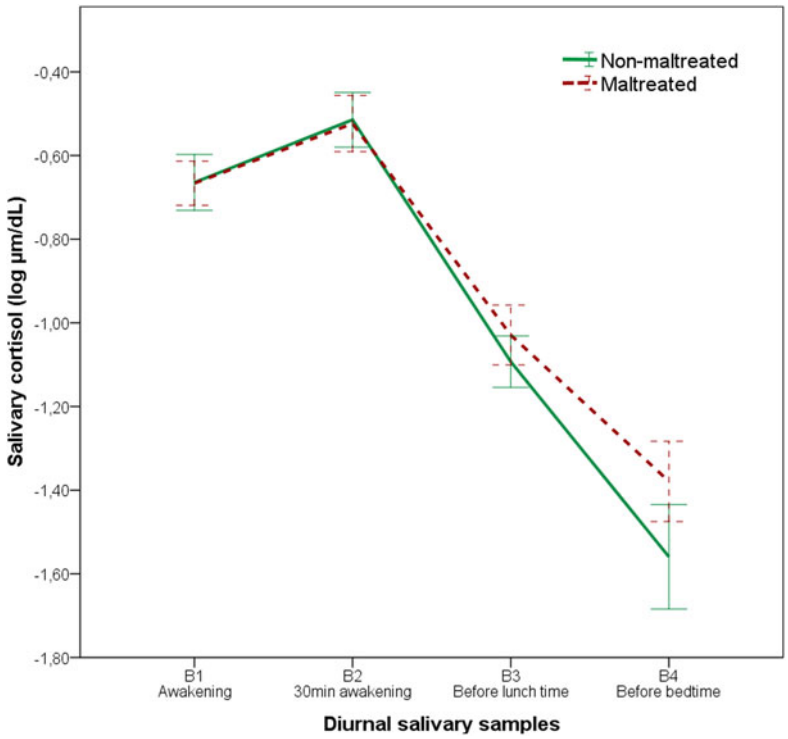

Fig. 2. Diurnal salivary cortisol in participants with and without CM. Exposure to CM significantly increased AUCg levels, indicating a higher total diurnal cortisol output. Specifically, youth exposed to CM showed increased cortisol levels before bedtime (B4). The analysis was adjusted for sex, pubertal stage, clinical status, time of the first cortisol sample collection (B1), psychopharmacological treatment, illegal drugs use, ethnicity, corticosteroid medication, oral contraceptive use, BMI, and socioeconomic status.

by a higher AUCg, $F_{(1,160)} = 12.244$, $p = 0.001$, $\eta_p^2 = 0.079$ (see Table 2 and Fig. 2). No significant interactions have been reported between CM and clinical status, pubertal stage, or sex. The effect of clinical status, pubertal stage, and sex on diurnal cortisol levels is reported in the online Supplementary material. Similar results were observed in the diurnal cortisol response when considering only subjects with confirmed CM (see online Supplementary material).

Neither the frequency nor the severity of CM was associated with diurnal cortisol slope during the day, $F_{\text{severity}} = 1.214$, $p = 0.300$; $F_{\text{frequency}} = 1.085$, $p = 0.372$, reflecting a similar cortisol diurnal trajectory between groups, also evidenced by AUCi, $F_{\text{severity}(2,160)} = 1.716$, $p = 0.184$, $\eta_p^2 = 0.024$; $F_{\text{frequency}(2,160)} = 1.276$, $p = 0.282$, $\eta_p^2 = 0.018$. However, the simple effect analysis revealed a significant interaction at B4 (before bedtime); participants exposed to moderate/severe CM experiences or often/frequently exposed to CM showed higher cortisol levels before bedtime when compared with subjects without CM ($p_{\text{severity}} = 0.020$; $p_{\text{frequency}} = 0.048$). The AUCg levels suggested a dose–response relationship between CM severity/frequency and total cortisol output during the day, $F_{\text{severity}(2,160)} = 6.349$, $p = 0.002$, $\eta_p^2 = 0.084$; $F_{\text{frequency}(2,160)} = 6.068$, $p = 0.003$, $\eta_p^2 = 0.081$. As expected, these results were even more significant when dichotomizing the sample according to the severity/frequency of CM as either: (1) no/low exposure or (2) moderate/severe exposure (see online Supplementary material).

Childhood maltreatment and salivary cortisol response during acute psychosocial stress (TSST-C)

Cortisol levels during the TSST-C significantly differed as a function of time ($F = 8.953$, $p < 0.001$), indicating the validity of this procedure to stimulate cortisol secretion in our cohort. A significant interaction between CM and time was identified ($F = 4.530$, $p = 0.002$), indicating a different trajectory of cortisol levels during the protocol between groups of CM. Specifically, the simple effects analysis in the context of mixed-effect model revealed a significant time point-specific interaction when comparing cortisol levels at T3 (immediately after the stressful situation) ($F = 4.993$; $p = 0.027$), at T4 (15 min after the stressful situation finished) ($F = 10.404$, $p = 0.001$), and at T5 (30 min after the stressful situation finished) ($F = 4.561$, $p = 0.034$). While in individuals without CM the cortisol levels increased after acute stress, there were no changes in cortisol concentration in subjects with CM (see Fig. 3*a* and Table 2). In line with this, participants with CM showed lower levels of AUCi than those without CM, $F_{(1,165)} = 4.779$, $p = 0.030$, $\eta_p^2 = 0.031$, reflecting fewer hormonal changes over time. In contrast, CM was not associated with a global difference in cortisol levels throughout the entire TSST-C procedure ($F = 3.015$, $p = 0.084$), as also indicated by AUCg, $F_{(1,165)} = 0.091$, $p = 0.763$, $\eta_p^2 = 0.001$. Similar results were observed in cortisol response during TSST-C when considering only subjects with a confirmed history of CM (see online Supplementary material). Sex, pubertal stage, and clinical status did not interact with CM, and none of these variables explained a different response pattern during the TSST-C. However, significant differences were observed in the overall cortisol levels according to pubertal stage and clinical status. Adolescents showed higher levels of cortisol (AUCg) when compared with children, and subjects with a current psychiatric diagnosis reported lower levels of cortisol (AUCg) when compared with healthy participants (further details in the online Supplementary material).

When the severity and frequency of CM were analyzed, significant interactions were again identified between CM and time ($F_{\text{severity}} = 2.773$, $p = 0.006$; $F_{\text{frequency}} = 2.665$, $p = 0.008$). Specifically, the simple effects analysis revealed a significant time point-specific interaction when comparing cortisol levels at T3 (immediately after the stressful situation) ($p_{\text{severity}} = 0.012$;

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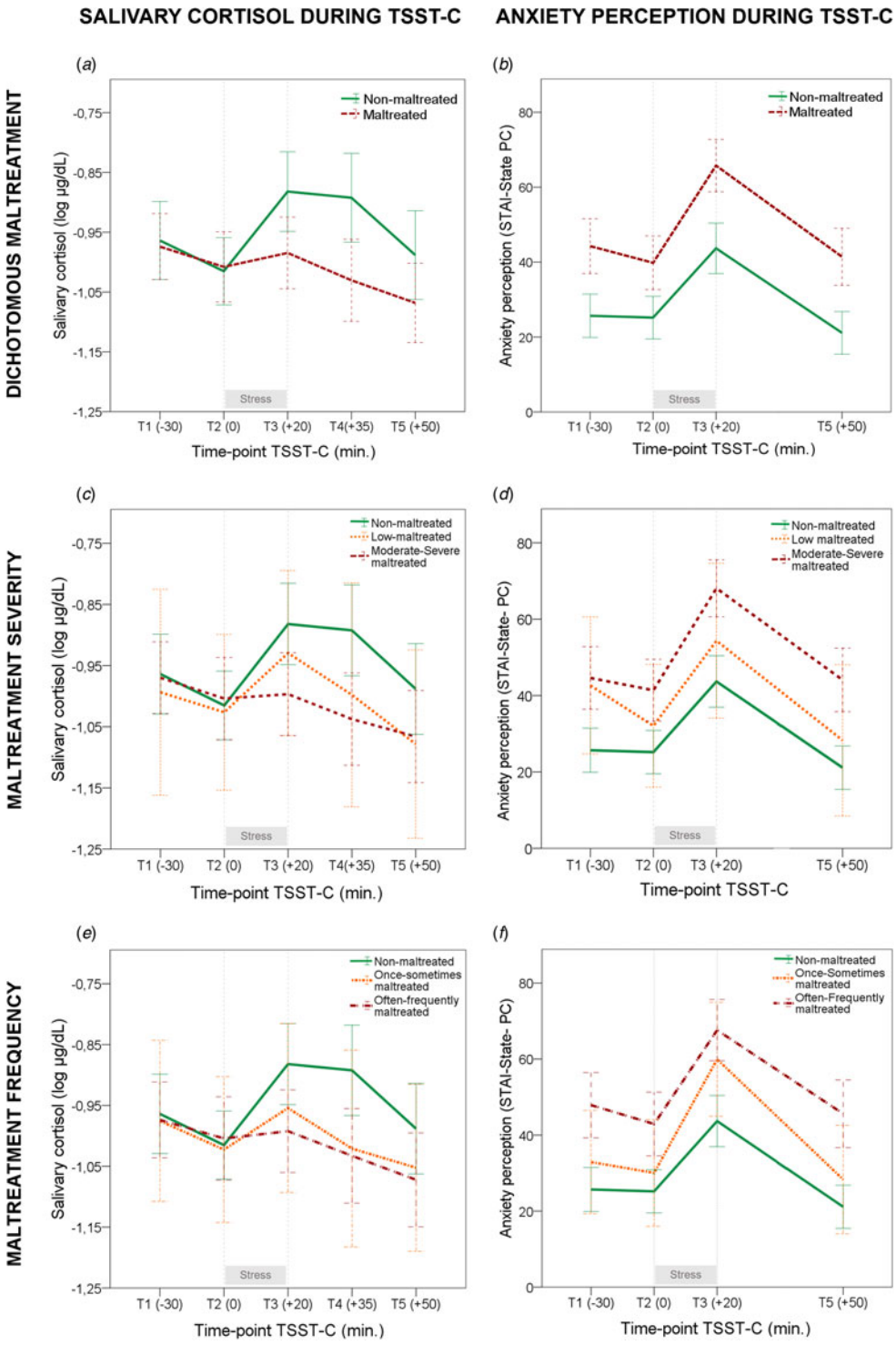

Fig. 3. Salivary cortisol response and anxiety perception during the Trier Social Stress Test for children (TSST-C) according to CM. (*a*) Subjects without CM had increased cortisol levels after exposure to acute psychosocial stress, while in those with a history of CM the cortisol levels remained stable. (*b*) Anxiety perception increased by the same magnitude in both participants with and those without a history of CM, after exposure to psychosocial stress. However, subjects with CM showed higher overall levels of anxiety during the protocol. (*c*) Participants without CM or low exposure to CM had a similar pattern of HPA-axis response during the TSST-C, increasing cortisol levels after acute stress. However, those exposed to moderate/severe CM showed a blunted cortisol response when faced with acute psychosocial stress, indicating hyporeactivity of the HPA-axis. (*d*) Anxiety perception increased by the same magnitude in all subjects, after exposure to psychosocial stress. However, youth with CM, both with low and moderate/severe exposure, had higher overall levels of anxiety during the protocol when compared with non-maltreated participants. (*e*) Subjects without CM and those who suffered CM once/sometimes had a similar pattern of HPA-axis response during the TSST-C. However, those exposed to CM often/frequently showed lower levels of cortisol after exposure to acute psychosocial stress, indicating hyporeactivity in the HPA-axis during acute psychosocial stress. (*f*) Anxiety perception increased by the same magnitude in all the subjects after exposure to psychosocial stress. However, youth with CM, both those who suffered CM once/sometimes and those who suffered CM often/frequently, had higher overall levels of anxiety. The analysis was adjusted for sex, pubertal stage, psychopathological diagnosis, psychopharmacological treatment, illegal drugs use, ethnicity, corticosteroid medication, oral contraceptive use, BMI, and socioeconomic status.

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$p_{frequency} = 0.026$), at T4 (15 min after the stressful situation finished) ($p_{severity} = 0.001$; $p_{frequency} = 0.001$), and at T5 (30 min after the stressful situation finished) ($p_{severity} = 0.033$; $p_{frequency} = 0.023$). While subjects without CM showed an increase in cortisol levels after the stressor, those exposed to moderate/severe or often/frequent CM were characterized by a blunted response, suggesting a dose–response relationship between CM severity/frequency and cortisol fluctuation during the TSST-C (see Fig. 3*c* and *e*). In this vein, participants exposed to moderate/severe and often/frequent CM displayed significantly lower values of AUCi than those without CM or exposed to low severity/frequency of CM, $F_{severity(2,165)} = 3.921$, $p = 0.022$, $\eta_p^2 = 0.052$; $F_{frequency(2,165)} = 3.194$, $p = 0.044$, $\eta_p^2 = 0.042$ (see Table 2). As expected, these results were even more significant when a new dichotomization was performed for severity/frequency of CM as either: (1) none or low and (2) moderate or severe exposure (for details see online Supplementary material). No significant differences in overall cortisol levels during the protocol were observed between severity/frequency groups of CM ($F_{severity} = 1.736$, $p = 0.179$; $F_{frequency} = 1.839$, $p = 0.162$), also evidenced by AUCg, $F_{severity(2,\ 165)} = 0.057$, $p = 0.945$, $\eta_p^2 = 0.001$; $F_{frequency(2,\ 165)} = 0.074$, $p = 0.929$, $\eta_p^2 = 0.001$.

Childhood maltreatment, anxiety trait, and anxiety perception during acute psychosocial stress (TSST-C)

Participants with CM exhibited significantly higher levels of anxiety trait than those without CM, $F_{(1,160)} = 9.129$, $p = 0.003$, $\eta_p^2 = 0.060$. The severity and frequency of CM were also associated with anxiety trait, $F_{severity(2,160)} = 5.109$, $p = 0.007$, $\eta_p^2 = 0.062$; $F_{frequency(2,160)} = 4.102$, $p = 0.019$, $\eta_p^2 = 0.056$, with the lowest anxiety trait levels exhibited by subjects none exposed to CM (see Table 2). No significant correlation between anxiety trait and overall diurnal cortisol levels was found (see online Supplementary material).

As seen in Fig. 3*b*, the TSST-C consistently increased perceived anxiety after acute stress in all the subjects ($F = 34.544$, $p < 0.001$). However, there were no interactions between time and CM ($F = 1.742$, $p = 0.160$), reflecting similar trajectories of perceived anxiety during the acute psychosocial stress in both subjects with and those without CM. Furthermore, those with CM showed higher overall perceived anxiety during the entire procedure than subjects without CM ($F = 23.836$, $p < 0.001$). Moreover, in youth without CM, anxiety perception during the TSST-C was negatively correlated with cortisol levels, but not in youth exposed to CM (see online Supplementary material). Subjects exposed to both low and high severity/frequency of CM showed higher overall levels of anxiety during the whole protocol than subjects without CM, $F_{severity} = 11.112$, $p < 0.001$; $F_{frequency} = 12.142$, $p < 0.001$ (see Fig. 3*c* and *d*). However, there were no differences between groups in the magnitude of the increase of perceived anxiety after the acute stressor, $F_{severity} = 1.670$, $p = 0.131$; $F_{frequency} = 1.240$, $p = 0.287$, $\eta_p^2 = 0.022$, with all groups exhibiting the same trajectory (see Table 2). Similar results were obtained when considering only subjects with a confirmed history of CM (for details see online Supplementary material).

Discussion

The present study elucidated how the proximal CM in children and adolescents impacts on HPA-axis functioning and on anxiety perception. In summary, youth exposed to CM, regardless of the presence of a current psychopathology, showed (i) a basal disruption of the HPA-axis circadian rhythm with increased daily cortisol levels, (ii) reduced HPA-axis reactivity during an acute psychosocial stress, and (iii) increased anxiety perception as a trait and during the whole psychosocial stress episode. Interestingly, all the subjects exposed to CM experienced heightened anxiety but only those exposed to more severe or frequent CM exhibited significant HPA-axis dysregulation. To the best of our knowledge, this is the first study to date to report the impact of CM severity measured as the gravity of the experiences suffered, rather than as the accumulation of different types of CM (e.g. pinch with momentary redness considered as low physical abuse, *v.* physical aggression that needs medical intervention considered as very severe).

Our results suggest that subjects who have suffered CM have higher overall diurnal cortisol levels. Specifically, the participants with CM were characterized by a blunted decline of cortisol levels from lunchtime to bedtime, compared with those without CM. This alteration of the circadian cortisol rhythm is consistent with the presence of hypercortisolism, as evidenced by higher AUCg scores in the group exposed to CM, especially those exposed to more severe and frequent CM. This may indicate a desynchrony trend in this intrinsic biological process, which has been described as a risk factor for rising mental health symptoms. Our findings are accordant with other studies focused on CM, which have reported both a blunted decline in HPA-axis activity throughout the day (Bernard, Zwerling, & Dozier, 2015) and higher overall cortisol output (Cicchetti & Rogosch, 2001). Our results could help to elucidate the co-occurrence of hypercortisolism and a flattened diurnal cortisol response, as high diurnal cortisol levels may be explained by an atypical diurnal decline. Similar findings have been reported in adults exposed to childhood adversities, suggesting the persistence of a less pronounced diurnal cortisol slope (Kuras et al., 2017). This HPA-axis dysregulation has important implications for other biological functions, as immune system (e.g. compromising the release of pro and anti-inflammatory substances) ultimately contributes to the increased risk of chronic disease later in life.

Although a recent meta-analysis (Bernard et al., 2017) reported no overall effect of CM on the diurnal cortisol slope, the authors also discussed the impact of many confounders. For example, age may influence the association between CM and cortisol rhythms; whereas cortisol levels could be elevated soon after the onset of a stressor (hypercortisolism), they could decrease over time, reflecting a pattern of hypocortisolism in adulthood (Miller, Chen, & Zhou, 2007). Although we did not observe this interaction between CM and pubertal stage, diurnal cortisol levels showed to be higher in adolescents when compared to children. Furthermore, our findings suggest that CM is associated with biological alterations also in youth without psychiatric disorders. In this regard, different approaches suggest that resilient subjects, who were exposed to CM but are asymptomatic, may present a particular neurobiological adaptive response, as brain connectivity changes to compensate for the alterations caused by abuse (Ohashi et al., 2019).

Secondly, regarding the HPA-axis response to acute psychosocial stress, consistent with the extant literature (Bunea et al., 2017), children and adolescents exposed to CM exhibited a blunted cortisol response during the TSST-C, compared with those without CM. While previous literature supports that the blunted cortisol response is better observed in adult populations (while arguing that smaller effects are seen in children and adolescents due to HPA-axis hyperactivation following immediate trauma), an early hypoactivation is already observed in our sample, as has been reported previously (MacMillan et al., 2009).

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Although subjects exposed to CM remained hyporeactive under acute stress, in terms of HPA-axis activity, they experienced a significant increase in perceived anxiety, equivalent to that experienced by those not exposed to CM. This reveals a clear dissociation between anxiety perception and the physiological response to stressful situations in young people with CM, which might impair their ability to manage appropriately and cope with everyday emotionally negative situations (Liu et al., 2012). Notably, emotion regulation deficits have been suggested as a key pathway linking CM with psychopathology (Dvir, Ford, Hill, & Frazier, 2014; Hart et al., 2018). Further studies are required to explore which biomarkers other than cortisol might be linked with heightened anxiety in subjects exposed to CM (Quidé et al., 2019). Our results further suggest that, although participants with a current psychopathology tended to have lower cortisol levels in general, the HPA-axis alterations in subjects exposed to CM were present in both subjects with and without a current psychopathology. Contrary to some previous findings, in our sample neither pubertal stage (Gunnar, Wewerka, Frenn, Long, & Griggs, 2009) nor sex (Trickett et al., 2014) interacted with CM to predict HPA-axis reactivity during the TSST-C.

Furthermore, besides the impact of CM on HPA-axis activity and increased levels of anxiety (trait and state), our findings also showed that the severity and frequency of CM play a key role, thereby supporting a dose–response relationship (Anda et al., 2006). Thus, in line with Trickett et al. (2014), subjects exposed to more severe or more frequent forms of CM manifested the most subdued HPA-axis responses under basal conditions and in response to psychosocial stress; notably, Trickett et al. considered severity as the accumulation of different types of CM, rather than according to the specific characteristics of the experiences suffered. These findings warn of the deleterious impact that milder forms of CM may have once they become chronic. This is important as children who experience mild CM are often not detected or receive less clinical and social care (Humphreys, 2020). Furthermore, it seems that these children start showing higher levels of perceived anxiety before there is a marked biological dysregulation, offering a window of opportunity for early detection and intervention. Hence, the use of accurate child screening instruments at subclinical stages should be generalized, since most children are only identified once they already have severe psychiatric symptomatology (Bailhache, Leroy, Pillet, & Salmi, 2013). Moreover, since dysfunction in neurobiological systems negatively impacts treatment outcomes, youth with CM may also require specific treatment adapted to their condition (Tyrka, Burgers, Philip, Price, & Carpenter, 2013).

The methodology used in the present study includes a wide range of CM experiences reported from different sources, since there is often a substantial gap between subjects identified in informant-based studies and self-report assessments (Baldwin, Reuben, Newbury, & Danese, 2019). Thus, our findings suggest that participants with a suspected history of CM identified by clinicians show the same HPA-axis dysfunctions as subjects with a confirmed history of CM. Likewise, given that CM studies may lack sensitivity when the experiences are not qualitatively assessed (via the severity and frequency of exposure), key information may be lost and findings distorted. This highlights the need for specific training of clinicians in child psychiatric and pediatric services, so CM assessment can be routinely implemented, despite the time and effort required to perform such complex assessments (Zeanah & Edm, 2018).

Although prior evidence suggests that exposure to CM during middle childhood has the greatest effects on emotional dysregulation (Dunn, Nishimi, Gomez, Powers, & Bradley, 2018), it is difficult to pinpoint the exact developmental period when HPA-axis functioning is disrupted. Future research should incorporate more detailed information about the timing and proximity of CM to delineate vulnerable periods (Andersen & Teicher, 2008). It would be interesting to study the clinical course of the children to identify possible risk and protective factors for the future onset of psychopathology. A more dimensional approach focused on symptom dimensions might reveal varying patterns of adrenocortical regulation (Cicchetti & Rogosch, 2001). It is important to note that CM is not a phenomenon that can be studied in isolation, since both its causes and consequences are systematic and there are many factors that must be taken into account in order to fully understand it.

The blunted reactivity observed in our study supports plausible habituation, i.e. chronic exposure to stress may be linked with an adaptive desensitization to new stressors over time (Murali & Chen, 2005). These latent neurobiological alterations could drive an increased vulnerability to psychopathology during childhood and adolescence (Busso et al., 2017), which may persist, leading to the onset of a wide range of psychiatric conditions in adulthood (Kudielka & Wüst, 2010). Other factors with the potential to moderate the consequences of CM should also be taken into account, such as the type of CM suffered, the relationship with the abuser, social support received, and coexistence of other types of trauma such as bullying (Arseneault, 2018), domestic violence (Osofsky, 2018), or recent stressful life events (March-Llanes, Marqués-Feixa, Mezquita, Fañanás, & Moya-Higueras, 2017).

One of the limitations of the current study is the methodology used for assessing the presence and characteristics of CM exposure. Widely used questionnaires such as the CTQ cannot be administered to children younger than 12 years; indeed, there is no validated questionnaire to assess the presence of CM in the 7–17 years range. The main reason behind this is that younger children have a limited understanding of their own exposure, since they are still cognitively immature. Thus, any assessment of CM in this vulnerable population needs to be adjusted to maximize the reliable information that can be captured from the different informants (not only the child) and, at the same time, to minimize the trauma that the interview itself can represent to a victimized child. Thus, use of TASSCV allows the proper assessment of children and adolescents exposed from milder to severe forms of CM, which would have otherwise not been identified. Unfortunately, use of TASSCV requires a longer time for a proper assessment together with the gathering of information from multiple informants, which might make it more challenging to use than simply relying on short self-administered questionnaires such as the CTQ or considering only the most severe children already detected by social services. Since most of the sample was recruited in psychiatric units, there is an unusually high proportion of ADHD cases in the non-CM group; thus, our findings might not be generalizable to other populations. At the same time, the majority of CM-exposed subjects suffered from some sort of psychiatric condition, while most participants non-exposed to CM had no psychopathological history. Further research including a higher proportion of subjects exposed to CM with no psychiatric symptomatology (i.e. resilient) is required to disentangle the role of CM in the development of HPA-axis disturbances and whether the later precede the onset of psychiatric disorders.

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Conclusions

CM affects multiple domains of life such as intimate relationships, violence and criminal offending, employment, drug abuse, and physical and mental health (Hughes et al., 2017). It is a serious global health problem with staggering long-term economic costs (Thielen et al., 2016). This study is intended to raise awareness of the biological and clinical repercussions of CM during or proximally to exposure, encouraging clinicians to ask patients about CM history and to respond accordingly, seeking therapeutic alternatives to manage acute stress better. Children exposed to CM and attended in child protection units, child psychiatric, or pediatric units are still at a sensitive period of neurological, cognitive, social, and emotional development, during which high-quality interventions can make an important difference and shift the balance between risk and protective factors (Chinitz, Guzman, Amstutz, Kohchi, & Alkon, 2017). Thus, family psychotherapeutic interventions have the potential to normalize HPA-axis function if implemented promptly (Gunnar, DePasquale, Reid, Donzella, & Miller, 2019).

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S003329172100249X

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EPI-Young Stress GROUP. María Martín[9], Eulalia Anglada[12], Pilar Santamarina[2,3,4], Patricia Rubio[6], Iría Méndez[2,3,4], Dolores Moreno[2,6], María Ramos[2,6], Jorge Vidal[2,6], Juanjo Carballo[2,6], Elena Font[2,3,4], Lydia Gayubo[11], Laura Colino[11], María Rodrigo-Yanguas[11], Maddi Laborde[8], Jaume March-Llanes[5].

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UNIVERSITAT DE BARCELONA

Dr. Lourdes Fañanás Saura
Section of Zoology and Biological Anthropology
Dept. Evolutionary Biology, Ecology and Environmental Sciences
Faculty of Biologia, Universitat de Barcelona

Supervisor's report on the contribution of the PhD applicant to the article.

Prof. Dr. Lourdes Fañanás Saura, full professor at the Department of Evolutionary Biology, Ecology and Environmental Sciences of the Faculty of Biology (Universitat de Barcelona) and supervisor of the present doctoral thesis by Águeda Castro Quintas, hereby certifies that Laia Marques Feixa included also the article "*Childhood maltreatment disrupts HPA-axis activity under basal and stress conditions in a dose-response relationship in children and adolescents*" on her doctoral thesis defended on the Universitat de Barcelona, and the participation of the PhD applicant in the article included the following tasks:

- Participation in the conception and design of the study
- Laboratory analyses
- Help with the statistical analyses
- Critical revision of the article for intellectual content

Signed by Prof. Lourdes Fañanás

Barcelona, 5th December 2023

3.5. Secretory immunoglobulin A (s-IgA) reactivity to acute psychosocial stress in children and adolescents: the influence of pubertal development and history of maltreatment

Laia Marques-Feixa*, **Águeda Castro-Quintas***, Helena Palma-Gudiel, Soledad Romero, Astrid Morer, Marta Rapado-Castro, María Martín, Iñaki Zorrilla, Hilario Blasco-Fontecilla, Maite Ramírez, María Mayoral, Íria Méndez, Nerea San Martín-González, María Rodrigo-Yanguas, José Luís Monteserín-García, Lourdes Fañanás y EPI-Young Stress GROUP

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Secretory immunoglobulin A (s-IgA) reactivity to acute psychosocial stress in children and adolescents: The influence of pubertal development and history of maltreatment

Laia Marques-Feixa [a,b,1], Águeda Castro-Quintas [a,b,1], Helena Palma-Gudiel [b,c], Soledad Romero [b,d,e], Astrid Morer [b,d,e], Marta Rapado-Castro [b,f,g], María Martín [h], Iñaki Zorrilla [b,i], Hilario Blasco-Fontecilla [b,j], Maite Ramírez [k], María Mayoral [b,f], Iría Mendez [d], Nerea San Martín-Gonzalez [a], María Rodrigo-Yanguas [j], José Luis Monteserín-García [a,b], Lourdes Fañanás [a,b,*], EPI-Young Stress GROUP

[a] Department of Evolutionary Biology, Ecology and Environmental Sciences, Faculty of Biology, University of Barcelona, Biomedicine Institute of the University of Barcelona (IBUB), Barcelona, Spain
[b] Network Centre for Biomedical Research in Mental Health (CIBERSAM), Spain
[c] College of Public Health and Health Professions, Department of Epidemiology, University of Florida, USA
[d] Department of Child and Adolescent Psychiatry and Psychology, 2017SGR88, Institute of Neuroscience, Hospital Clínic de Barcelona, Barcelona, Spain
[e] Institut d'Investigacions Biomediques August Pi i Sunyer (IDIBAPS), Barcelona, Spain
[f] Department of Child and Adolescent Psychiatry, Institute of Psychiatry and Mental Health, Hospital General Universitario Gregorio Marañón, School of Medicine, Universidad Complutense, IiSGM, Madrid, Spain
[g] Melbourne Neuropsychiatry Centre, Department of Psychiatry, The University of Melbourne & Melbourne Health, Victoria, Australia
[h] Hospital Benito Menni, Adolescent Crisis Unit, Sant Boi de Llobregat, Spain
[i] Hospital Santiago Apostol, Department of Psychiatry, Vitoria-Gasteiz, Spain
[j] Department of Psychiatry, Puerta de Hierro University Hospital-Majadahonda, Autonoma University, ITA Mental Health, Madrid, Spain
[k] Day Hospital for Adolescents Barrualde-Galdakao, Child and Adolescent Mental Health, Galdakao, Spain

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ABSTRACT

Background: Mucosal secretory immunoglobulin A (s-IgA) is an antibody protein-complex that plays a crucial role in immune first defense against infection. Although different immune biomarkers have been associated with stress-related psychopathology, s-IgA remains poorly studied, especially in youth.
Objectives: The present study investigated how s-IgA behaves in front of acute psychosocial stress in children and adolescents, including possible variability associated with developmental stage and history of childhood maltreatment (CM).
Methods: 94 children and adolescents from 7 to 17 years (54 with a current psychiatric diagnostic and 40 healthy controls) drawn from a larger Spanish study were explored (EPI-Young Stress Project). To assess biological reactivity, participants provided five saliva samples during an acute laboratory-based psychosocial stressor, the Trier Social Stress Test for Children (TSST-C). Samples were assayed for s-IgA, as well as for cortisol. Pubertal development was ascertained by Tanner stage and CM following TASSCV criteria.
Results: We observed s-IgA fluctuations throughout the stressor, indicating the validity of TSST-C to stimulate s-IgA secretion ($F(4,199) = 6.200$, $p < .001$). Although s-IgA trajectories followed a reactivity and recovery pattern in adolescents, children exhibited no s-IgA response when faced with stress ($F(4,197) = 3.406$, $p = .010$). An interaction was found between s-IgA and CM ($F(4,203) = 2.643$, $p = .035$). Interestingly, an interaction between developmental stage, CM history and s-IgA reactivity was identified ($F(12,343) = 2.036$, $p = .017$); while children non-exposed to maltreatment exhibited no s-IgA changes to acute stress, children with a history of CM showed a similar response to adolescents, increasing their s-IgA levels after the psychosocial stressor.

* Corresponding authors at: Faculty of Biology, University of Barcelona, Av Diagonal 643, 2n A, Barcelona, Spain.
E-mail address: lfananas@ub.edu (L. Fañanás).
[1] These authors contributed equally to this work.

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Conclusion: Acute psychosocial stress stimulates s-IgA secretion, but only after puberty. However, children with a history of maltreatment exhibited a response resembling that of adolescents, suggesting an early maturation of the immune system. Further studies are needed to clarify the validity of s-IgA as an acute stress biomarker, including additional measures during stress exposure.

1. Introduction

Exposure to stress leads to activation of various biological processes that are aimed at mounting an effective response to a threatening situation and to later restore homeostasis once the stressor has ended. Physiological changes involved in stress response are fundamentally orchestrated by the sympathetic nervous system (SNS) and the hypothalamic–pituitaryadrenal (HPA) axis. Each of these systems involves a quick adaptive response, within minutes or hours, which is known as "fight or flight response". This response prepares the system to detect danger as well as to provide the energy required to survive (Sapolsky et al., 2000; Segerstrom and Miller, 2004). Among others, the SNS activates the immune system, characterized by the activation of inflammatory processes, which could accelerate wound repair and help prevent infections from taking hold (Godoy et al., 2018).

In controlled settings, several studies have documented an increase in certain inflammatory biomarkers such as cytokines following laboratory-induced psychological stress (Steptoe et al., 2007). Although blood sampling is the gold standard to determine levels of inflammatory biomarkers, there is an increasing interest in the ability to assess biological markers of stress reactivity in saliva, a less invasive, cheaper and safer biospecimen that enables sample collection many times per day (Szabo et al., 2020). Salivary levels of pro-inflammatory cytokines such as interleukin (IL)-6, tumor necrosis factor (TNF)-α, and IL-1β have already been found to increase in response to acute stress (Slavish et al., 2015). In this context, secretory Immunoglobulin A (s-IgA), the predominant immunoglobulin in mucosa, has emerged as a promising psychological biomarker of stress exposure due to its key role as a fast first-line immune defense that also provides oral protection from pathogens (Nurkka et al., 2003; Staley et al., 2018).

S-IgA secretion is under strong neuroendocrine control. Several studies support that, in adult populations, s-IgA increased after acute stress exposure (Campisi et al., 2012; Trueba et al., 2012). Specifically, Benham (2007) observed that s-IgA reached a significant increase 6 min after an acute psychological stressor and decreased during the first minutes of the recovery period, while cortisol was still increasing. This rapid response could be explained by an activation of the sympathetic nerves that innervate salivary glands, which enhances s-IgA output. However, very little research on s-IgA has explored antibody release during earlier stages of life such as childhood and adolescence (Castro-Quintas et al., 2022), when s-IgA levels have not yet reached those of adulthood (Sonesson et al., 2011). Additionally, the crosstalk between the neuroendocrine and the immune systems (e.g. cortisol reactivity) is still developing and under the influence of the psychosocial environment during this period (Gunnar et al., 2009).

The most common laboratory-induced psychosocial acute stress protocols may include mental arithmetic tasks, public speaking or cognitive interference tasks. In the case of children and adolescents, the Trier Social Stress Test for Children (TSST-C) is the protocol for inducing stress most recognized and widely used, and it has been shown to reliably trigger the activation of different biological systems (Allen et al., 2017; Wu et al., 2019). However, only one study in the literature explored s-IgA reactivity during TSST-C in children and adolescents. This study supported that youths (from 7 to 17 years old) displayed s-IgA reactivity to and recovery from acute stress (Laurent et al., 2015).

Moreover, when a stress stimulus is prolonged in time, a dysregulation of biological systems may occur leading to brain alterations and physiological disruptions that negatively impact health. This exposure can be particularly harmful during early stages of life leading to more profound and long-lasting effects on the regulation of stress response systems further influencing the vulnerability to develop mental disorders (Oh et al., 2018). Also, individuals experiencing chronic stressors have less effective immune functioning, experiencing nonspecific inflammation, having higher susceptibility to adverse health outcomes, such as vascular disease, autoimmune disorders, and premature mortality (Miller et al., 2011; Wan et al., 2022).

There are several potential pathways leading to a pro-inflammatory state after the exposure to stressors during young age, such as childhood maltreatment (CM) (Danese et al., 2017). Hunter et al., (2011) described an increase of cortisol reactivity in infants (0–5 years) exposed to adverse experiences. Conversely, chronic stressors dysregulate the acute stress response, leading, for example, to a blunted cortisol response. However, less is known about s-IgA alteration after adverse experiences.

This study intends to characterize the variability in s-IgA responses to psychosocial stressors from childhood to adolescence and aims to explore the influence of developmental stage and history of CM on s-IgA response to stress. We hypothesize that adolescents will show higher s-IgA levels than children throughout TSST-C and that participants exposed to CM will show a blunted response to TSST-C compared to non-exposed to CM, following a similar pattern to their cortisol response during TSST-C. We also hypothesize that s-IgA increase and recovery pattern will both be faster than cortisol's.

2. Materials and Methods

2.1. Sample and procedure

Participants were 94 youths aged 7–17 (54 had been diagnosed with a current psychiatric disorder and 40 were healthy controls). Participants in this study were a subset of a larger study cohort (*EPI_young_stress project*) recruited from April 2016 to March 2020 (Marques-Feixa et al., 2021). Participants were eligible for the subset analysis based on availability of data on primary predictors and outcomes of interest. Youths with a current psychiatric diagnosis were recruited from six child and adolescent mental health units in Spain. Healthy controls were recruited at the University of Barcelona or in the psychiatric units via advertisements, primary healthcare centres, schools and other community facilities. Exclusion criteria for all participants included diagnosis of an autism spectrum disorder, an eating disorder with Body Mass Index (BMI) < 18, intellectual disability (IQ < 70), current drug dependence, not being fluent in Spanish, extreme premature birth (<1500 g), head injury with loss of consciousness, and severe neurological or other pathological conditions (such as epilepsy, cancer or autoimmune diseases). The Ethical Review Board of each hospital and university involved in the project approved this study.

Families were explicitly informed of the voluntary nature of the study, their rights, and the procedures, risks and potential benefits involved. Written consent was required from parents/legal guardians. The children provided written assent after the nature of the procedure had been fully explained. Participants and their parents or legal guardians were interviewed separately, face to face, by a trained psychologist or psychiatrist to obtain sociodemographic and medical data, and to explore the CM history. A second appointment on a later date was scheduled at 4 PM to perform the Trier Social Stress Test for Children (TSST-C) at each corresponding research centre. Further details about the nature of the study have been described elsewhere (Marques-Feixa et al., 2021).

2.1.1. Trier social stress Test for children (TSST-C)

The TSST-C is the acute psychosocial stress protocol most widely used in children and adolescents, and it has been shown to reliably trigger the activation of different biological systems (Buske-Kirschbaum et al., 1997). To avoid circadian rhythm variability in biomarkers, participants were scheduled at 4:00 pm (Kudielka et al., 2004). Briefly, upon arrival at the research center each participant rested for 30 min in a quiet room accompanied by a familiar researcher. After this resting period, the participant entered an experimental room where a panel of two unfamiliar judges (a woman and a man) wearing lab coats awaited sitting behind a table. The judges were instructed to maintain a neutral stance throughout the TSST-C and to avoid giving any kind of positive feedback to the participants. The judges explained the nature of the tasks to the participant, highlighting that they would be videotaped to analyze their performance afterwards, and that they were expected to be the best. During the first task (speech task), the participants had 5 min to think of an end of a story explained by experts and 5 min for freely telling their end for the story in front of a microphone. The second task (arithmetic task) consisted of a five-minute long serial subtraction (2 from 421 in children from 7 to 12 years old, and 3 from 758 in adolescents from 13 to 17 years old). Whenever a participant made a mistake, a judge asked them to start over. Participants spent around 20 min in the experimental room. After the stress tasks, participants returned to the quiet room with the familiar researcher for an additional 30-minute recovery period. The entire procedure lasts 80 min (further details can be found in the Supplementary Material of Marques-Feixa et al. (2021)).

Five saliva samples were collected during this procedure: 30 min before the stressor (T1), immediately before the stressor (T2), immediately after the stressor (T3), 15 min after the stressor (T4), and 30 min after the stressor (T5) (see Fig. 1). All the participants were given a series of instructions to avoid factors that have been reported to influence biomarkers levels. Specifically, they were told to refrain from eating or drinking (with the exception of water) for two hours before the TSST-C; to refrain from intense physical activity for 24 h, and not to take benzodiazepines that day; to refrain from smoking for 1 h before; not to consume alcohol or caffeine in the 24 h preceding the TSST-C (Kudielka et al., 2009). The day of the protocol participants were asked about their current health status.

2.2. Measures

2.2.1. Developmental stage and current psychopathology

Pubertal development was ascertained by Tanner stage questionnaire (Morris and Udry, 1980), which was used to classify the participants as either children (Tanner stages 1–3) or adolescents (Tanner stages 4–5). Psychopathology was ascertained using the Spanish version of the Schedule for Affective Disorders and Schizophrenia for School-Age Children: Present and Lifetime Version DSM-5 (K-SADS-PL-5) (APA: American Psychiatric Association, 2013; De la Peña et al., 2018). Diagnoses dimensions are depicted in Table 1.

Table 1
Sociodemographic and anthropometric data of participants (n = 94).

Variables		Value
Age - mean (Sd) [range]		13.8 (2.4) [7–17]
Sex – n (%)	Female	56 (60%)
	Male	38 (40%)
Pubertal stage – n (%)	Child (Tanner stage 1–3)	47 (50%
	Adolescent (Tanner stage 4–5)	47 (50%)
Cultural origin– n (%)	European	78 (83%)
	Others [a]	16 (17%)
Socioeconomic status (SES)- mean (Sd) [range] [b]		40.4 (17.9) [8–66]
Current psychiatric diagnosis status – n (%)	Subjects without current psychiatric diagnosis	40 (43%)
	Subjects with current psychiatric diagnosis [c]	54 (57%)
History of childhood maltreatment (CM) – n (%)	Without history of CM	44 (47%)
	With history of CM	50 (53%)
Current infection – n (%)	No	78 (83%)
	Ambiguous	9 (10%)
	Sick or cold	7 (7%)
Body mass index (BMI) [d] mean (Sd) [range]		21.1 (4.3) [12–34]
BMI-for-age percentile [e] – n %	Underweight	4 (4.6%)
	Healthy weight	59 (67.8%)
	Overweight	10 (11.5%)
	Obesity	14 (16.1%)

[a] Other cultures included Latin American (69%), Maghrebin (19%), and others (12%).
[b] Socioeconomic status (SES) was assessed based on the Hollingshead Four-Factor Index (Hollingshead, 1975), ranging from 8 to 66, with higher scores reflecting higher SES. This analysis was conducted with 92 subjects.
[c] Diagnoses dimensions of the primary psychiatric disorder: Attention-deficit/hyperactivity disorder (27%), Affective disorders (24%), Trauma and stress-related disorders (19%), Anxiety disorders (13%), Behavioral disorders (9%), Psychotic disorders (6%) and Eating disorders (2%).
[d] This analysis was conducted with 87 subjects.
[e] BMI-for-age percentile was calculated based on clinical growth charts for children and teens aged between 2 and 19 years. For calculating it, we considered the precise months of age. Following clinical growth chart criteria participants were classified considering their percentile as: <5th, underweight; ≥5th to 84th, healthy weight; ≥85th to 94th, overweight, and ≥ 95th, obese. This analysis was conducted with 87 subjects.

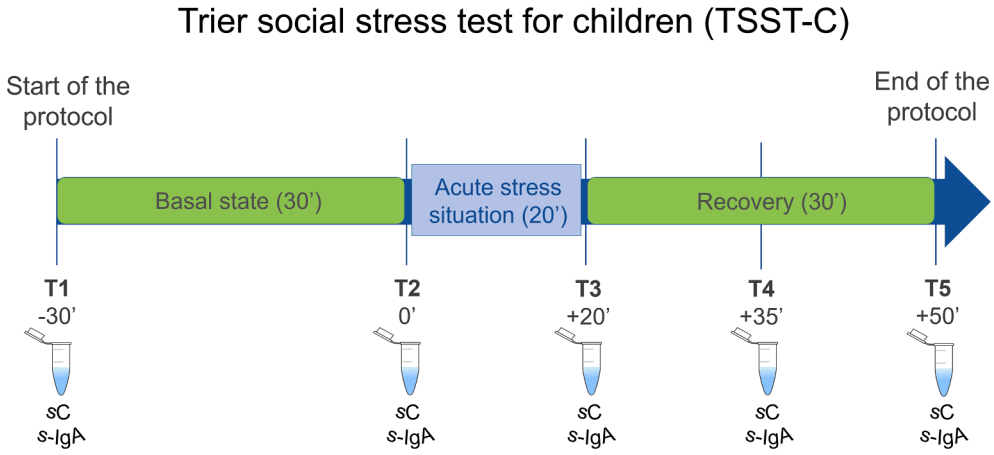

Fig. 1. Summary of the Trier Social Stress Test for Children (TSST-C) protocol.

2.2.2. Childhood maltreatment (CM)

The participants and their parents/legal guardians were evaluated by trained psychologists by means of an exhaustive interview following the criteria of the instrument "Tool for assessing the severity of situations in which children are vulnerable" (TASSCV) (CARM, 2012) (available online in Spanish). Previously, reports from social services or teachers were reviewed, where applicable. In addition, the information was ascertained through questionnaires answered by participants. Adolescents who were older than 12 were administered the self-report versions of the Childhood Trauma Questionnaire short version (CTQ-SF) (Bernstein et al., 2003) and the Childhood Experience of Care and Abuse Questionnaire (CECA-Q2) (Kaess et al., 2011), while participants aged 7–11 answered an adapted *ad-hoc* hetero-administered questionnaire (for details see Supplementary Material of Marques-Feixa et al., (2021)). The CTQ and CECA-Q2 were used as complementary information to determine presence and type of CM.

In summary, CM history was coded by clinicians according to the TASSCV criteria. Every subtype of CM included in the present study (emotional neglect, physical neglect, emotional abuse, physical abuse and sexual abuse) was coded as either: i) non-existent (no indicators of risk for a vulnerable situation), ii) suspect (when there was no conclusive evidence, but there were clear signs of risk that arouse suspicion), or iii) confirmed (clear evidence of it). Confirmed and suspected histories of CM were combined into the same category for downstream analysis.

2.2.3. s-IgA And cortisol determination

Saliva samples were collected by cotton oral swabs (Salimetrics) and were immediately stored at −20 °C for a maximum of 3 months. Before s-IgA and cortisol determination, the tubes were thawed and centrifuged, following the manufacturer's instructions, to remove debris from the saliva. Salivary s-IgA and cortisol concentration were determined using a high sensitivity enzyme-linked immunosorbent assay (ELISA) (commercial kit Salimetrics, LLC, State College, PA). Samples were tested in duplicate and the mean was calculated (μg/dL). The lower limit of sensitivity of s-IgA was 0.025μg/dL and of cortisol was 0.007μg/dL. Cortisol concentrations at any timepoint with a coefficient of variation (%CV) higher than 30% were determined in duplicate for a second time. Whenever this happened, the final cortisol value used for downstream analysis was the mean of the two measurements obtained in the duplicate (i.e., initial measurements were disregarded due to high variability). Two samples out of 470 (0.4%) still had CV > 30% after performing duplicates. Regarding s-IgA, only 10 samples had CV > 15%, of which only 2 had CV > 30%. No s-IgA duplicates were performed. For more details in sample %CV, please see Supplementary Table S3.

2.3. Data analysis

Analyses were conducted using SPSS 26.0. Salivary concentration of both s-IgA and cortisol were log10 transformed to fulfill the requirements for normal distribution in statistical analyses.

To determine the effect of developmental stage and CM in s-IgA fluctuation during TSST-C, mixed-effects models with a random effect of intercept and a random slope of time, were employed (Model 1). Time factor had five categories (time-points) and the interaction with time was considered the main effect of interest of the model. In addition, simple effects tests were performed to evaluate the specific timepoint interaction between groups. In a second step, a post-hoc analysis (Model 2) was conducted to test differential effect of CM history according to the developmental stage, entering a new factor that combines the developmental stage and the history of CM: (1) non-maltreated children, (2) children exposed to CM, (3) non-maltreated adolescents, and (4) adolescents exposed to CM. Considering that cortisol strongly influences s-IgA levels (Guzmán-Mejía et al., 2021; Stojanović et al., 2021); cortisol measures were included in the mixed model as covariates to adjust for cortisol levels at each corresponding time-point during TSST-C. Thus, to account for the possible confounding influence of cortisol variability, sex, current psychopathological status, and current infection (none, ambiguous or definitely sick-cold), these covariates were included in both statistical models. There were not missing data in any of the variables of interest. We have also included results of s-IgA fluctuations without cortisol correction, detailed in Supplementary material.

To determine the effect of developmental stage and CM in cortisol fluctuation during TSST-C, the same analyses were conducted (Model 3 and Model 4). The s-IgA was not considered as covariate since s-IgA secretion is limited to mucosal tissues and cortisol production occurs in the adrenal gland, so we did not consider that s-IgA influenced cortisol. These two analysis are detailed in Supplementary material, as cortisol fluctuations during TSST-C are described in detailed in a previous study (Marques-Feixa et al., 2021). All tests were two-tailed with significance defined as p-value < 0.05.

3. Results

Sociodemographic and anthropometric data of participants are presented in Table 1.

As depicted in Fig. 2, the s-IgA levels fluctuated significantly during the TSST-C ($F(4,199) = 6.200, p \leq 0.001$), indicating the validity of this acute psychosocial stressor to stimulate s-IgA secretion in the present sample (Model 1). Developmental stage was significantly associated with overall s-IgA levels ($F(1,82) = 6.710, p = .011$), reflecting higher s-IgA concentrations throughout the entire TSST-C procedure in adolescents when compared to children (similarly to higher overall cortisol levels observed in adolescents, detailed in Supplementary material). Furthermore, a significant interaction between developmental stage and time was identified ($F(4,197) = 3.406, p = .010$), indicating different trajectories of s-IgA levels between children and adolescents (not observed in cortisol fluctuations, see Supplementary material). Specifically, the simple effects analysis of s-IgA revealed a timepoint-specific interaction at T2 (immediately before the stressful situation) ($F = 8.545, p = .004$), T3 (immediately after the stressful situation) ($F = 12.429; p = .001$), T4 (15 min after the stressful situation finished) ($F = 4.89, p = .029$) and at T5 (30 min after the stressful situation finished) ($F = 4.647, p = .033$). In adolescents, s-IgA levels started to increase immediately before the acute stress, and continued rising immediately after the end of the stress task to finally return to basal s-IgA levels during the recovery period, while children showed no s-IgA changes throughout the protocol. Regarding s-IgA fluctuation through TSST-C, children did not show significant differences. However, adolescents showed a significant increase between T1- T2 (p = .033) and T1-T3 (p < .001), although not significant differences were observed between T2-T3. Between T3-T4 (during the 15 min after the end of the stressor) s-IgA decreased (p < .001) (see Fig. 2 and Table 2).

Additionally, a significant interaction between time and maltreatment was observed ($F(4,203) = 2.643, p = .035$). However, simple effects test did not reveal any significant timepoint-specific interaction. Thus, a second approach (Model 2) was performed to explore simultaneously developmental stage and maltreatment history. A different s-IgA trajectory across the TSST-C was observed between groups ($F(12,343) = 2.096, p = .017$) (see Tab. 2 and Fig. 3). Specifically, in T2 (immediately before stressor) children (both exposed and non-exposed to maltreatment) showed lower s-IgA levels when compared with adolescents without maltreatment ($p = .021$, $p = .004$, respectively). However, after the acute stressor only children non-exposed to maltreatment showed lower s-IgA levels compared with all other groups [children exposed to maltreatment, adolescents exposed to maltreatment and adolescents non-exposed to maltreatment, respectively (T3 ($p = .039$, $p = .001$, $p < .001$) and T4 ($p = 0.50$, $p = .012$, $p = .013$))]. In addition, in T5 non-maltreated adolescents showed higher s-IgA levels when compared with non-maltreated children ($p = .014$). Furthermore, regarding s-IgA fluctuation throughout TSST-C, children non-exposed to CM did not show significant differences, while children exposed to CM had a non-significant increase of s-IgA between T2 and T3 (p = .070).

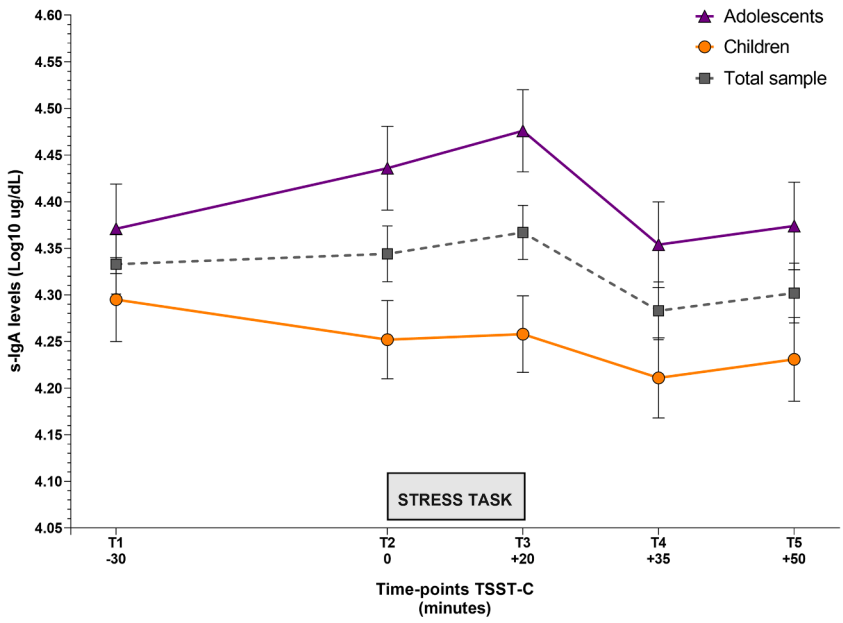

Fig. 2. s-IgA fluctuations during TSST-C in whole sample and according to developmental stage (Model 1). Error bars SE.

Table 2
Mixed-model analysis for s-IgA levels (Model 1 and Model 2).

		Developmental stage				Developmental stage according to CM history					
		Children (n = 47) (Mean, SD)	Adolescents (n = 47) (Mean, SD)	*F* (*p*) [a]	*F* (*p*) [b]	Non-maltreated children (n = 25) (Mean, SD)	Children exposed to CM (n = 22) (Mean, SD)	Non-maltreated adolescents (n = 19) (Mean, SD)	Adolescents exposed to CM (n = 28) (Mean, SD)	*F* (*p*) [a]	*F* (*p*) [b]
s-IgA levels during TSST-C (μg/dL log-transformed)	T1	4.30 (0.045)	4.37 (0.048)	3.406 ** (0.010)	1.274 (0.261)	4.30 (0.062)	4.30 (0.066)	4.36 (0.071)	4.37 (0.066)	2.096* (0.017)	0.361 (0.789)
	T2	4.25 (0.042)	4.44 (0.045)		8.545** (0.004)	4.23 (0.057)	4.27 (0.061)	4.49 (0.065)	4.39 (0.061)		3.292* (0.023)
	T3	4.26 (0.041)	4.48 (0.044)		12.429*** (0.001)	4.18 (0.056)	4.35 (0.061)	4.51 (0.065)	4.46 (0.061)		5.905*** (0.001)
	T4	4.21 (0.043)	4.35 (0.046)		4.899* (0.029)	4.13 (0.060)	4.30 (0.065)	4.36 (0.069)	4.36 (0.064)		3.022* (0.032)
	T5	4.23 (0.045)	4.37 (0.047)		4.647* (0.033)	4.17 (0.062)	4.29 (0.067)	4.41 (0.071)	4.35 (0.065)		2.275 (0.082)

CM: childhood maltreatment.
[a] Mixed-model.
[b] Simple effects tests in the context of mixed-model.
p values: *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001.

Adolescents non-exposed to CM showed an increase before the stress, between T1-T2 (p =.021), after the TSST-C, specifically between T1-T3 (p =.006) but not between T2-T3 and a decrease between T3-T4 (p =.003). Similarly, adolescents non-exposed to CM showed and increase between T1-T3 (p =.045), a tendency to increase between T2-T3 (0.064) and a decrease between T3-T4 (p =.015), although they did not show an increase before the stressor, between T1-T2. With the exception of cortisol measures throughout TSST-C, none of the covariates (sex, current psychopathology and current infection) were significant in either Model 1 or Model 2 (for more information, see Supplementary material). Similar results were obtained in the analyses non—adjusted for cortisol levels (see Supplementary material).

4. Discussion

The present study indicates that s-IgA measurement constitutes a feasible biomarker to explore peripheral immunological reactivity to stress in young populations. In particular, we observed that, although children and adolescents showed similar s-IgA basal levels, their s-IgA stress reactivity seemed to differ. Adolescents showed an increase after the stressor and a rapid recovery, while children did not show an s-IgA response. Nevertheless, we observed that children exposed to CM exhibited an s-IgA pattern more similar to that of adolescents. To the best of our knowledge, this is the second paper to assess s-IgA response to stress in children and teens and the first one to do so in a young population exposed to CM. Therefore, evidence of s-IgA functioning in young populations is scarce and warrants further inquiry (Castro-Quintas et al., 2022).

Our findings are consistent with the only existing study exploring acute stress response in children and adolescents (Laurent et al., 2015). However, this previous research did not directly compare s-IgA reactivity between children and adolescents. In this regard, our study reveals that there is no s-IgA response to psychosocial stress before puberty. Differences observed could be due to the stressor task not being powerful enough for children to activate their s-IgA secretion. However, a perceived anxiety test administered in this sample during the TSST-C

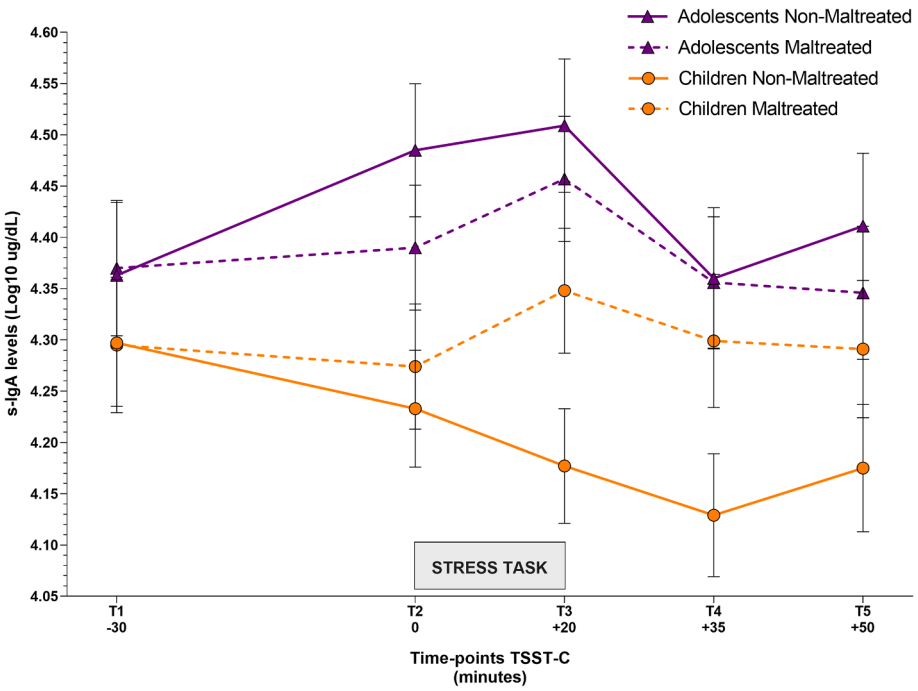

Fig. 3. s-IgA trajectories according to the developmental stage, and the history of CM (Model 2): (1) non-maltreated children, (2) children exposed to CM, (3) non-maltreated adolescents, and (4) adolescents exposed to CM. Error bars SE.

procedure, revealed that participants rated the session as equally stressful independent of developmental stage (Marques-Feixa et al., 2021). Accordingly, literature supports that puberty is one of the most sensitive periods of life with regard to immune system reprogramming by stress (Csaba, 2020), since children are born with an undeveloped and adaptable immune system, which matures and acquires memory as they grow (Simon et al., 2015). However, little is known about when this system becomes responsive to psychosocial cues. Our results here suggest that children's immune system may not respond to acute stress, in comparison to adolescents, although their self-perceived stress or their HPA axis may (Marques-Feixa et al., 2021). Interestingly, the functioning of biological systems are known to be mediated by both intrinsic and environmental factors (Seltzer et al., 2010). Accordingly, Ulmer-Yaniv et al., (2018) supports that during early infancy, children's immune system regulation relies on maternal health and interaction rather than on other environmental signals. Additionally, it has been proposed that biological response to stress may be associated to general cognitive functioning and the development of social cognition (Van Den Bos et al., 2016). It would be of great interest to understand how the brain, depending on the developmental stage, detects psychosocial stress signals and activates different biological systems to respond accordingly.

In this regard, history of CM seems to alter s-IgA response during the TSST-C. Danese et al. (2017) observed that CM is a threatening situation that can be linked to danger and may co-occur with physical abuse, which can facilitate pathogen infection that, in turn, can induce inflammation and damage. Specifically, we observed that children exposed to CM showed a heightened immunological stress response with a pattern equivalent to that exhibited by adolescents. Although the ability to deal with threatening situations is a hallmark of adolescence, CM could make children more aware of potentially dangerous situations leading to an early activation of their stress response. Thus, the apparent advancement of s-IgA reactivity to psychosocial stress observed in children exposed to CM is consistent with accelerated biological aging in this group, as revealed by the epigenetic clock, telomere length and advanced pubertal timing (Chen et al., 2021; Colich et al., 2020). This is in line with human development theories that argue that early adverse environments may accelerate the onset of puberty to increase the opportunity for reproduction prior to possible mortality (Belsky, 2012); e.g., girls who are victims of sexual abuse have been described to experience a precocious puberty (Noll et al., 2017). However, in our study no differences in s-IgA response according to CM history were observed in the adolescent group. This is in contrast with previous studies reporting heightened inflammation in subjects exposed to early adversity and might reflect unique features of s-IgA as opposed to other immune markers such as C-reactive protein or interleukins.

Additionally, our findings suggest that HPA axis and the immune system follow independent maturation processes, since the HPA axis response to TSST-C in the same sample follows a similar pattern in children and adolescents (Marques-Feixa et al., 2021) contrary to our findings on s-IgA reactivity. Adolescents have higher s-IgA and cortisol levels when compared to children, suggesting an influence of pubertal hormones in overall immunoendocrinological levels. However, while cortisol response throughout TSST-C is fundamentally modified by CM, s-IgA response to the acute stressor is modified by developmental stage. Adolescents non-exposed to CM showed both cortisol and s-IgA responses. Both children and adolescents exposed to CM exhibited s-IgA response in front of stress, but no change in cortisol levels. Children non-exposed to CM did show a response for cortisol but they did not show a response for s-IgA. Further research is needed to clarify whether these changes are unique to maturation or may be indicative of early evidence of reprogramming due to stress.

Our findings also suggest that s-IgA increases in a short period after an acute psychosocial stress, highlighting its possible use as a non-invasive immune biomarker in youths. Specifically, we observed an s-IgA increase 20 min after the psychosocial stress was initiated followed by a fast return to basal levels 35 min after the beginning of the stressor. These results contrast with those found in our previous work on this sample, in which cortisol levels remained high after 35 min (Marques-Feixa et al., 2021). This is in line with a previous study based on undergraduate students exposed to the TSST, which reported that cortisol remained high 30 min after completing the stress task, but s-IgA levels had fully recovered by then (Campisi et al., 2012). This might indicate that the s-IgA response is released prior to cortisol and that it follows a faster fashion as reflected by its rapid increase and return to basal levels.

Thus, s-IgA and cortisol might be independent biomarkers providing complementary information that, when studied together, offer a comprehensive view of the stress response in humans. In future studies, it may be interesting to evaluate both cortisol and s-IgA simultaneously.

Some limitations should be noted. First, it would be interesting to increase the number of samples collected during the stressor in order to better understand the pattern of s-IgA response, since Benham (2007) described a peak of s-IgA levels 6 min after stress onset in young adults. Moreover, the only study based in youths found a peak of s-IgA levels 10 min after the stressor start (Laurent et al., 2015). Unfortunately, these intermediate measures were not collected in our study, which could have allowed us to better define s-IgA dynamics. Second, the methodology used to assess CM exposure (TASSCV) requires extensive interviews with multiple informants, and a longer time for administration when compared with the most used questionnaires in the field, which might not always be possible for clinicians in a daily setting. Of note, younger children have a limited understanding of their own exposure due to their cognitive immaturity. Additionally, widely used questionnaires, such as the CTQ or CECA-Q2, can not be administered to children younger than 12 years; indeed, there is no validated questionnaires to assess the presence of CM in the 7 to 17 years range. Thus, use of the TASSCV allowed the proper assessment of different types of CM exposure in the whole age range included in our study, which would have otherwise not been possible to explore. Third, the majority of participants with a history of CM also had a current psychiatric disorder, while most participants non-exposed to CM had no psychopathological history. Further research including a higher proportion of resilient youth (exposed to CM with no psychiatric disorders) would help disentangle the effect of both variables in the biomarkers analyzed. Fourth, although the TSST-C difficulty adaptation was determined by age, the analysis were conducted based on puberty development.

The inclusion of additional SNS biomarkers, such as alpha amylase, and epigenetic measures, such as DNA methylation, could provide a more comprehensive understanding of the complex crosstalk between the neuroendocrine and the immune systems (Martins et al., 2021). Furthermore, the study of other systemic inflammation biomarkers, such as CRP or interleukins, could help to elucidate the biological mechanisms that are responsible for linking higher inflammation to CM (Coelho et al., 2014; Entringer et al., 2020). Moreover, other stress biomarkers, such as cortisol, have been described to follow a non-linear pattern of stress reactivity through development (Gunnar et al., 2009). Thus, further studies should explore s-IgA reactivity patterns across all five Tanner stages to disentangle immune maturation across pubertal transition. Since it has been suggested that youth with more externalizing behaviors were characterized by attenuated and less dynamic s-IgA responses (Laurent et al., 2015), it could be interesting to include different diagnosis as a potential mediator of this relationship in future studies (Cicchetti et al., 2015).

Finally, it might be interesting to explore how the age of exposure to CM, its proximity or its chronicity can influence the resulting s-IgA reactivity to psychosocial stress to determine the most critical developmental periods (Slopen et al., 2013). Similarly, the nature of the adversity (e.g. neglect vs abuse; or physical vs emotional) has a differential impact in the biological deregulation observed (Baumeister et al., 2016; Sumner et al., 2019). Further studies are needed to explore whether social support or secure attachment could buffer the effects of CM on immune dysregulation. However, maternal secure attachment and social support could buffer the impact of CM in early stages of life (Sung et al., 2016).

5. Conclusions

The present study found evidence of an increased s-IgA reactivity to stress only after puberty onset, supporting that the immune system gradually matures from birth to late life (Simon et al., 2015). However, children previously exposed to CM may exhibit an advance of this response, activating their immune system when faced with psychosocial stressors at earlier stages of development. This phenomenon would be in line with widespread theories defending that individuals exposed to a wide range of pernicious exposures (from either psychosocial or chemical nature) experience what is known as accelerated biological aging. Further studies are required to elucidate the role of CM and developmental stage in immune system regulation in young participants.

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Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2022.04.010.

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UNIVERSITAT DE BARCELONA

Dr. Lourdes Fañanás Saura
Section of Zoology and Biological Anthropology
Dept. Evolutionary Biology, Ecology and Environmental Sciences
Faculty of Biologia, Universitat de Barcelona

Supervisor's report on the contribution of the PhD applicant to the article.

Prof. Dr. Lourdes Fañanás Saura, full professor at the Department of Evolutionary Biology, Ecology and Environmental Sciences of the Faculty of Biology (Universitat de Barcelona) and supervisor of the present doctoral thesis by Águeda Castro Quintas, hereby certifies that Laia Marques Feixa included also the article "*Secretory immunoglobulin A (s-IgA) reactivity to acute psychosocial stress in children and adolescents: the influence of pubertal development and history of maltreatment*" on her doctoral thesis defended on the Universitat de Barcelona, and the participation of the PhD applicant in the article included the following tasks:

- Participation in the conception and design of the study
- Laboratory Analyses
- Statistical analyses and data interpretation
- Writing of the first manuscript draft
- Critical revision of the article for intellectual content

Signed by Prof. Lourdes Fañanás

Barcelona, 5th December 2023

3.6. Salivary secretory immunoglobulin A as a putative biomarker of psychosocial stress response during the first stages of life: A systematic review

Águeda Castro-Quintas, Helena Palma-Gudiel, Nerea San Martín-González, Javier Rubén Caso, Juan Carlos Leza, Lourdes Fañanás

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Salivary secretory immunoglobulin A as a potential biomarker of psychosocial stress response during the first stages of life: A systematic review

Águeda Castro-Quintas [a,b,c], Helena Palma-Gudiel [b,d], Nerea San Martín-González [a,b,c], Javier R. Caso [b,e], Juan C. Leza [b,e], Lourdes Fañanás [a,b,c,*]

[a] Department of Evolutionary Biology, Ecology and Environmental Sciences, University of Barcelona, Spain
[b] Network Centre for Biomedical Research in Mental Health (CIBER of Mental Health, CIBERSAM), Institute of Health Carlos III, Spain
[c] Institute of Biomedicine of the University of Barcelona (IBUB), Barcelona, Spain
[d] Department of Epidemiology, University of Florida, United States
[e] Department of Pharmacology and Toxicology, Faculty of Medicine, Complutense University of Madrid, Imas12. IUIN, Madrid, Spain

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ABSTRACT

Mucosal secretory immunoglobulin A (s-IgA) has been recognized as a key component of human first line defense against infection. However, its reactivity to psychosocial stressors is poorly understood. This systematic review aimed to explore whether s-IgA levels changed after psychosocial stress in subjects under the age of 18. Fifteen articles were included. s-IgA basal levels are increased in children older than 9 years old exposed to stress. Furthermore, s-IgA seems to follow a circadian rhythm, which is altered under stress conditions. Finally, the collective evidence suggests that salivary s-IgA rapidly increases under acute stress after puberty. Overall, our review indicates that s-IgA could be considered a potential psychosocial stress biomarker of interest for pediatric and child-juvenile psychiatric population. Further studies are needed to validate the role of s-IgA circadian rhythm and basal levels as psychosocial stress biomarkers and disentangle the role of age and type of stressor.

1. Introduction

The ability to efficiently detect and respond to potential threats is essential for survival. The brain coordinates the stress response to keep the balance in physiological processes and maintain homeostasis after any perturbation. Activation of the sympathetic nervous system (SNS) and the hypothalamus–pituitary–adrenal (HPA) axis under psychological challenges causes respectively epinephrine (adrenal medulla) and cortisol (adrenal cortex) release into the bloodstream (Carrasco & Van de Kar, 2003; Godoy et al., 2018), and a rapid physiological adaptation to maintain vigilance and to regulate energy expenditure, such as glucose storage (Sapolsky et al., 2000; Selye, 1936). Along with those two systems, acute stress also activates the innate immune system as a countermeasure to the potential exposure to pathogens in a "fight or flight" scenario.

From an evolutionary perspective, life-threatening situations can lead to injury. Consequently, the sentinel immune system (innate) activates in response to any situation that is encoded as threatening, regardless of its type (pathogen, non-pathogen or other damaging signals, including chemical, physical or psychological stress), first recognizing the threatening signal and releasing multiple inflammatory proteins and oxido/nitrosative mediators into the bloodstream and mucosal surfaces (Miller & Raison, 2016; Segerstrom & Miller, 2004). This response orchestrates a network of regulatory pathways that prevents over activation of the immune system, which could be deleterious (Rook, 2013). Improved sanitation of urban environments has critically reduced the infectious challenges that were primary sources of mortality across most of human evolution (Miller & Raison, 2016). However, according to literature, social daily situations that are experienced as stressful can induce the activation of the stress response and the immune system in the absence of infectious agents, increasing the individual's susceptibility to chronic inflammatory diseases (Herr et al., 2018; Segerstrom & Miller, 2004). Moreover, factors such as stressful events, urban setting, and the exposure to some infections have been considered

* Corresponding author.
E-mail address: lfananas@ub.edu (L. Fañanás).

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as risk factors for developing mental disorders including schizophrenia spectrum disorders (Arango et al., 2021).

The immune system provides defense from pathogens via a vast network of cells and tissues, performing a complex and orchestrated attack once the intruder is detected. The immune response has two major components: the innate and the adaptive immune systems. Innate immunity is the first line of defense, it remains active in the span of minutes to hours after infection; and after recognizing signature molecules of pathogens through pattern-recognition receptors (Kumar et al., 2009), it gives rise to the release of cytokines, small proteins important in cell signaling, and the activation of the complement system, a regulated network of proteins involved in a sequential cascade of enzymatic reactions resulting in the opsonization of the pathogen (Sarma & Ward, 2010; Segerstrom & Miller, 2004). Meanwhile adaptive immunity is directly activated by pathogens and may last for a few weeks, months or even for a person's entire life. Particularly, the adaptive immunity response is specific and involves specialized immune cells, such as lymphocytes, and immunoglobulins (Ig), Y-shaped proteins that bind to an antigen preventing disease development. There are five different types of immunoglobulins (Igs) (IgA, IgD, IgE, IgG, and IgM), which differ in their biological features, structure, target specificity and distribution.

A particular form of IgA, secretory-IgA (s-IgA), is found in mucosal secretions of gastrointestinal tract and in other secretions, including saliva and breast milk, acting as a first line barrier against invading pathogens (Carpenter, 2020). The s-IgA is constituted by two IgA molecules and a secretory component. This component is added to the dimeric IgA when it is carried across the epithelium before mucosal lumen release. The s-IgA prevents the passage of microorganisms into the circulatory system (Bosch et al., 2002) and plays a key-role in the maintenance of oral health, among others (Gutierrez-Corrales et al., 2017). Although at birth no salivary s-IgA can be detected, infants' s-IgA is already present at one week of age. (Haworth & Dilling, 1966). In the first year of life, s-IgA reaches 30% of adult levels (Fagerås et al., 2011), and these levels continue to increase until 20 years old (Jafarzadeh et al., 2010).

Furthermore, s-IgA is under strong neuroendocrine control (Fig. 1). Specifically, the autonomic nerves innervating the salivary glands robustly influence s-IgA production, thus, sympathetic nervous system activation enhances preformed-IgA release by plasma B cells and its epithelial translocation (Teeuw et al., 2004). This mechanism may explain why some environmental challenges perceived as acute stressors (e.g., academic exams, public speech or standardized stressful laboratory conditions such as an arithmetic task or a simulated job interview) could increase salivary s-IgA within minutes after its initiation (Ohira, 2004; Takatsuji et al., 2008; Trueba et al., 2012) and with a rapid decrease during recovery (Campisi et al., 2012; Tsujita & Morimoto, 1999). In fact, s-IgA is released following circadian rhythms, with a peak in the morning just after waking up (Nader et al., 2010), followed by a sharp decline the next four hours and reaching the lowest value just before bedtime (Shirakawa et al., 2004), in a similar way to cortisol, suggesting a possible synchrony on both responses (Hucklebridge et al., 1998).

However, under prolonged or chronic psychological stress (e.g.,

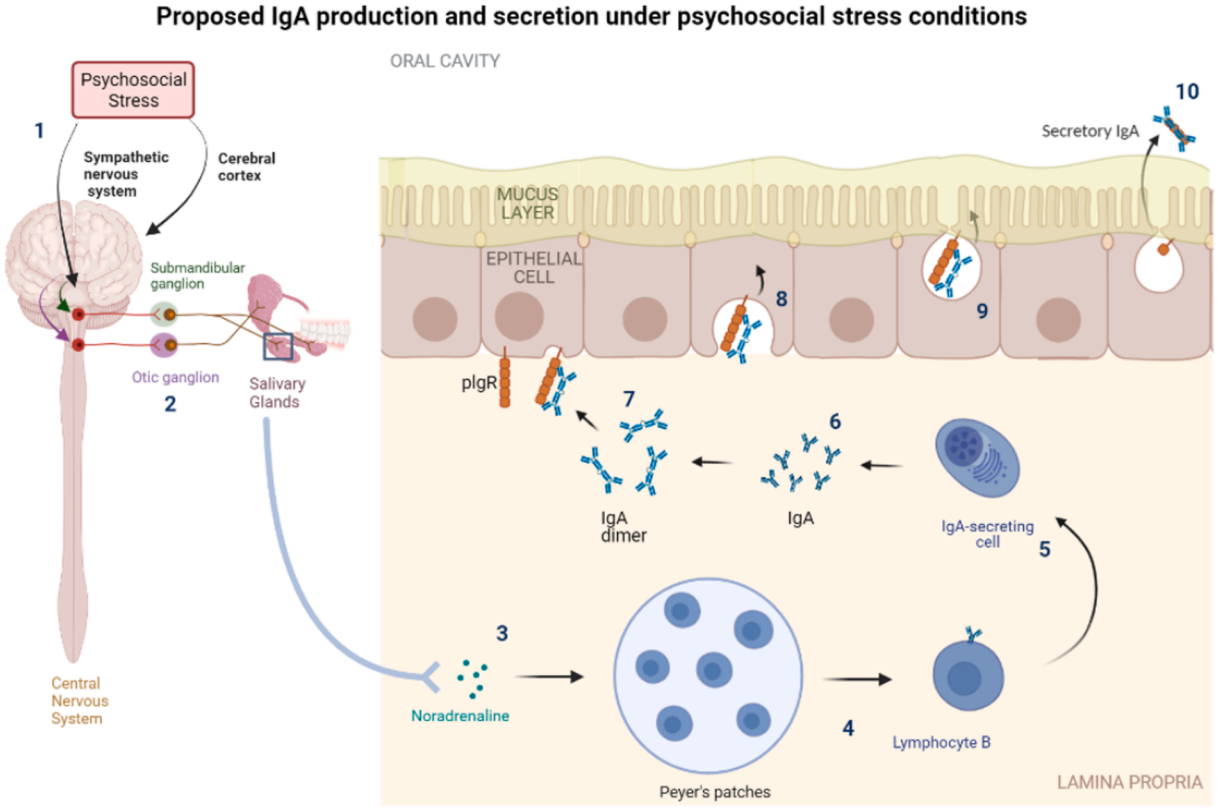

Fig. 1. Proposed IgA production and secretion under psychosocial stress conditions. (1) Psychosocial stress activates sympathetic nervous system. (2) Then, preganglionic nerves of the thoracic segment of the spinal cord are activated, which synapse with postganglionic nervous in both submandibular and otic ganglions. (3) Consequently, neurotransmitter norepinephrine in salivary glands that (4) started salivary immune response in Peyer's patches, aggregated lymphoid nodules, by activating unspecific lymphocyte B. (5) Along with signals elicited by specific proteins binding, IgA-secreting cells formation is induced. (6) As soon as monomeric IgA are released in the lamina propria, (7) they are joined in pairs by the J chain to form dimeric IgA (8) which is captured by the polymeric immunoglobulin receptor (pIgR). (9) The dimeric IgA-pIgR complex is internalized and transported by transcytosis to the oral cavity. (10) The former is then released as secretory component bound to dimeric IgA to yield secretory IgA (s-IgA). s-IgA secreted into the mucus layer prevents the direct adhesion to the epithelium of pathogenic agents. Created with BioRender.com

major adverse life events, poor care during childhood and maternal distress exposure during pregnancy) s-IgA response seems to be suppressed (Kang et al., 2018; Phillips et al., 2006; Vermeer et al., 2012), and this suppression in turn predicted higher susceptibility to different illnesses such as upper respiratory tract infections (Welch, 2018), or orthodontic pain and caries (da Silva Campos et al., 2010). Although previous studies have identified specific psychosocial stressors or challenges that are associated with changes in s-IgA concentration, especially in saliva, most of them have only examined adult populations, leaving unanswered questions regarding s-IgA dynamics under acute or chronic psychosocial stress during childhood and adolescence. Noticeably, child nervous system nor the immune system are fully developed at birth, but rather continue to mature in response to the postnatal environment. An increased vulnerability to immune system perturbations due to early exposure to stress spans prenatal life and infancy but also childhood and adolescence, periods characterized by the acquisition of immune memory. A two-way interaction between the brain and the immune system makes it possible for prenatal, childhood and adolescent psychosocial stressors to have a major and long lasting impact on both systems (McCrory et al., 2010) increasing susceptibility to several diseases, including mental disorders (O'Connor et al., 2014).

The fact that s-IgA quickly rises after acute stress exposure in saliva, an accessible and non-invasive sample (Engeland et al., 2019), together with its putative detectability throughout all lifespan, could make it a promising biomarker of acute and chronic psychosocial stress reactivity in young population. Therefore, the objective of the present systematic review is to examine the existing studies measuring mucosal secretory IgA levels after psychosocial stress exposure in infancy, childhood, and adolescence and to discuss its utility as a possible biomarker of altered psychosocial stress immunity response. Finally, we suggest future directions for this body of research.

2. Material and methods

2.1. Eligibility criteria

PRISMA guidelines for conducting and reporting systematic reviews were followed in the elaboration of the current systematic review (Page et al., 2021). The following inclusion criteria were considered: (1) human studies, (2) studies in infants and adolescents (under 18 years old), and (3) studies addressing the association between psychosocial stress and IgA response in mucosal tissues (saliva, tears, intestinal secretions or urine). Additionally, the following exclusion criteria were considered: (4) studies in which the stressor addressed was either *in vitro* or non-psychological in nature (i.e. physical exercise), and (5) studies in which the measured IgA was specific to a particular antigen. The search was limited to articles written in English. There was no limitation regarding publication time. With regard to the age of the participants, studies reporting mean age instead of age range were included whenever the mean age plus the standard deviation was lower than 18 years old, although we acknowledge that, some of the studies reviewed might include a minority of older subjects.

2.2. Search strategy

Research was conducted in the databases PubMed, PsycINFO and Web of Science on November 8th, 2022 using the Spanish Foundation for Science and Technology (FECYT in its Spanish acronym) interface. The search terms were the following: "immunoglobulin A or IgA", "trauma, adverse, stress or maltreatment" and "baby or toddler or infant or child* or adolescent or teen or young". All the citations found in each database were imported to EndNote X9 and de-duplicated.

2.3. Study selection

The titles, abstracts and full texts of all papers retrieved were independently screened by two authors for eligible articles. In case of disagreement, a consensus between the authors was reached. In some instances, a third author helped to take an agreement.

2.4. Data collection process and risk of bias assessment

Data was extracted by one author and later checked by a second author. Extracted data included first author, year of publication, the number of participants, the age and sex of the participants, the procedure for inducing stress, stress outcomes, psychiatric outcomes, timing of sample collection, the source tissue, the methodology employed for determining s-IgA concentration, other measures determined, and the main findings of each study (Table 1).

The risk of bias of all included studies was evaluated following the "Newcastle - Ottawa Scale" (NOS) for quality assessment of cohort studies and case-control studies (Wells et al., 2009). Adaptations of NOS were made considering the methodological design of the studies, in order to better assess the quality of the information that was relevant for this review. To that aim, 6 items were considered and evaluated from 0 to 2, where 2 is the best score. The items considered were: 1) the population sample size, 2 was given when the sample size was higher than 97, calculated considering unlimited population with a confidence level of 0.95 and an error of 0.10 (Marrugat & Vila, 2012); 1 was given when the sample size was between 69 and 97, calculated considering an infinite population with a confident level of 0.90 and an error of 0.10; 0 was given when the sample size was between 1 and 69; 2) quality of stress definition (2, stress induction or direct measures of participants stress; 1, standardized environmental stress measures; 0, non-standardized measure); 3) quality of sample collection (2, samples collected by professionals following adequate instructions; 1, self-collection following adequate instructions; 0, self-collection with no previous instructions) 4) Timing of sample collection (2, sample collection throughout the day and at least one at awakening and one before going to bed [s-IgA circadian rhythm] or in the evening [s-IgA reactivity to acute stress or basal levels]; 1, sample collection throughout the day [s-IgA circadian rhythm] or in the morning [s-IgA reactivity to acute stress or basal levels]; 0, the time was not indicated); 5) Adequate number of samples (2, at least two [s-IgA basal levels], three [s-IgA reactivity to acute stress] or four [s-IgA circadian rhythm] samples per day; 1, repeated measures across the study [s-IgA basal levels] or two [s-IgA reactivity to acute stress] or three [s-IgA circadian rhythm] samples per day; 0, one sample across the study [s-IgA basal levels] or one [s-IgA reactivity to acute stress] or two [s-IgA circadian rhythm] samples per day); 6) Control by covariates (2, control by sex, age and other variables related to the sampling;1, control by sex and age; 0, not controlling by covariates). An overall score was calculated for each study by summing the scores of each criterion and expressing it as a percentage, considering the total possible score. The studies with a total score lower than 50% were classified as low-quality, those between 50% and 74% as mid-quality and those equal or higher than 75% as good-quality. Although this risk of bias score evaluates different important methodological aspects, this measure should only be taken as a general approximation of the quality of each study.

3. Results

3.1. Study selection

The process followed for article selection is presented in Fig. 2. A total of 4746 articles were identified: 2362 in PubMed, 272 in PscyINFO and 2111 in Web of Science. After removing duplicates, we proceeded to a screening phase, reading 4366 titles and abstracts and 83 articles were selected for eligibility. A total of 15 articles were included in this review.

Table 1

Outline of papers reviewed (n = 15) including subjects information (number of subjects, ethnicity, sex and age); type of stress induced, and the protocol followed; psychological and psychiatric outcomes that could been measured; procedure, time and tissue of extracted sample for s-IgA determination; statistic approach and the reported s-IgA dara; main biological variables assessed apart from s-IgA and main s-IgA findings. The articles are sorted by alphabetical order and reference country information is also reported.

Authors (Year) Country	Sample Ethnicity	Age[a] Sex Research design	Stressors *Measures*	s-IgA measurement Procedure of sample collection[b, c]	Time of sample extraction (hours)	Statistic approach Reported s-IgA data[d]	Other measures: Biological and Psychological	Findings[e]
Abraham et al. (2021) Israel	47 children and their parents 100% Caucasian	3.37 ± 0.40 years 55.3% male Longitudinal	Parenting style *CIB* *Parent-infant synchrony* Fear eliciting task ("Mask")	Basal s-IgA Three salivary samples (*cotton swab*): 1. 10 min after arrival to infant's home 2. 20 min after the end of the masks assessment 3. 35 min after the end of the mask assessment	Between 16:00 and 19:00	Correlations *Raw data and AUCg calculations* s-IgA (ru/ml) = 3442.9 ± 279.9	Cortisol	• Reduced parental synchrony was associated with higher child s-IgA levels (p =.03). • Child's high levels of self-regulation were significantly associated with lower s-IgA levels (p =.001).
Byrne et al. (2017) USA	102 healthy children NA	9.50 ± 0.34 years 46% male Cross sectional	Parenting style *APQ*	Basal s-IgA One salivary sample (*passive drool*)	At waking	Linear regressions (Adjusted for flow rate) *Log transformations* s-IgA (μg/mL) = 4.79 ± 11.06	BMI CRP	• APQ poor monitoring and supervision by parents was associated with significantly higher levels of s-IgA in their children (p =.016). • Higher children's s-IgA levels were associated with a higher BMI(p =.005).
Ellberg et al. (2019) USA	51 toddlers/ mother dyads Caucasian 90.2%	At 1, 3 and 6 months (longitudinal) 53% males Longitudinal	Pre-postnatal maternal distress *EPDS* *PSS* *PANAS*	Basal s-IgA Three salivary samples (One sample/time point) (*cotton swab*): 1. 1 month 2. 3 months 3. 6 months	Around 16:00	ANCOVAS *Log transformations* s-IgA (μg/mL) 1 month = 170.87 ± 181.33 3 months = 51.24 ± 42.22 6 months = 41.40 ± 22.29	Breastfeeding Infant's length Infant's weight	• Higher maternal distress during the postnatal period was associated with reduced infant s-IgA (p =.03). • Maternal prenatal distress did not predict infant s-IgA changes across the assessments (p >.05).
Kang et al. (2020) Canada	1043 Children/ mother dyads NA	3.7 ± 1.1 (2–8) months 53.4% male Longitudinal	Pre-postnatal maternal distress *PSS* *CES-D scale*	Basal s-IgA One fecal sample	NA	Multiple linear and logistic regression models *Log transformation* Median s-IgA (μg/mg) = 6.1 (2.9–11.0)	Antibiotic exposure Breastfeeding Infant's length Infant's weight	• Maternal depressive symptoms in prenatal period and in both pre and postnatal periods were significantly associated with reduced infants' fecal s-IgA concentrations (p <.05). • These lower s-IgA levels yielded a large effect size in older infants (p <.05). • None associations were observed for maternal stress symptoms neither in pre nor postnatal periods.

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Table 1 (*continued*)

Authors (Year) Country	Sample Ethnicity	Age[a] Sex Research design	Stressors *Measures*	s-IgA measurement Procedure of sample collection[b, c]	Time of sample extraction (hours)	Statistic approach Reported s-IgA data[d]	Other measures: Biological and Psychological	Findings[e]
Laurent et al. (2015) USA	82 healthy children and adolescents 73% Caucasian 27% NA	7–17 years 49% male Cross sectional	Induced Psychosocial stress- 62% of the participants: *TSST adaptation* (speech, mental arithmetic, mirror tracing; 20 min) - 38% of the participants: Interpersonal stress session (three exclusion challenges; 20 min)	Stress s-IgA reactivity Six salivary samples (*passive drool*): 1. Before the induced stress (-25 min) 2. After task 1 (+10 min) 3. After task 2 (+15 min) 4. After task 3 (+20, +40, +60 min)	Between 14:00 and 17:00	Hierarchical linear modeling *Raw datas* s-IgA (μg/mL) = 155.03 ± 112.48	CBCL Cortisol sAA STAI-C	• S-IgA levels significantly increased during acute stress and started to decrease immediately after the task ended ($p < .001$). • There were differences in s-IgA responses related to youth externalizing behavior ($p = .010$).
Ma et al. (2018) Canada	Study 1: 115 children (wave 1) Study 2: 103 children (waves 1, 2 and 3) 95% Caucasic 5% Latin American, Haitian or other ethnics	Wave one: 10.79 ± 0.92 (9–12) years Wave two: 13.62 ± 1.10 (12–15) years Wave three: 16.95 ± 1.27 (15–18) years 47% male Longitudinal	Exposure to diurnal anxiety and depressive symptoms *CDI* *RCMAS*	s-IgA circadian rhythm 48 salivary samples (eight samples in two consecutive school days per each wave) (*cotton swab*): 1. At awakening 2. 30 min post awakening 3. Every 2 h until bedtime	Throughout the day, from awakening to bedtime	Hierarchical Linear Modelling *Log transformations* s-IgA (μg/mL) Wave one = 1.55 ± 0.33 Wave two = 1.49 ± 0.52 Wave three = 1.76 ± 0.28	Frequency and type of physical illness Medication	• Children's diurnal s-IgA rhythm showed a gradual increase within the first 4 hr post-awakening, followed by a gradual decrease toward the afternoon and a final increase before bedtime ($p < .001$). • Children with higher anxiety had steeper increases in s-IgA compared to those with lower anxiety ($p < .05$). • Higher total anxiety and worries in children between ages 9–12 were associated with diminished global s-IgA levels when they were 12–15 years of age ($p < .05$). Lowered s-IgA levels in turn predicted higher total anxiety symptoms and worries between ages 15–18 ($p < .05$). • In late adolescence, higher total anxiety was associated with lower s-IgA levels. • s-IgA levels at 9–12 years old were associated with s-IgA levels at 12–15 ($p < .01$) but not at 15–18 years old ($p > .05$).
Marques-Feixa et al. (2022) Spain	94 children and adolescent 83% European 17% Latin American,	13.80 ± 2.40 (7–17) years 40% male Cross sectional	Induced Psychosocial stress *TSST-C:* -Habituation period (30 min) - TSST (20 min) -	Stress s-IgA reactivity Five salivary samples (*cotton swab*): 1. Before the task (-30 min)	TSST-C protocol started at 16:00	Mixed model and simple effects test *Log transformations* s-IgA (μg/dL) Children	Cortisol Current infectionK-SADS-PL-5 (Current Psychopathology) Tanner Stage	• Adolescents showed an increase after the stressor and a rapid recovery, while children did not show an s-IgA

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Table 1 (*continued*)

Authors (Year) Country	Sample Ethnicity	Age[a] Sex Research design	Stressors *Measures*	s-IgA measurement Procedure of sample collection[b, c]	Time of sample extraction (hours)	Statistic approach Reported s-IgA data[d]	Other measures: Biological and Psychological	Findings[e]
	Maghrebin and others		Recovery (30 min) History of childhood maltreatment	2. When the task started (0 min) 3. At the end of the task (+20) 4. During the recovery period (+35, +50 min)		Adolescents T1 = 4.30 ± 0.045 T1 = 4.37 ± 0.048 T2 = 4.25 ± 0.042 T2 = 4.44 ± 0.045 T3 = 4.26 ± 0.041 T3 = 4.48 ± 0.044 T4 = 4.21 ± 0.043 T4 = 4.35 ± 0.046 T5 = 4.23 ± 0.045 T5 = 4.37 ± 0.047		response (p =.010). • However, children exposed to maltreatment exhibited an s-IgA pattern more similar to that of adolescents when compared to non maltreated children (p =.017).
Molnar et al. (2018) USA	50 children/ mother dyad NA	5.68 ± 0.26 years 58% male Longitudinal	Pre-postnatal substance exposure Maternal cigarette and/or cannabis consumption during pregnancy TLFB	Basal s-IgA One salivary sample (*passive drool*)	Between 13:00 and 17:00	Hierarchical Multiple Regression *Raw datas* s-IgA (μg/mL) Control group = 101.77 ± 26.75 Cigarette group = 149.60 ± 91.76 Cannabis group = 166.85 ± 81.88	Cotinine Nicotine OHCOT THC	• Children with postnatal cigarette or pre and postnatal cannabis exposure did not have differences in their s-IgA levels (p =.419). • Prenatal cigarette exposure and the combination of cigarette and cannabis in both pre and postnatal timings was correlated with the higher levels of s-IgA (p =.047).
Noakes et al. (2007) Australia	82 infants NA	At 3 and 12 months NA Longitudinal	Prenatal tobacco exposure: Maternal cigarette consumption during pregnancy	Basal s-IgA Two salivary samples (*specimen suction set*): 1. At 3 months old 2. At 12 months old	NA	Mann-Whitney *U* test, Wilcoxon signed Rank tests or Spearman correlation coefficients *Log transformation* Median s-IgA (μg/mL) (approximation) 3 months: 12 months: Non exposed ≈ 10 Unexposed ≈ 5 Exposed ≈ 10 Exposed ≈7	Allergen SPT Specific IgA to pneumococcal PS serotype 14 Urinary cotinine	• Infants exposed to maternal cigarette smoke in pregnancy had significantly higher total IgA levels at 12 months old (p =.026), but not at 3 (0.722) when compared to non exposed. • IgA levels decreased with age in both groups.
Reindl et al. (2022) Germany	Initial sample: 253 (94 children in foster care and 157 biological children) Final sample: 232 (84 children in foster care and 146 biological children) NA	T1: Children in foster care: 3.80 ± 1.57 Biological children: 4.10 ± 1.46 T2: After 6.45 ± 1.59 months T3: After 6.04 ± 1.49 months 50% male in both groups	Caregiving - Foster care vs biological parental care: *Caregiver-child interactions* *Relationship quality* *History of maltreatment and/or neglect*	Basal s-IgA Three salivary samples (*cotton swab*): 1. At T1 2. At T2 (+6 months) 3. At T3 (+1 year)	Between 13:00 and 16:00	Linear mixed models *Log transformation* Total s-IgA (μg/mL) (approximation) Foster care Foster care higher caregiving lower caregiving T1 ≈ 3.75 T1 ≈ 4.00 T2 ≈ 3.80 T2 ≈ 3.80 T3 ≈ 3.85 T3 ≈ 3.75	Cortisol DHEA Progesterone	• No differences were found for s-IgA between foster care and biological care children (p =.50). • However, caregiving quality modulated s-IgA concentrations: children in foster care of lower caregiving quality showed decreasing s-IgA concentrations across the study period (p =.028)

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Table 1 (*continued*)

Authors (Year) Country	Sample Ethnicity	Age[a] Sex Research design	Stressors *Measures*	s-IgA measurement Procedure of sample collection[b, c]	Time of sample extraction (hours)	Statistic approach Reported s-IgA data[d]	Other measures: Biological and Psychological	Findings[e]
		Longitudinal						when compared with children in foster care of higher caregiving quality, which showed s-IgA increments ($p = .049$).
Ulmer-Yaniv et al. (2018a) Israel	125 children NA	9.63 ± 0.65 years NA Longitudinal	Maternal depression Parenting style: *CIB*	Basal s-IgA Three salivary samples (*cotton swab*): 1. After 10 min of acquaintance (baseline) 2. After testing (+80 min from baseline) 3. After CIB (+155 min from baseline)	Between 16:00 and 19:00	Pearson correlations *Raw data and AUCg calculation* Total diurnal s-IgA (NA) Non exposed = $1288.54*10^3 \pm 469.39*10^3$ Exposed = $1893.06*10^3 \pm 451.84*10^3$	CBCL Cortisol	• Children of depressed mothers had higher s-IgA levels than children of non-depressive mothers ($p < .01$). • Children internalizing and externalizing symptoms were significantly associated with higher s-IgA levels ($p < .01$).
Ulmer-Yaniv et al. (2018b) Israel	Initial sample: 232 children (148 war-exposed) Final sample: 177 children (101 war-exposed) NA	9–11 years 48% male Longitudinal	War-exposure Parenting style: *CIB*	Basal s-IgA Nine salivary samples (three samples/time point) (*cotton swab*): 1. Following acquaintance (baseline) 2. After testing (+60 min from baseline) 3. After CIB (+84 min from baseline)	Between 15:00 and 19:00	Pearson correlations *Raw data and AUCg calculation* Child s-IgA (NA) Not exposed = 618.72 ± 413.49 Exposed = 909.03 ± 518.46	DAWBA Oxytocin SCARED	• War-exposed children had higher s-IgA levels ($p < .01$) and anxiety-related symptoms ($p < .01$). • Higher s-IgA levels were associated with lower maternal sensitivity ($p > .05$).
Vermeer et al. (2012) Netherlands	68 children NA	2.5 ± 0.48 years 57% male Cross sectional	Caregiving - Center childcare vs family childcare: *Emotional support provided by the caregiver*	s-IgA circadian rhythm Eight salivary samples (four samples/day) (*cotton swab*): On one childcare days (by the center or the family care) , and on one day at home	Around 7:00, 11:00, 15:00 and 18:00	AUCg calculation and MANCOVAS *Log transformations* s-IgA (μg/mL) Child Care Home Care 7 AM = 4.63 ± 0.89 7 AM = 4.33 ± 0.87 11 AM = 3.48 ± 0.7 11 AM = 3.55 ± 0.61 3 PM = 3.86 ± 0.77 3 AM = 4.06 ± 0.79 6 PM = 3.49 ± 0.69 6 PM = 3.57 ± 0.67	Child use of medicine, mood, naps and food on the collection day	• The diurnal pattern of s-IgA in toddlers showed a steep fall in the morning followed by a flattening out starting at mid-morning, both at childcare and at home ($p < .001$). • Lower caregiver sensitivity was associated with lower s-IgA levels in all time-points ($p < .05$).
Watamura et al. (2010) USA	79 healthy children 64% Caucasian 20% Asian American 9% Black 7% American Indian	3 – 5 years 54% male Longitudinal	Caregiving - Center childcare during the week vs parental care in the weekend: *Number of stressors in the past 6 months*	s-IgA circadian rhythm Twelve salivary samples (three samples/day on two childcare days, and on two weekend days) (*cotton swab*): 1. Mid-morning 2. Mid-afternoon 3. Evening	At 10:30, 15:30 and 20:00	Bivariate correlations and ANOVAS (Adjusted for flow rate) *Log transformations* s-IgA (μg/mL) (approximation) Child Care Day Weekend	Cortisol Parental report of illness frequency	• There was a clear s-IgA diurnal rhythm only on the weekends characterized by s-IgA dropped levels from the afternoon to the evening ($p = .013$), but not on care days ($p > .05$). • Younger children had higher s-IgA

(*continued on next page*)

Table 1 (*continued*)

Authors (Year) Country	Sample Ethnicity	Age[a] Sex Research design	Stressors *Measures*	s-IgA measurement Procedure of sample collection[b, c]	Time of sample extraction (hours)	Statistic approach Reported s-IgA data[d]	Other measures: Biological and Psychological	Findings[e]
						Mid-Morning 35.50 40.98 Mid-Afternoon 34.34 38.37 Evening 35.70 32.04		during child care days evenings compared to older children (p =.007). • Older children presented a diurnal decline both in the week and the weekend (p =.048).
Yirmiya et al. (2018) Israel	111 children (58 war-exposed) from a cohort of 232 children NA	11.66 ± 1.23 years 40% male Longitudinal	War-exposure Parental style *CIB*	Basal s-IgA Three salivary samples (*Cotton swab*): 1. Upon arrival to the lab (baseline) 2. After testing (+10 min from baseline) 3. After CIB (+100 min from baseline)	NA	Pearson correlations *Raw data and AUCg calculations* s-IgA (NA) Not war exposed = $5050.09*10^3 \pm 267.20*10^3$ War exposed = $6205.58*10^3 \pm 360.17*10^3$	Cortisol SCARED	• War-exposed children had significantly higher s-IgA levels than non-exposed (p <.05). • Elevated children's s-IgA levels were correlated with reduced social collaboration (p <.05) and more anxiety symptoms (p <.05).

Abbreviations: APQ, Alabama Parenting Questionnaire; AUC, Area Under the Curve; BMI, Body Mass Index; CBCL, Child Behavior Checklist; CDI, Children's Depression Inventory; CES-D, Center for Epidemiological Studies Depression Scale; CIB, Coding Interactive Behavior Manual; CRP, C-Reactive Protein; DAWBA, Developmental and Well-Being Assessment; DHEA, dehydroepiandrosterone; ELISA, Enzyme-Linked Immunosorbent Assay; EPDS, Edinburgh Postnatal Depression Scale; K-SADS-PL-5, Kiddie-Schedule for Affective Disorders & Schizophrenia, Present & Lifetime Version V; OHCOT, trans-3′-hydroxycotinine; PANAS, Positive and negative Affect Schedule; PSS, Perceived Stress Scal; RCMAS, Revised Children's Manifest Anxiety Scale; sAA, Alpha-Amylase; SCARED, Screen for Child Anxiety Related Emotional Disorders; SPT Skin-Prick Test; STAI, State-Trait Anxiety Inventory; STAI-C, STAI for Children; THC, Tetrahidrocannabinol; TLFB, Timeline Follow Back; TSST-C, Trier Social Stress Test for Children.

[a] Age is specified as either mean age of the sample ± standard deviation (SD) (if available), or age range.

[b] When the stressor was induced, 0 min refers to the starting point of the induced stress task.

[c] s-IgA was determined in all the studies using a enzyme-linked immunosorbent assay (ELISA) kit.

[d] s-IgA concentrations are specified as Mean ± SD of the total sample or the different groups (with the exception of two studies, that reported Median ± SD). When the data were extracted from a graphic, approximated values were reported.

[e] p values are indicated whenever the paper reported them.

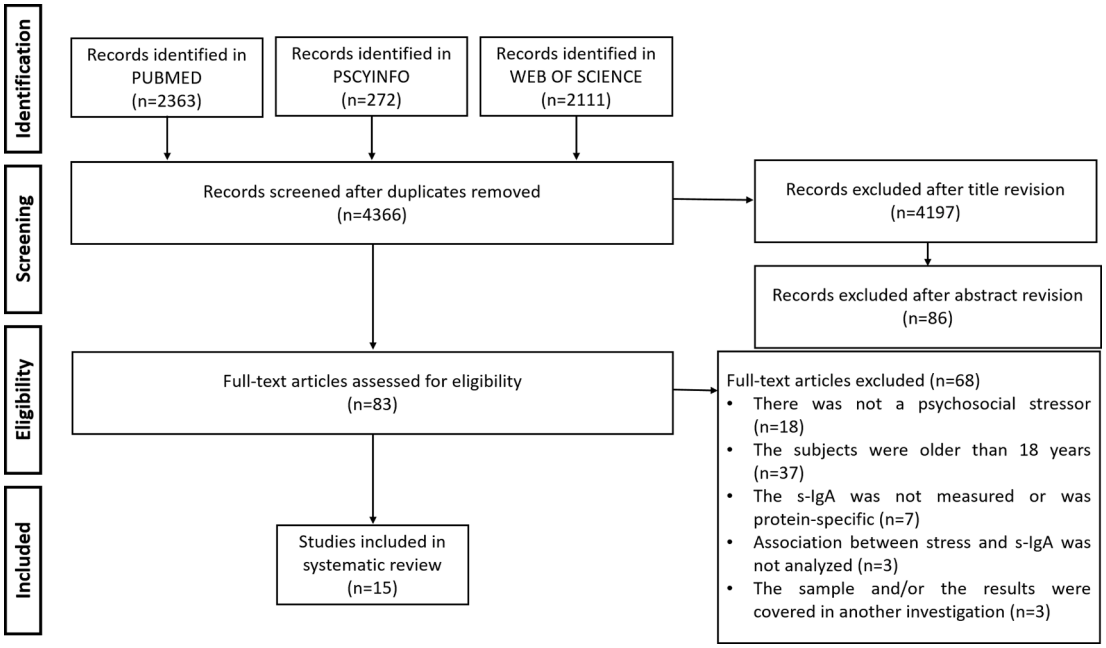

Fig. 2. PRISMA flowchart detailing the filtering steps undertaken to select the studies.

3.2. General characteristics of included studies

The selected studies were conducted in eight different countries: USA (5), Israel (4), Canada (2) Australia (1), Germany (1), Netherlands (1) and Spain (1), and they were published between 2007 and 2022 (Fig. 3 a).

From all evaluated studies, four (27%) were cross sectional and eleven (73%) were longitudinal. Studies characteristics are presented in Table 1.

The number of participants in each study ranged from 47 to 253 with an outlier of 1043 (median = 94) and seven studies assessed more than 100 subjects (Fig. 3 b). The age ranged between 0 and 18 years. Three studies evaluated infants from 0 to 1 year old (Ellberg et al., 2019; Kang et al., 2020; Noakes et al., 2007), five studies included children between 1 and 5 years (Abraham et al., 2021; Molnar et al., 2018; Reindl et al., 2022; Vermeer et al., 2012; Watamura et al., 2010) and seven studies included children and adolescents from 7 to 18 years old (Byrne et al., 2017; Laurent et al., 2015; Ma et al., 2018; Marques-Feixa et al., 2022; Ulmer-Yaniv et al., 2018a,b; Yirmiya et al., 2018). Regarding sex, thirteen studies included both female and male participants (half-half proportion), and two studies did not report the sex of the participants. Six studies reported information about ethnicity, showing a Caucasian predominance.

3.3. Nature of the stressors in the studies reviewed

The nature of the psychosocial stressors described in the included articles is diverse. According to the duration and chronicity of the stressors, they were classified as isolated short-term stressors or acute stressors (e.g., mathematical test, speaking test) and severe long-term exposure stressors or chronic stressors (e.g., parental style, maternal prenatal distress, war exposure).

Ten studies evaluated the impact of only one stressor. Specifically, nine studies included chronic stressors as pre and postnatal maternal distress measured with depression and stress scales (Ellberg et al., 2019; Kang et al., 2020); maternal drug consumption during pregnancy (Noakes et al., 2007) and during pregnancy and postpartum (Molnar et al., 2018); diurnal anxiety and depressive symptoms (Ma et al., 2018); parenting style measured with parent infant-synchrony and interaction (Byrne et al., 2017); and childcare (e.g. center care, family care [family home who carried a license for caring 3 or 4 children], foster care or biological parental care (Reindl et al., 2022; Vermeer et al., 2012; Watamura et al., 2010). One article studied s-IgA reactivity under an induced acute stressor using a performed-orientated task based on an adaptation of the Trier Social Stress Test for Children (TSST-C), consisting on a combination of a speaking, a mental arithmetic and a mirror-tracing task in the 62% of the participants and a interpersonal stress task, consisting on three exclusion challenge, in the 38% of the participants (Laurent et al., 2015).

Additionally, five studies evaluated the impact of two stressors. Three studies included the combination of two chronic stressors as maternal depression and parenting style (Ulmer-Yaniv et al., 2018a) and war and parenting style (Ulmer-Yaniv et al., 2018b; Yirmiya et al., 2018). Finally, two studies included a chronic stressor and an induced acute stressor. Marques-Feixa et al. (2022) performed the TSST-C in children-young population exposed and non-exposed to childhood maltreatment. The TSST-C, is a standardized tool for inducing stress in a controlled environment in which participants are instructed to continue a story and to perform a mental arithmetic task. Before beginning, they are told that the tasks will be recorded and rated by a group of public speaking experts. Abraham et al. (2021), included parenting style but also applied the Fear Eliciting Task ("Mask") to 3 years old children. In this task an experimenter used four increasingly fear-eliciting masks: rabbit, lion, alligator and monster (15 s per mask), while calling children by name and moving her head slowly from side to side. The parents are present but adopt a passive role during the test. In this study, although authors employed diverse tasks to evaluate infants' emotionality and self-regulation abilities, they were not considered as a protocol of stress induction. See Table 1 for more information regarding the specific test questionnaires and measurements used for each stressor.

3.4. Study design and biological samples collection

Every unspecific form of s-IgA assessed in mucosa was considered for this systematic review, however, only one article used feces for s-IgA analysis (Kang et al., 2020), the other 14 studies used saliva.

For the purpose of this review, articles were classified according to the specific aspect of s-IgA functioning that they measured as: measures of basal s-IgA levels, measures of s-IgA circadian rhythm and measures of s-IgA reactivity to acute stress.

There was a high heterogeneity between studies regarding the number of biological samples obtained for each participant, which ranged from one to twelve with an outlier of forty-eight samples per subject. Ten articles assessed s-IgA basal levels, collecting one to three saliva samples throughout a day (Abraham et al., 2021; Byrne et al., 2017; Ellberg et al., 2019; Kang et al., 2020; Molnar et al., 2018; Noakes et al., 2007; Reindl et al., 2022; Ulmer-Yaniv et al., 2018a,b; Yirmiya et al., 2018) Three articles measured s-IgA circadian rhythm by collecting three to eight samples throughout a day (Ma et al., 2018; Vermeer et al., 2012; Watamura et al., 2010). Finally, for assessing s-IgA reactivity to acute stress, two articles collected five to six samples throughout the laboratory stress task (Laurent et al., 2015; Marques-

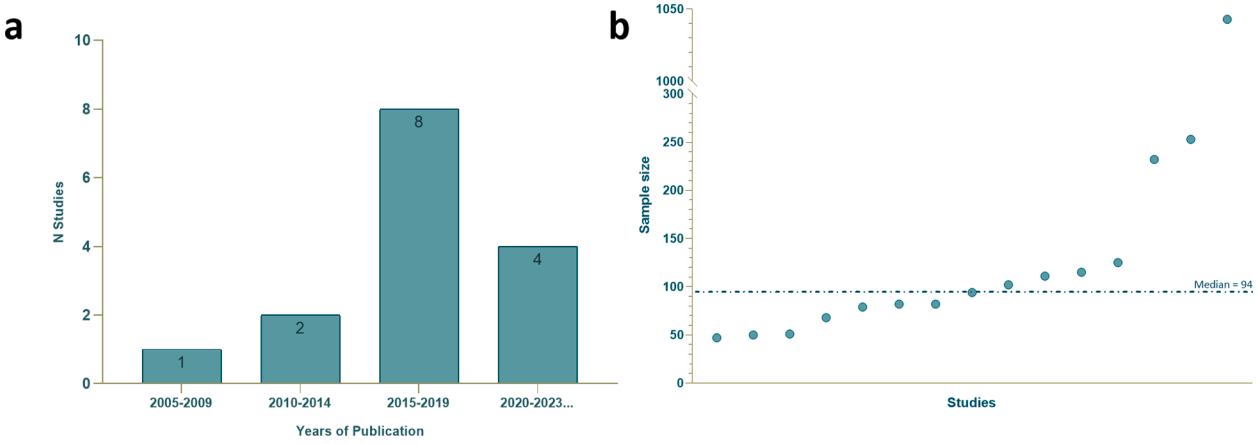

Fig. 3. (a) Year of publication of the selected studies. The studies are grouped in ranges of five years. The last bar represents only the last two years. **(b) Sample Size of the studies reviewed.**

Feixa et al., 2022). Seven articles repeated the collection protocol in an interval of time that varied from one day to six years (Ellberg et al., 2019; Ma et al., 2018; Noakes et al., 2007; Reindl et al., 2022; Ulmer-Yaniv et al., 2018b; Vermeer et al., 2012; Watamura et al., 2010).

Eight of the studies took place in the afternoon (between 3 PM and 6 PM) (Abraham et al., 2021; Ellberg et al., 2019; Laurent et al., 2015; Marques-Feixa et al., 2022; Molnar et al., 2018; Reindl et al., 2022; Ulmer-Yaniv et al., 2018a,b), one took place in the morning (at waking) (Byrne et al., 2017) and in three studies, samples were collected across the day (Ma et al., 2018; Vermeer et al., 2012; Watamura et al., 2010). Three studies did not report timing of sample collection (Kang et al., 2020; Noakes et al., 2007; Yirmiya et al., 2018).

Determination of the s-IgA in the laboratory was performed using an enzyme-linked immunosorbent assay (ELISA) in all the studies. Information of s-IgA concentration is reported in Table 1.

3.5. Additional measures reported in the included articles

Different additional measures were reported together with s-IgA determinations in all the reviewed studies.

Five of the studies reported psychiatric outcomes of their sample including infant emotional and behavior problems evaluated using the Child Behavior Checklist (CBCL), the Kiddie-Schedule for Affective Disorders & Schizophrenia (K-SADS-PL-5) the Developmental and Well-Being Assessment (DAWBA) or the Screen for Child Anxiety Related Emotional Disorders (SCARED) (Laurent et al., 2015; Marques-Feixa et al., 2022; Ulmer-Yaniv et al., 2018a,b; Yirmiya et al., 2018).

Eleven out of the fifteen studies reported information about additional biomarkers (73%). Salivary cortisol concentration was investigated in seven of the fifteen studies (47%), being the most common additional biomarker (Abraham et al., 2021; Laurent et al., 2015; Marques-Feixa et al., 2022; Reindl et al., 2022; Ulmer-Yaniv et al., 2018a; Watamura et al., 2010; Yirmiya et al., 2018). Alpha amylase (sAA) (Laurent et al., 2015), dehydroepiandrosterone (DHEA) and progesterone (Reindl et al., 2022) were explored together with cortisol. Additionally, C-reactive protein (CRP), Body Max Index (BMI) (Byrne et al., 2017) and oxytocin (Ulmer-Yaniv et al., 2018b) were measured. Moreover, when prenatal maternal smoking exposure was studied, cotinine and nicotine were assessed (Molnar et al., 2018; Noakes et al., 2007).

When prenatal maternal distress was assessed, infant birth weight and length were reported (Ellberg et al., 2019; Kang et al., 2020). In this line, two studies explored child use of medicine (Vermeer et al., 2012) and infant illness frequency (Watamura et al., 2010) and one study reported information regarding children medication and frequency and type of physical illness (Ma et al., 2018).

3.6. Main findings

The main findings of the studies are summarized in Fig. 4.

Firstly, ten studies that evaluated basal s-IgA levels observed differential s-IgA directions. Specifically, 7 studies reported increased s-IgA levels under stressful conditions (Abraham et al., 2021; Byrne et al., 2017; Molnar et al., 2018; Noakes et al., 2007; Ulmer-Yaniv et al., 2018a,b; Yirmiya et al., 2018), 2 studies reported a decrease on s-IgA basal levels (Ellberg et al., 2019; Kang et al., 2020) and one study did not observe differences among groups (Reindl et al., 2022). Thus, these results suggest that basal s-IgA levels under stress may depend on participant's age, the type of stressor and the methodological design employed.

Secondly, three studies evaluated the impact of caregiving type (Vermeer et al., 2012; Watamura et al., 2010) and diurnal anxiety (Ma et al., 2018) on s-IgA circadian rhythm, pointing out to the existence of s-IgA diurnal pattern characterized by a gradual decrease throughout the day. However, authors differed on the age of appearance and the environmental conditions.

Finally, two studies evaluated s-IgA reactivity under a psychosocial acute stressor in children and adolescents employing a laboratory-based task (Laurent et al., 2015; Marques-Feixa et al., 2022). Both studies indicated that salivary s-IgA shows reactivity to acute stress, characterized by a fast increase upon the beginning of the stress task followed by a fast decline on s-IgA levels during the recovery period.

3.7. Risk of bias

The risk of bias of the 15 articles reviewed was assessed according with the criteria mentioned in the section "2.4. Data collection process and risk of bias assessment". A summary of the risk of bias present in each manuscript can be found in Table 2. Two studies presented scores below 50% and were considered of low-quality (Molnar et al., 2018; Noakes et al., 2007; Watamura et al., 2010), six studies achieved a percentage of risk of bias between 50% and 74% and were considered of

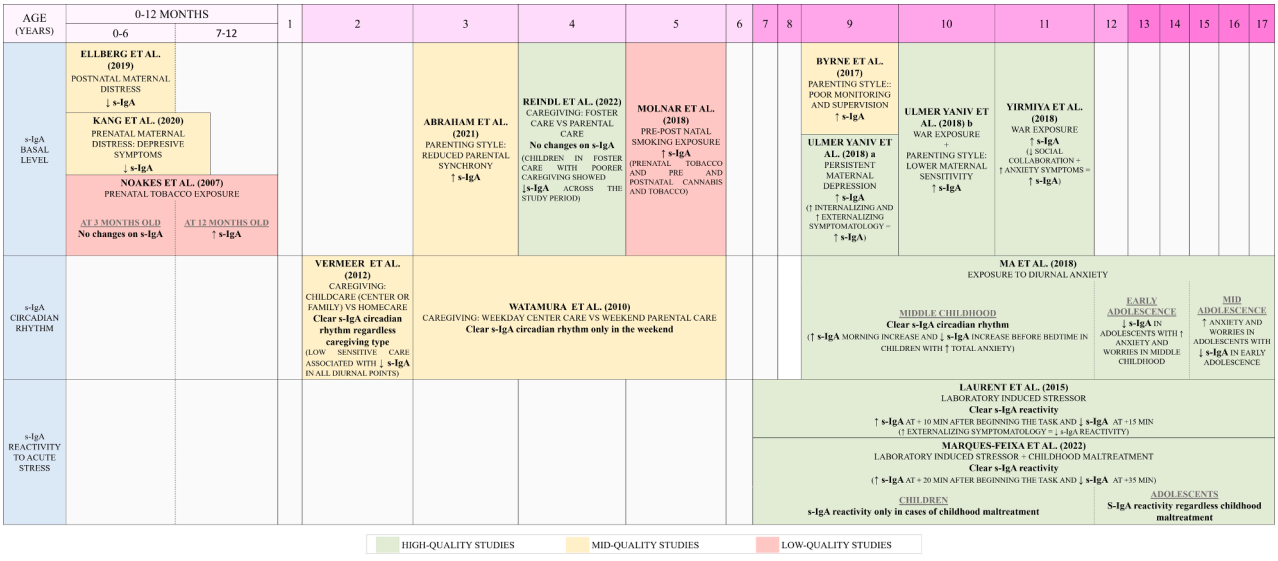

Fig. 4. Summary of the main findings of the reviewed studies. Studies are classified according to the age of the subjects (0–18 years) and the aspect of s-IgA functioning measured (basal level, circadian rhythm and reactivity to acute stress). The quality of the study extracted from the risk of bias is indicated in colors (high-quality, green; mid-quality, yellow; low-quality, red).

Table 2
Quality of the reviewed articles (n = 15). Items are scored as 2 (best possible score), 1 (medium score), or 0 (worst score) and the sum of the 6 items represent the final punctuation for each article. An overall score was calculated for each study by summing the scores of each criterion and expressing it as a percentage, considering the total possible score. The quality of the study was classified, as low when the total score was lower than 50%, as mid when the total score was between 50% and 74% and as good when the total score was equal or higher than 75%.

	Selection		Outcome Assessment			Comparability	Total (%)
	Population sample size[a]	Quality of stress definition[b]	Quality of sample collection[c]	Timing of sample collection[d]	Adequate number of samples[e]	Control for covariables[f]	
Abraham et al., 2021	0	2	2	2	2	0	67
Byrne et al., 2017	2	1	2	1	0	2	67
Ellberg et al., 2019	0	1	2	2	1	2	67
Kang et al., 2020	2	1	2	0	0	2	58
Laurent et al., 2015	1	2	2	2	2	2	92
Ma et al., 2018	2	2	1	2	2	2	92
Marques-Feixa et al., 2022	2	2	2	2	2	2	100
Molnar et al., 2018	0	0	2	2	0	0	33
Noakes et al., 2007	1	0	2	0	1	1	42
Reindl et al., 2022	2	1	2	2	1	2	83
Ulmer-Yaniv et al., 2018a	2	1	2	2	2	0	75
Ulmer-Yaniv et al., 2018b	2	1	2	2	2	1	83
Vermeer et al., 2012	0	1	1	1	2	2	58
Watamura et al., 2010	1	1	1	1	1	1	50
Yirmiya et al., 2018	2	1	2	2	2	1	83

[a] Population sample size punctuation: 2, a sample size higher than 97, calculated considering unlimited population with a confident level of 0.95 and an error of 0.10 (Marrugat & Vila, 2012); 1, a sample size between 69 and 97, calculated considering an infinite population with a confident level of 0.90 and an error of 0.10; 0, a sample size between 1 and 69.

[b] Quality of stress definition punctuation: 2, laboratory stress induction or direct measures of participants stress; 1, standardized environmental stress measures; 0, non-standardized measure.

[c] Quality of sample collection punctuation: 2, samples collected by professionals following adequate instructions; 1, self-collection following adequate instructions; 0, self-collection with no previous instructions.

[d] Timing of sample collection punctuation: 2, sample collection throughout the day and at least one at awakening and one before going to bed (s-IgA circadian rhythm) or in the evening (s-IgA reactivity to acute stress or basal levels); 1, sample collection throughout the day (s-IgA circadian rhythm) or in the morning (s-IgA reactivity to acute stress or basal levels); 0, the time was not indicated.

[e] Adequate number of samples punctuation: 2, at least two (s-IgA basal levels), three (s-IgA reactivity to acute stress) or four (s-IgA circadian rhythm) samples per day; 1, repeated measures across the study (s-IgA basal levels) or two (s-IgA reactivity to acute stress) or three (s-IgA circadian rhythm) samples per day; 0, one sample across the study (s-IgA basal levels) or one (s-IgA reactivity to acute stress) or two (s-IgA circadian rhythm) samples per day.

[f] Control by covariates punctuation: 2, control by sex, age and other variables related to the sampling;1, control by sex and/or age; 0, not controlling by covariates).

mid-quality (Byrne et al., 2017; Ellberg et al., 2019; Kang et al., 2020; Vermeer et al., 2012) and seven of them presented a percentage equal or higher than 75%, so they could be considered of high-quality (Abraham et al., 2021; Laurent et al., 2015; Ma et al., 2018; Marques-Feixa et al., 2022; Reindl et al., 2022; Ulmer-Yaniv et al., 2018a,b; Yirmiya et al., 2018).

4. Discussion

To the best of our knowledge, this is the first systematic review to date that explores s-IgA reactivity to psychological stress during the first stages of life including 15 empirical studies. The different articles evaluated: i) s-IgA basal level, ii) s-IgA circadian rhythm and iii) s-IgA reactivity to acute stress.

4.1. s-IgA basal level

Results from the ten articles evaluating the impact of stress on s-IgA basal levels seem to indicate that consequences may vary depending on the type of stressor and children age (Abraham et al., 2021; Byrne et al., 2017; Ellberg et al., 2019; Kang et al., 2020; Molnar et al., 2018; Noakes et al., 2007; Reindl et al., 2022; Ulmer-Yaniv et al., 2018a,b; Yirmiya et al., 2018).

When exploring tobacco exposure during pregnancy, Noakes and colleagues (2007) did not find significant differences on s-IgA basal levels between exposed and non-exposed infants at 3 months old. Curiously, exposed infants showed significant higher s-IgA levels at 12 months of age when compared with non-exposed children. In this line, Molnar et al. (2018) reported increased s-IgA levels among 5-year-old children born to mothers who smoked tobacco during pregnancy or combined tobacco and cannabis during pregnancy and postnatal period. Higher s-IgA levels in children exposed during pregnancy could be due to the immunogenic components of the cigarette, which could cross the placental barrier and have a direct effect in the fetal immune system, which could be reflected in later children's s-IgA reactivity and health (Sandin et al., 2011). Interestingly, as reported by Noakes et al. (2007), children exposed to maternal tobacco presented a higher prevalence of chest infections, which could explain the increased s-IgA levels. Although the prenatal period seems to be a sensitive stage for the immune system programming, other factors as breastfeeding should be considered, since maternal milk is a source of different immunoglobulins that protect infants against pathogens (Rio-Aige et al., 2021). According to Noakes and colleagues (2007), the length of breastfeeding was shorter in the group of smokers, impacting on their susceptibility to infections and their postnatal immune system development. However, these results should be interpreted with caution taken in to account some methodological limitations of both studies.

Secondly, with regard to perinatal maternal distress, Ellberg et al. (2019) and Kang et al. (2020) reported lower s-IgA levels in 1 to 8 months old infants exposed to different forms of pre or postnatal maternal distress. Specifically, Kang and colleagues (2020) observed that prenatal and persistent depression are associated with reduced s-

IgA levels in offspring's feces. Interestingly, when analyzing the impact of infant's age, s-IgA concentrations were only significantly lower in infants older than 4 months whose mothers showed depression during pregnancy. These results suggest that maternal depressive status during pregnancy could be a risk factor for the optimal fetal immunity system development. Different maternal immune and neuroendocrine mediators have been proposed as mechanisms underlying these associations (Merlot et al., 2008). Furthermore, maternal and infant gut microbiome has been also suggested to be modified under maternal distress, which might affect infant's intestinal s-IgA production (Jašarević et al., 2015; Mu et al., 2021).

On the other hand, Ellberg and colleagues (2019), employing a composite measure of depression and stress, proposed that maternal postnatal distress was associated with lower s-IgA levels in infants between 1 and 6 months of age. In accordance with previous literature, maternal well-being and caregiving in early stages of life might influence infant emotional and physiological functioning (Barclay et al., 2022; Buhler-Wassmann & Hibel, 2021). Moreover, these results are in line with other studies included in this review, which point out that poor family caregiving downregulates s-IgA diurnal secretion (Vermeer et al., 2012; Watamura et al., 2010). Notably, further studies must deeply explore the role of s-IgA levels in maternal breast milk, since those levels could be altered in the presence of different psychosocial stressors.

Contrary, Ulmer-Yaniv et al. (2018a) reported that maternal depression at any time-point of child's life was associated with increased evening s-IgA basal levels at 10 years old. Interestingly, higher children s-IgA levels were also associated with more internalizing and externalizing symptoms. This is in line with previous studies indicating that both early life adversity and psychopathological symptoms are associated with increased activity of the immune system (Coelho et al., 2014; Mitchell & Goldstein, 2014; Slopen et al., 2013). Beyond increased s-IgA levels, Ulmer-Yaniv and colleagues (2018a) observed elevated cortisol levels in children from depressed mothers, suggesting a crosstalk between neuroendocrine and immune system. These results are in great accordance with previous literature indicating the existence and HPA axis-related immune modulation (Mueller et al., 2022; Ziemssen & Kern, 2007).

In regard to parenting and caregiving, worse parenting resulted in increased s-IgA basal levels in the studies of Abraham et al. (2021) and Byrne et al. (2017) while not differences were found in Reindl et al. (2022) study. In line with the study by Ulmer-Yaniv et al. (2018a), Abraham et al. (2021) found that poor parental caregiving was associated with increased levels of both s-IgA and cortisol in three-year-old children, highlighting once again that parents play an important role on infant stress regulation. Moreover, children with better self-regulation abilities showed lower s-IgA basal levels, as reported in previous studies which claim that personality traits modulates the impact that stress have on immune system functioning (Kubitz et al., 1996; Segerstrom & Miller, 2004). In this line, Byrne and colleagues (2017) identify that poor monitoring and supervision predict higher s-IgA and C-Reactive Protein (CRP) levels in nine-year-old children, suggesting an hyper activation of the immune system under situations that can be interpreted as social rejection. On the other hand, Reindl et al. (2022) indicated that familial foster care might mitigate the impact of early life adversity since no differences on s-IgA and cortisol levels were found between 4-year old children in foster and biological parental care. Contrary to expected, authors found that children in foster care with higher caregiving quality showed an increasing s-IgA pattern across the one-year study period. Meanwhile, children in foster care with lower caregiving quality showed decreasing s-IgA concentrations, suggesting that long-term stressors could downregulate s-IgA production. Further studies are needed to determine whether poor caregiving disrupts the expected age-related increase on s-IgA levels.

Finally, the studies by Ulmer-Yaniv et al. (2018b) and Yirmiya et al. (2018) indicated that war exposure increased basal s-IgA levels in nine- to eleven-year old children. Furthermore, both studies observed that increased s-IgA levels lead to higher anxiety symptoms and reduced social collaboration skills, indicating that s-IgA could be a biomarker of perceived anxiety in children exposed to chronic stress. Moreover, when studying the impact of maternal caregiving among children exposed to war, greater maternal sensitivity was associated with lower s-IgA levels in both studies. These results are in line with previous literature claiming that mothers might buffer children stress response throughout multiple physiological and social cues (Ruttle et al., 2011).

Therefore, the above-mentioned studies highlight the difficulty of exploring the impact of different stressors on s-IgA basal levels. Most of the studies, including those with high quality, point out to increased s-IgA basal levels after exposure to stressors, especially in children older than 9 years old. However, it should be noticed that there are several confounding factors that might be mediating this association. In this line, the nature and chronicity of stressors might differentially affect immune system functioning. Moreover, it should be explored whether participation in the study is acting as an acute stressor that rapidly increases s-IgA levels. Importantly, other aspects as age and sex must be consider in further studies as it has been well stablished that s-IgA levels increased throughout life span in a sex specific way. Finally, considering that s-IgA secretion varies throughout the day, timing of sampling may contribute to the inconsistency of these findings.

4.2. *s-IgA circadian rhythm*

The three studies exploring the impact of parenting, caregiving and psychopathological symptoms on s-IgA circadian rhythm suggest that these stressors could modulate the existing diurnal pattern depending on children's age (Ma et al., 2018; Vermeer et al., 2012; Watamura et al., 2010).

With regard to s-IgA rhythmicity, the three reviewed studies identified the existence of a diurnal pattern, although the curves presented noticeable differences between studies. The only similarity identified in all the studies was the decrease of s-IgA levels during late afternoon and evening. However, it is important to highlight that the methodological differences regarding the number of samples and collection time hinder the comparison of the patterns across studies. For instance, despite evaluating children around 3 years old with similar stressors, Watamura et al. (2010) and Vermeer et al. (2012) obtained contradictory findings. Specifically. Vermeer and colleagues (2012) determined that 2- to 3-year old children presented circadian rhythm both during weekdays and weekends characterized by a morning decrease, a slight afternoon increase and a final evening decline. Contrary, Watamura and colleagues (2010) only reported rhythmicity on the weekends. This circadian pattern was characterized by a gradual decrease from mid-morning to evening, with a sharper decline on mid-afternoon. These discrepancies may be explained by the methodological design, especially related to differences on the time of sample collection. On the other hand, Ma and colleagues (2018), employing a high-quality design, reported the existence of a well-defined s-IgA circadian rhythm in 9-to-12 year old children. According to these authors, s-IgA presented a slight increase within the first four hours post-awakening and a gradual decrease toward the afternoon followed by a final increase before bedtime. This study presents the greatest reliability as eight saliva measures per day were obtained, in two consecutive days, starting at awakening, 30 min after and then collecting samples every two hours until bedtime. This approach seems to be more suitable for studying s-IgA diurnal rhythmicity since it considers children's awakening time and has continued measures.

In spite of the above-mentioned methodological differences between the studies, the type of stressor also seems to play a role on s-IgA rhythmicity. Regarding caregiving, both Vermeer and colleagues (2012) and Watamura and colleagues (2010) agree that s-IgA circadian pattern is better defined in the weekends, when children are cared for at home. Additionally, according to Vermeer and colleagues (2012) lower caregiver emotional support is associated with lower s-IgA levels in all

daytime points during weekdays and weekends. These findings suggest that children could benefit from a warm atmosphere and secure environments to self-regulate their s-IgA diurnal secretion. In this line, Watamura et al. (2010) reported an increase on this immunoglobulin in the weekdays evening in young children when they are at home with their parents. This result is in great accordance with the increased s-IgA levels during the afternoon that Vermeer et al. (2012) reported. However, the mediating role of children's age should not be underestimated. Particularly, Watamura et al. (2010) observed that 3- to 4- year old children only presented the expected s-IgA circadian rhythm during weekends, while 4- to 5- year old children showed rhythmicity independently of the day. Thus, while the ability to regulate s-IgA secretion seem to depend on environmental clues in young children, older children s-IgA rhythmicity could be independent of the received care. Interestingly, when studying cortisol profiles, Watamura et al. (2010) observed that young and old children presented a well defined circadian rhythm in both weekdays and weekends, suggesting that s-IgA rhythmicity presents greater differences according to children's age.

Furthermore, in regard with children's anxiety, Ma and colleagues (2018) observed that psychological anxiety symptoms, including worries and social concern, predicted a higher s-IgA level's increase in the morning and lower s-IgA evening levels in 9 to 12 year old children. However, physiological anxiety symptoms (e.g. nausea, headaches, and muscle tension) were not associated with s-IgA diurnal slopes, suggesting that cognitive anxiety might have great impact on biological systems at these ages. Nevertheless, the effect of anxiety on s-IgA seems to be mediated also by age. Specifically, authors reported the existence of a continuous loop between anxiety and s-IgA levels in the different stages. Higher total anxiety and worries in children in late childhood (between 9 and 12 years old) were associated with diminished global s-IgA levels when they reached the adolescence (between 12 and 15 years old), which in turn seems to predict higher total anxiety symptom and worries in late adolescence. Furthermore, in late adolescence (between 15 and 18 years old), higher total anxiety was associated with lower s-IgA levels. S-IgA levels in late childhood were significantly associated with s-IgA levels in early adolescence, but these levels were not associated with those presented in late adolescence. These results indicate that children with persistent worries over time may be at risk for compromised mucosal immunity, reflected by lowered overall levels of s-IgA. The findings further illustrate the mutual regulation between chronic anxiety symptoms and immunity over several years. These results, together with previous evidence, point out that stress produces maladaptive responses that lead to the downregulation of the immune system, especially when it is chronic. It has been also proved that chronic stress dysregulates cortisol secretion which in turn leads to altered immune responses (Johnston-Brooks et al., 1998; Juster et al., 2010).

Due to the methodological differences, the nature of the explored stressors and the age of the participants, it is not possible to conclude that stress modifies s-IgA rhythmicity in a specific way. Further studies are needed to determine s-IgA rhythmicity and possible psychological stress related disturbances. Moreover, an optimal design would include at least four saliva samples to measure s-IgA circadian rhythm according with participant's awakening time and routines.

4.3. S-IgA reactivity to acute stress

The two studies assessing s-IgA reactivity under acute stress using variations of the TSST-C observed that s-IgA levels significantly increased during the stress task and then gradually decreased until the end of the acute stress protocol in participants between 7 and 17 years (Laurent et al., 2015; Marques-Feixa et al., 2022).

Both studies analyzed s-IgA in combination with other biomarkers, i.e., cortisol (Laurent et al., 2015; Marques-Feixa et al., 2022) and salivary alpha amylase or SAA (Laurent et al., 2015). The results of both studies reinforce the idea that, when faced with acute stress, s-IgA levels increase faster when compared to both cortisol and SAA. In fact, s-IgA peaks around 5 to 6 min after facing the stressor (Benham, 2007) and this response ends approximately 30 min after the exposure. Notably, cortisol, which is the most widely used stress biomarker, peaks around 20 to 30 min after exposure to the stressor (Giacomello et al., 2020). Interestingly, Laurent et al. (2015) reported that s-IgA and cortisol were positively associated, suggesting a cross-system linkages in humans.

Although the immediacy of the s-IgA response makes it a good candidate for exploring acute stress responses, some confounding variables should be considered. Firstly, it should be highlighted that s-IgA reactivity seems to vary according to the developmental stage of the participants. In that sense, Marques-Feixa et al. (2022) observed that s-IgA levels increased after the stress task in adolescents, although this response was not observed in children between the ages of 7–11, suggesting this population group might not be able to release s-IgA after a stressor. These results are in line with previous reports indicating that puberty is a key period for the reprograming of the immune system. Interestingly, the immune system is immature in the first stages of life and goes throughout different changes during childhood and puberty in order to adapt and optimize its function to the environment (Simon et al., 2015).

Despite the importance of age, early life adversities seem to advance the abovementioned immune changes and modulate s-IgA reactivity prematurely. According to Marques-Feixa et al (2022) children with a history of childhood maltreatment did show a moderate s-IgA reactivity, similar to the one observed in adolescents, suggesting a precocious adaptive immunological response. Additionally, authors claimed that adolescents who have suffered maltreatment showed a slightly flattened s-IgA response, which may also be indicative of an immune dysregulation associated with chronic relational trauma. In accordance with these results, Laurent et al. (2015) reported that youth behavioral problems also have the ability to downregulate s-IgA reactivity. Specifically, youth with high externalizing symptoms exhibited lower s-IgA responses during the stressor when compared with youth with low externalizing behaviors. However, the impact of the developmental stage was not explored in this study. These results are in accordance with previous literature indicating that rumination and neuroticism traits flatten s-IgA stress response in adulthood (Reza et al., 2016). Alterations in other biological systems have been also reported in people exposed to early adverse experiences; for instance, the anticipation of menarche (Boynton-Jarrett et al., 2013), increased cellular aging (Colich et al., 2020) and epigenetic aging (Palma-Gudiel et al., 2019). Furthermore, cortisol reactivity in front stress seems to be strongly regulated by childhood maltreatment and psychopathological status, but not by pubertal stages (Laurent et al., 2015; Marques-Feixa et al., 2022). These changes might have a major and long lasting impact on both brain and immune system (McCrory et al., 2010), contributing significantly to a poorer mental and physical health later in adulthood (O'Connor et al., 2014). Thus, these findings suggest that the combined use of different biomarkers, particularly cortisol, could provide complementary information regarding acute stress response in humans.

To sum up, s-IgA reactivity patterns vary throughout the ontogenic stages, although early adverse events dysregulate this response. The robustness of the two high-quality studies reviewed (Laurent et al., 2015; Marques-Feixa et al., 2022) highlights s-IgA as a potential non-invasive salivary acute stress biomarker to be used in pediatric and childhood-adolescent psychiatry research. For an optimal design, we recommend the use of standardized stress protocols, the collection of different salivary samples across the test, taking into consideration that s-IgA peaks around 6 min. Conducting the study in the afternoon or evening is also recommended in order to avoid possible bias related to s-IgA circadian rhythm.

5. Conclusion

The present review highlights that s-IgA basal levels, s-IgA circadian rhythm and s-IgA reactivity to acute stress could be used as biomarkers

of psychosocial stress in childhood. However, the variety of stressors together with the different group of ages evaluated makes it difficult to obtain generalizable conclusions.

Firstly, the reviewed studies suggest that s-IgA basal levels is an accurate approach to study the impact of psychosocial stress in late childhood (Abraham et al., 2021; Byrne et al., 2017; Ellberg et al., 2019; Kang et al., 2020; Molnar et al., 2018; Noakes et al., 2007; Reindl et al., 2022; Ulmer-Yaniv et al., 2018a,b; Yirmiya et al., 2018). Specifically, stress generally resulted on increased s-IgA levels in children older than 9 years old. Secondly, the studies that explored s-IgA rhythmicity suggest that s-IgA levels varied throughout the day with a tendence to decrease throughout the afternoon and the evening, although the curves were slightly different between studies (Ma et al., 2018; Vermeer et al., 2012; Watamura et al., 2010). Noticeably, rhythmicity seems to be better defined in older children, suggesting the possible existence of reprogramming processes at certain ages. Furthermore, reported changes on s-IgA rhythmicity due to psychosocial stressors, although the methodological differences do not allow establishing a specific directionality. Finally, regarding s-IgA reactivity under acute stress, the studies by Laurent et al. (2015) and Marques-Feixa et al. (2022 agreed on the existence of a fast s-IgA response after stress exposure, especially in adolescents. However, this reactivity pattern seems to be modified by childhood maltreatment and current psychopathological status, leading to diminished s-IgA responses after puberty and anticipated responses in childhood.

Independently of the s-IgA measure employed, this review highlights that age influences s-IgA functioning. Previous studies have described an increase on s-IgA levels across the ontogenic periods (Weemaes et al., 2003) but our review additionally suggest that there are different immunological reprogramming periods across lifespan. During the first years of life, s-IgA levels are more dependent on the maternal health and bond, especially due to breastfeeding, while older children rely on themselves to regulate the s-IgA responses to the environmental conditions. Finally, the abovementioned reprograming seems to be especially relevant during pubertal when s-IgA becomes clearly reactive to acute stressor. The findings of the reviewed studies suggest that this age related s-IgA changes could be compromised in the presence of adverse events during these sensitive reprogramming periods.

On the other hand, the type of the stressor evaluated also seems to play a key role on s-IgA dysregulation. The fact that stressors differently affect children's s-IgA responses indicate that stressors could be targeting different biological pathways. For example, tobacco exposure clearly increases the risk of infection and the consequent s-IgA secretion, while relational trauma during infancy seems to be related to glucocorticoid dysregulation that latter compromise immunological functioning. Therefore, the study of additional biomarkers, especially cortisol, would provide a better understanding of the complex nature of stress response in humans and the cross talk of different biological systems.

However, there are several concerns regarding methodological designs that should be addressed in future studies. On the one hand, standardized protocols for salivary collection are needed in order to facilitate comparisons between studies. Moreover, reporting raw s-IgA levels would improve the understanding of the obtained results and would enable to perform meta-analytic approaches. Finally, we consider of great importance to understand how s-IgA reactivity, rhythmicity and basal levels behave in the unexplored ontogenic stages.

To sum up, the present evidences indicate that s-IgA can be considered a reliable biomarker of acute stress. However, further research is needed to determine how psychosocial stress impacts on s-IgA circadian rhythm and basal levels.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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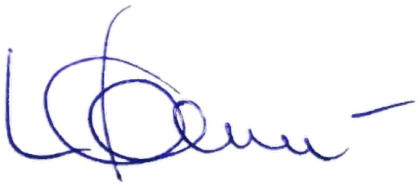
UNIVERSITAT DE BARCELONA

Dr. Lourdes Fañanás Saura
Section of Zoology and Biological Anthropology
Dept. Evolutionary Biology, Ecology and Environmental Sciences
Faculty of Biologia, Universitat de Barcelona

Supervisor's report on the contribution of the PhD applicant to the article.

Prof. Dr. Lourdes Fañanás Saura, full professor at the Department of Evolutionary Biology, Ecology and Environmental Sciences of the Faculty of Biology (Universitat de Barcelona) and supervisor of the present doctoral thesis by Águeda Castro Quintas, hereby certifies that the participation of the PhD applicant in the article "*Salivary secretory immunoglobulin A as a putative biomarker of psychosocial stress response during the first stages of life: A systematic review*" included the following tasks:

- Participation in the conception and design of the study.
- Writing of the first manuscript draft.
- Critical revision of the article for intellectual content.



Signed by Prof. Lourdes Fañanás

Barcelona, 5th December 2023

4. Global summary of the results

The main hypothesis was tested throughout the six independent studies mentioned above.

Regarding the first specific hypothesis, maternal distress increased the risk of poor offspring outcomes by means of HPA axis sensitization and immune system interaction, as revealed by the three first publications presented in this dissertation.

I. Exploration of maternal cortisol levels throughout pregnancy revealed that cortisol pattern is preserved in all trimesters of pregnancy, although cortisol levels gradually increased across gestation. However, women with depressive symptoms showed a blunted circadian rhythm in mid-pregnancy, characterized by higher evening levels. Furthermore, higher cortisol levels in mid-late pregnancy were associated with prematurity and infant's lower weight percentile at birth. Thus, checking for any symptoms of depression during pregnancy, regardless the past or current history of mental disorders, is important, as they might have consequences on the mother and the infant wellbeing.
II. Increased maternal cortisol levels during pregnancy were associated with higher DNA methylation signatures at *NR3C1* exon 1_D and lower DNA methylation signatures at *FKBP5* intron 7, in placental chorionic villi; meanwhile, higher DNA methylation at *FKBP5* introns 1 and 7 in the maternal decidua were associated with lower gestational age at birth, suggesting that maternal distress during pregnancy could influence placental epigenetic signatures differently depending on the time of exposition, the placental layer and the gene of study, particularly with more pronounced effects in those related to stress response, potentially impacting pregnancy development and newborn outcomes.
III. Infants born to mothers exposed to SARS-CoV-2 showed poorer responses in items related to regulation and motor system domains at two months old, particularly when mothers were infected in the third trimester. These results highlight the importance of testing children prenatally exposed to SARS-CoV-2 for future neurodevelopmental disorders, regardless the severity of the infection.

Regarding the second specific hypothesis, endocrine and immune effectors involved in the acute stress response were dysregulated in children and adolescents with maltreatment.

IV. Children and adolescents with a history of childhood maltreatment showed Hypothalamic-Pituitary-Adrenal (HPA) axis dysregulations with i) blunted cortisol circadian rhythm, and ii) absence of cortisol response in front to

psychosocial stress (TSST-C). Furthermore, a dose-response relationship between the frequency and severity of the maltreatment and cortisol dysregulation was observed: children and adolescents exposed to moderate/severe maltreatment experiences or with an often/frequent exposition had higher HPA axis dysregulations. However, children and adolescents with maltreatment had a greater perceived anxiety during acute stress exposure. These associations were observed regardless the current presence of psychiatric diagnosis.

V. Acute psychosocial stress (TSST-C) stimulates the secretion of the secretory immunoglobulin A (s-IgA) (a mucosal immunoglobulin of the immune system) but only after puberty. However, while children without history of childhood maltreatment did not showed changes on s-IgA levels across the stressor, children with history of childhood maltreatment showed a similar response to adolescents, increasing their s-IgA after the acute stressor. This result could be suggesting that complex trauma could anticipates the immune system maturation.

VI. A systematic review on the reliability of salivary s-IgA as a new biomarker of psychosocial stress in young population evidenced that s-IgA can be consider a good biomarker in front of acute stress, especially after puberty. However, further research is needed to specifically determine how psychosocial stress impacts on s-IgA circadian rhythm and basal levels and disentangle the possible role of age and type of stressor.

5. Discussion

The main objective of this thesis has been to study the impact of psychosocial stressors during the prenatal period, childhood, and adolescence and how endocrine and immune systems are primarily involved in the response to these stressors.

At the beginning of this thesis, we wondered if the depressive symptoms of the mother during pregnancy were associated with a dysregulation of daily cortisol secretion and if this could have an impact on the placental epigenetic signatures and on the perinatal outcome of the offspring.

For these purposes, we had access to a cohort of 112 healthy pregnant primiparous women drown from the general population and their offspring.

For the first purpose, we have had the opportunity to analyze the diurnal oscillations of the HPA axis at very different moments during pregnancy, obtaining the circadian cortisol pattern for each trimester individually (Castro-Quintas, Eixarch, et al., 2023). Although the circadian rhythm was more or less preserved in all gestational trimesters, the presence of subclinical depressive symptoms, measured with EPDS, altered this circadian profile in early to mid-pregnancy. These alterations are characterized by a lack of elevation in morning cortisol (CAR) and by not decreasing cortisol levels in the evening. Our results are in line with previous literature reporting flatter diurnal cortisol patterns across all trimesters among pregnant women with anxious depressive disorders or exposed to early adverse life events (Murphy et al., 2022; O'Connor et al., 2014). However, we demonstrated that this disruption of the diurnal rhythm of the HPA axis can occur not only in women with a depression diagnosis but also in those presenting subclinical traits or symptoms.

Despite subtle alterations observed in cortisol rhythmicity, depressive symptoms did not fully dysregulate HPA axis function in early pregnancy. Hormonal fluctuations observed during this phase may contribute to a comparable cortisol circadian profile in both groups of women, indicating that relevant hormonal changes that also influences fetus, as cortisol levels, start at the beginning of gestation. Advanced pregnancy stages, driven by placental activity, can exert a notable influence on maternal physiological changes, including cerebral reorganization (Servin-Barthet et al., 2023). These psychological changes may contribute to a higher risk of maternal depression in mid-late pregnancy. Moreover, in late pregnancy, numerous hormonal changes prepare for fetal maturation before birth, including a significant increment of cortisol facilitated by the elevated placental CRH production (Kammerer et al., 2006). This may suggest that late-

stage HPA axis functioning in pregnant women is more influenced by hormonal changes associated to the perinatal period than by maternal stress conditions itself, possibly explaining the absence of differences in HPA axis rhythmicity regarding the presence of depressive symptoms in late pregnancy reported in our sample.

According to our results, the total maternal cortisol levels secreted throughout a day increase over the course of pregnancy; although this increase appears gradual in women without depressive symptoms, while in women with depressive symptoms, it becomes more pronounced between early and mid-pregnancy. Cortisol plays a crucial role during pregnancy (Austin & Leader, 2000), so its levels increase in a controlled manner to meet the varying needs of the developing fetus at different stages. However, high levels of cortisol are neurotoxic and could potentially harm both other biological systems of the mother and the developing fetus, thereby affecting delivery and newborn conditions. Therefore, the presence of maternal depressive symptoms in the middle of pregnancy may pose a risk to the health of the mother-child dyad.

The second trimester, in particular, stands out as a crucial period for fetal brain maturation, marked by the intricate process of neurons reaching their final destinations within the brain (Turk et al., 2019). Furthermore, the fetus's own HPA axis, as it begins to function autonomously during this trimester (Howland et al., 2017). Thus, disruptions during this phase could potentially impact the delicate balance of cortisol requirements, having putative consequences on infant early neurobehavioral milestones and stress response. While it is true that the placenta harbors protective mechanisms, such as the enzyme 11βHSD2, there are instances when this safeguard may not be fully operational. This could occur during the early stages of pregnancy, when the placenta is not yet fully mature (Mayer & Joseph, 2013) or be associated with less expression of the *HSD11B2* gene in placenta, leading to an increased transfer of cortisol to the fetus (Monk et al., 2016). In fact, elevated cortisol levels in mid-late pregnancy of our mothers to be appeared to elevate the risk of low weight percentile at birth and prematurity.

On the other hand, the observed weak could be related with other pathways, possibly involving inflammatory outcomes. While changes in the maternal immune system during pregnancy typically do not impact the fetus (Abu-Raya et al., 2020), severe maternal depression during pregnancy could potentially lead to increased inflammatory cytokines (IL-6, IL-10) together with raised diurnal cortisol secretion, inducing a proinflammatory status in both the mother and the developing fetus (Osborne et al., 2018). Further exploration of these complex interactions is essential for a comprehensive

understanding of the multifaceted factors influencing proinflammatory maternal states and newborn's outcomes at birth.

The postnatal period is also crucial for infant development, with heightened prenatal distress amplifying vulnerability to adverse postnatal experiences and emphasizing the dynamic nature of early brain development. Maternal care during the initial postnatal phases is particularly important (Hartman et al., 2023). However, the occurrence of depression in the mother during pregnancy heightens the likelihood of its continuation into the postnatal period (Jahan et al., 2021). This not only impairs maternal-fetal bonding (Dubber et al., 2015) but also influences playtime, routines, and diminishes breastfeeding duration (Dias & Figueiredo, 2015; McLearn et al., 2006). Consequently, it increases the child's susceptibility to adverse situations, suggesting a potentially unfavorable prognosis. Despite the severe consequences of prenatal depression and the possibility that proper screening and treatment may help to reduce adverse birth outcomes, most research has concentrated exclusively on postpartum depression (Johnston et al., 2021). Indeed, there is a lack of consensus among European countries regarding clinical recommendations for screening, diagnosing, and managing peripartum depression (Motrico et al., 2022). The findings of this study suggest that it is crucial to establish effective strategies for detecting depressive symptoms at least once each trimester. Additionally, it emphasizes the use of cortisol as a biomarker for screening at-risk mothers, measuring either their diurnal rhythmicity or, at a minimum, morning, or evening levels.

As has been reported in the introduction section, the placenta, acting as a crucial intermediary between maternal and fetal stress. This temporal organ plays a pivotal role in mitigating the potential adverse consequences of maternal psychological and physiological conditions during the prenatal period, creating a supportive environment that fosters optimal development and well-being for the growing fetus.

For this reason, in the second study presented in this thesis, we aimed to investigate the potential impact of maternal depressive symptoms, and the associated elevation of cortisol levels, during pregnancy in the epigenetic signatures in genes involved in cortisol regulation: *HSD11B2*, *NR3C1* and *FKBP5*. To account for potential differential reactivity of the placental layers to maternal stress, given their distinct origins, we examined the methylation patterns of these genes separately in the chorionic villi and the maternal decidua. Additionally, we explored whether these epigenetic patterns were linked to gestational age at birth, birthweight, postnatal cortisol regulation, and

neurobehavioral dimensions of the newborn at two months of age studied with the NBAS scale (Castro-Quintas, Palma-Gudiel, Eixarch, et al., 2023).

Initially, our findings indicate a noteworthy correlation between heightened maternal diurnal cortisol levels during the first trimester of pregnancy and an increase in DNA methylation at exon 1D of the placental *NR3C1* gene, coupled with a decrease in methylation at intron 7 of the *FKBP5* gene, particularly in chorionic villi. Elevated methylation in the promoter region of *NR3C1*, where exon 1_D is located, has been previously linked to reduced expression of the glucocorticoid receptor (GR) in individuals facing adverse conditions (Watkeys et al., 2018). Consequently, a reduced presence of the GR suggests that cortisol will have less effective binding, leading to its failure to enter the nucleus and carry out its gene-regulating functions. In this context, FKBP5 functions as a cochaperone by binding to the GR in the cytoplasm and inhibiting cellular cortisol responses. Intriguingly, intron 7 has been identified to interact with the transcriptional start site of *FKBP5* through chromatin confirmation capture. As a result, the methylation of intron 7 has been found to alter FKBP5 induction. (Klengel et al., 2013). Thus, changes in the methylation patterns of both genes could potentially disrupt their interaction, compromising the placenta's ability to appropriately respond to maternal stress signals mediated by cortisol. Consequently, this disruption could result in heightened and prolonged exposure of the developing fetus to an excess of maternal cortisol.

It is noteworthy that the distinctive methylation patterns of these stress-related genes have been observed exclusively for cortisol measures during the first trimester of pregnancy in our sample. We hypothesize that there could be a potentially unique role of the placenta during early pregnancy in response to stress. In this gestational stage, the placenta performs essential functions for maintaining pregnancy, such as maternal endometrium invasion (Nelissen et al., 2011). This invasion requires high trophoblast proliferation during the first trimester of pregnancy, with trophoblasts being the main cells in the chorionic villi (Schroeder et al., 2013). As the second trimester begins, trophoblast growth in the villi decelerates, and vascularization becomes favored in the other placental layers. This may explain why we observed the methylation signatures in FKBP5 and NR3C1 associated with cortisol dysregulation only during the first trimester of pregnancy and specifically in the chorionic villi.

The absence of observations for maternal depressive symptoms associated with methylations signatures suggests that more severe pathologies may be necessary to

induce relevant changes in placental gene methylation, given that our sample consists of healthy pregnant women from the general population. Indeed, in other studies, conditions such as preeclampsia or eclampsia have been linked to changes in *FKBP5* and *NR3C1* methylation (Czamara et al., 2021; Hogg et al., 2013). Additionally, other studies have associated elevated perceived stress, but not depression, with methylation status (Capron et al., 2018; Monk et al., 2016). Our selected genes have an intrinsic nature of responding to cortisol-mediated processes, making them potentially more sensitive to alterations due to the stress perceived in the environment rather than the individualized nature of depressive symptoms. It is also conceivable that depression may alter other genes and pathways in the placenta. For instance, Czamara et al. (2021) observed that placental demethylation of *FKBP5* was associated with increased expression of genes upregulated in preeclampsia and linked to inflammatory and immune response pathways. Hence, it is imperative to explore various dimensions of maternal stress and other genes to comprehend how distinct stressors might manifest as epigenetic modifications in the placenta.

In the maternal decidua, we observed a robust association between the methylation level of intron 7 of *FKBP5* and the anticipation of delivery. A similar, albeit less robust, association was also noted for intron 1 of *FKBP5*. As mentioned above, the unique conformation of intron 7 can induce gene transcription (Klengel et al., 2013). Likewise, intron 1 contains a glucocorticoid-responsive element, and its methylation may similarly impact FKBP5 induction (Czamara et al., 2021). Methylation in both regions seems to contribute to a reduction in FKBP5 expression, enhancing the activation of the GR gene. Importantly, cortisol plays a pivotal role in parturition, promoting contractions. Therefore, an active GR, influenced by the reduced FKBP5 expression due to methylation, could amplify cortisol actions, ultimately facilitating the delivery process. Notably, the maternal decidua, being the placental layer closest to the uterus, provides a potential explanation for the specificity of our observations within this layer.

Finally, no association was observed between maternal stress and *HSD11B2* methylation signatures. Despite the crucial role of the enzyme expressed by this gene in cortisol regulation, in our study it appears less sensitive to subtle changes in maternal stress conditions, as its primary function is unrelated to stress. Notably, we observed a general hypomethylation in the two regions studied, a pattern also noted by Monk et al in their studied sample. (2016). This suggests that *HSD11B2*'s function remains preserved during pregnancy, effectively buffering cortisol levels. Consequently, the cortisol levels received by infants during the prenatal period may be optimal, with no

observed associations with their neurobehavioral milestones assessed by the NBAS scale. Additional analyses with larger samples of mothers from the general population exposed to moderate and chronic stress, as well as studies focusing on high-risk pregnancies, may clarify the existence of a dose-effect relationship between maternal psychological stress and the observed epigenetic changes in stress-regulating genes.

Furthermore, this study highlights the critical need to evaluate the methylation patterns across various layers of the placenta, given that the epigenetic profile seems to exhibit distinct functionalities contingent upon the placental layer and the trimester of pregnancy.

Building upon the potential effects of maternal stress on offspring, the third study presented in this thesis aims to delve into the consequences of maternal exposure to SARS-CoV-2 during pregnancy on early neurodevelopmental outcomes of offspring. The study compares the NBAS performance of six-week-old infants in two groups: those whose mothers were exposed to SARS-CoV-2 during pregnancy (with a positive serology) (clinical group, n=21) and those whose mothers were non-exposed (with a negative serology) (control group, n=21) (Ayesa-Arriola et al., 2023). It is important to note that, in this cohort, none of the mothers presented a severe clinical condition that required hospitalization.

Studies addressing the specific relationship between exposure to maternal infection and early unspecific risk markers on the newborn are still limited and their conclusions are contradictory. In our study we observed that performance of the item "cuddliness", which belongs to the "States Regulation domain" of the NBAS scale showed lower scores in infants whose mother were exposed to SARS-CoV-2 during pregnancy. This association is particularly pronounced in children exposed during the third trimester. Notably, none of the infants whose mothers were exposed to SARS-CoV-2 in the first trimester of pregnancy completed the item response to bell. The necessity for children to be in state 1 (deep sleep) for administration suggests a potential excess of residual excitation, indicating a heightened level of arousal unrelated to environmental triggers. Belot and colleagues (2020) suggest that difficulties in states regulation are related to higher excitation and motor agitation levels, potentially stemming from physiological dysregulation in the nervous system. This heightened arousal may be associated with increased activation of infants' HPA axis, potentially linked to prenatal stress exposure, thereby raising the risk of psychopathology (van Bodegom et al., 2017).

Noticeably, Provenzi et al. (2021) identified regulatory capacity alterations in 3-month-old infants whose mothers were pregnant during the COVID-19 pandemic period compared to those whose mothers were pregnant before this period. This discovery underscores the significance of psychosocial stress in shaping the functioning of children's HPA axis, particularly in the third trimester when this system is already established. These findings align with the research by Shuffrey et al. (2022), which observed developmental changes in social and motor skills in children born to mothers pregnant during the COVID-19 pandemic but not in those prenatally exposed to SARS-CoV-2 maternal infection.

Furthermore, it is essential to consider the disparity observed in the 'pull-to-sit' item between infants born to mothers exposed and non-exposed to SARS-CoV-2, which is related to head control. This outcome may suggest motor immaturity and sensory differences, commonly observed in children with neuropsychiatric disorders (Dickstein et al., 2005). Therefore, this finding could be regarded as a potential proxy marker for underlying neuropsychological deficits.

While there is a gap in studies linking prenatal infections to children's self-regulation abilities, previous research has connected a suboptimal response to cuddliness in infants exposed to adverse postnatal factors, such as maternal depression, indicating the potential influence of psychosocial factors on infants' arousal (Hernandez-Reif et al., 2006). Social interactions, particularly those with caregivers, depend on face-to-face exchanges and close physical contact that foster attachment (Markova et al., 2019), which were greatly disrupted during the COVID-19 pandemic. In fact, a recent review has pointed out that maternal status and environmental conditions may be more explanatory of the offspring's neurodevelopment than the history of viral infections during pregnancy (San Martín-González et al., 2023). In this regard, it will be of great importance to disentangle the contribution of prenatal viral infections and pre and postnatal psychosocial factors.

Additional research is imperative to unravel the impact of SARS-CoV-2 infection and the stress associated with the COVID-19 pandemic during pregnancy on offspring development. Given our findings and the magnitude of COVID-19 pandemic, it is recommended that children born to mothers infected during pregnancy, especially in late pregnancy, undergo extended longitudinal screening for neurodevelopmental milestones.

Expanding upon this early life perspective, the second part of this thesis centered on childhood and adolescence population. Considering that the development of the brain is not completed until well into adolescence (Knuesel et al., 2014), stressors experienced during childhood and adolescence can also have consequences on development, increasing the risk of develop psychiatric disorders (Heim et al., 2019). Hence, in the final three articles, we explore the potential consequences of psychosocial stress during childhood on key biological stress regulatory mechanisms related with the HPA axis and immune system.

The fourth manuscript explored the function of the HPA axis under baseline conditions and in response to an acute psychosocial stressor (the TSST-C), examining its potential association with a history of maltreatment (Marques-Feixa et al., 2021). Regarding the basal function responsible for maintaining the circadian rhythm, our observations indicate that children and adolescents with experiences of maltreatment, regardless of current psychopathology, exhibit elevated cortisol levels throughout the day, especially during the night. These findings align with a recent meta-analysis investigating the impact of child maltreatment on HPA axis functioning, which also identified a blunted evening slope (Schär et al., 2022). This dysregulation in evening cortisol production can adversely affect the quality of sleep, contributing to sleep disturbances. The interplay between heightened nighttime cortisol and compromised sleep quality may consequently lead to behavioral consequences, including increased irritability, difficulty concentrating, and comorbid internalizing and externalizing symptoms (Cicchetti & Rogosch, 2001).

However, conflicting results have been reported by other studies that showed lower levels of morning cortisol in subjects exposed to childhood maltreatment, suggesting hypocortisolism (Bernard et al., 2017). The discrepancies with previous studies could potentially be explained by differences in the type of abuse suffered by the child. Specifically, lower morning cortisol levels have been linked to instances of physical abuse and physical neglect, while children subjected to a multitude of abuse types, such as sexual and physical abuse, as well as severe emotional abuse, exhibit elevated morning cortisol levels (Bruce et al., 2009; Cicchetti & Rogosch, 2001).

On the other hand, concerning the HPA-axis response to acute psychosocial stress, children and adolescents exposed to childhood maltreatment exhibited a clear blunted cortisol response during the TSST-C, compared with those without childhood maltreatment. This finding is in line with previous research that indicates a blunted

response pattern during the TSST in adults exposed to early adversity (Bunea et al., 2017). Therefore, our result emphasizes that HPA axis hypoactivation is already present in exposed infants. Importantly, as these alterations have been observed independently of the presence of a current mental diagnosis, we demonstrate that these dysfunctions could be present before infants develop mental health problems (Murphy et al., 2022). However, the most paradoxical aspect observed is that children with maltreatment experiences exhibit heightened levels of anxiety, indicating a clear dissociation between their perceived stress and their biological response. Since the HPA axis is critical for activating the brain and other systems under stress conditions, individuals may find it considerably more challenging to manage everyday stressful situations emotionally and behaviorally. Consequently, these individuals might more readily resort to maladaptive self-regulation strategies, such as substance use, self-harm, or suicidal attempts, thereby increasing their risk of developing a mental disorder (Baldwin et al., 2023). Indeed, this dissociation between the biological response and the emotional interpretation should be considered, as it can influence the effectiveness of pharmacological treatments in individuals with clinical diagnoses who have a history of childhood maltreatment (Nemeroff et al., 2003).

Our findings highlight the pivotal role of both the severity and frequency of childhood maltreatment in shaping alterations within the HPA axis. This impact is evident in both its diurnal basal functioning and its response to stress, emphasizing a discernible dose–response relationship. Specifically, mild experiences of maltreatment correspond to a subtle disruption of the HPA axis, whereas chronic and enduring maltreatment can lead to a complete dysregulation of the HPA axis over time. Additionally, our study revealed that children exposed to milder forms of childhood maltreatment tend to perceive higher anxiety compared to their non-maltreated counterparts, although their anxiety levels are lower than those observed in children exposed to severe forms of maltreatment. This underscores a significant window of opportunity for early intervention, given that the HPA axis has the potential to recalibrate after adverse childhood experiences, especially with enhanced environmental support (Gunnar et al., 2019).

The impact of childhood maltreatment on HPA axis function can also hold significant implications for the immune system, since the HPA axis and glucocorticoids play a major role in regulating immunity (Danese & McEwen, 2012). Notably, childhood maltreatment has been linked to heightened inflammation in the adult population, evident in increased levels of CRP (Baumeister et al., 2016). Furthermore, adults who

experienced abuse as children face an elevated risk of diseases with potential inflammatory origins (Dong et al., 2004). Despite the wealth of evidence in the adult population, there remains a limited exploration of immune alterations in children and adolescents with more recent experiences of maltreatment.

Therefore, in the fifth manuscript included in this thesis, we aimed to explore the immune system response to an acute stressor (TSST-C) in children and adolescents exposed and not exposed to maltreatment, using a new immune biomarker highly abundant in mucous membranes, and thus, prominently present in saliva, the s-IgA (Marques-Feixa et al., 2022). This is an immunoglobulin that plays a fundamental role in the first line of the immune defense by secreting rapidly, making it a promising biomarker that, however, has been scarcely studied in the child and adolescent population (Laurent et al., 2015).

Our study initially demonstrated that s-IgA fluctuated across the TSST-C, confirming its validity as a biomarker of acute psychosocial stress in the young population. However, this reactivity seems to depend on the pubertal stage. While adolescents showed a rapid increase and recovery of s-IgA in response to the acute stressor, children did not exhibit a significant change in their s-IgA levels. Moreover, adolescents had higher overall levels of s-IgA, suggesting that hormonal changes may influence the immune response to environmental threats. This aligns with literature supporting that puberty is one of the most sensitive periods of life regarding immune system reprogramming due to stress (Csaba, 2020). Furthermore, it is worth noting that the ability to perceive a stressor as a potential threat depends on brain maturation, which could also explain the differential response between children and adolescents. Children are highly dependent on maternal protection, while adolescents are more independent and have more tools to defend themselves (Ulmer-Yaniv, Djalovski, Yirmiya, et al., 2018; van den Bos et al., 2016).

Our findings highlight the significant role of maltreatment in the s-IgA response to acute stress, with this role appearing to depend on the pubertal stage. Specifically, our observations revealed that children with a history of childhood maltreatment exhibited an elevated immunological stress response, resembling the pattern seen in adolescents. This suggests that childhood maltreatment may heighten children's sensitivity to potentially dangerous situations, given their awareness of a lack of adult protection, leading to an early activation of their immune stress response. Consequently, the observed advancement in s-IgA reactivity to acute psychosocial stress in children

exposed to childhood maltreatment aligns with evidence of accelerated biological aging in this group, as indicated by the epigenetic clock, telomere length, and advanced pubertal timing (Chen et al., 2021; Colich et al., 2020). This is also consistent with theories proposing that adverse experiences accelerate puberty onset to maximize the reproductive period before potential mortality (Belsky, 2012), as observed in girls who are victims of sexual abuse (Noll et al., 2017). However, the acceleration of puberty could have adverse health effects, potentially increasing vulnerability to the development of mental disorders (Hamlat et al., 2020).

Comparing the maturation processes of the HPA axis and the immune system, we observed that the cortisol response to the TSST-C remained consistent across both children and adolescents, while the s-IgA response was greatly influenced by pubertal maturation. However, when examining the history of maltreatment, both cortisol and s-IgA responses to stress appear to be altered. This result underscores the enduring alterations associated with childhood maltreatment, encompassing changes in different biological systems implicated in the stress response, such as the HPA axis and the immune system. Such persistent alterations may contribute to heightened susceptibility to diseases and a more challenging disease course (Baldwin et al., 2023; Lippard & Nemeroff, 2020). This complex interplay highlights the multifaceted impact of early-life experiences on physiological systems, potentially influencing long-term health outcomes.

Our findings also suggest that s-IgA increases in a brief period following acute psychosocial stress, underscoring its potential use as a noninvasive immune biomarker in youths. Specifically, we observed a rapid s-IgA increase 20 minutes after the initiation of psychosocial stress, followed by a swift return to basal levels 35 minutes after the commencement of the stressor. This pattern contrasts with the cortisol response, which begins to increase 35 minutes after the start of the stressor. The rapid response of s-IgA suggests its release is under a strong neuroendocrine control and precedes that of cortisol (Teeuw et al., 2004) indicating that cortisol and s-IgA could serve as complementary biomarkers, providing distinct information.

Finally, our observation that s-IgA rapidly increases in saliva after acute stress exposure and its potential complementarity with cortisol information prompted us to conduct a systematic review exploring existing studies measuring mucosal s-IgA levels following psychosocial stress exposure in infancy, childhood, and adolescence. The ultimate goal was to determine its utility as a potential biomarker for altered psychosocial stress response (Castro-Quintas, Palma-Gudiel, San Martín-González, et al., 2023).

Our findings indicate that s-IgA basal levels appear to increase after exposure to stressors, particularly in children older than 9 years old. However, the nature and chronicity of stressors may variably impact immune system functioning. For instance, war exposure was clearly associated with higher basal s-IgA levels (Ulmer-Yaniv, Djalovski, Yirmiya, et al., 2018; Yirmiya et al., 2018), whereas s-IgA levels varied with age after exposure to maternal depressive symptoms. It was lower in exposed infants under six months old (Ellberg et al., 2019) and higher in older children (Ulmer-Yaniv, Djalovski, Priel, et al., 2018). These results also suggest a potential crosstalk between the infant and maternal immune systems, indicating that mothers may buffer children's stress response through various physiological and social cues (Ruttle et al., 2011).

The studies investigating s-IgA rhythmicity suggest that s-IgA levels vary throughout the day, with a tendency to decrease in the afternoon and evening, although the curves slightly differ between studies. Notably, the circadian rhythm of s-IgA appears to be more pronounced in older children. In young children, the s-IgA pattern is more defined on days spent at home with their parents, suggesting that children may benefit from a warm atmosphere and secure environments for the self-regulation of s-IgA diurnal secretion (Vermeer et al., 2012; Watamura et al., 2010). Additionally, Ma and colleagues (2018) observed that adolescents experiencing higher anxiety and persistent worries over time may be at risk for compromised mucosal immunity, reflected by lower overall levels of s-IgA. This finding, in conjunction with previous evidence, underscore that stress can induce maladaptive responses leading to the downregulation of the immune system, particularly when it is chronic.

Regarding s-IgA reactivity under acute stress, reviewed studies unanimously agreed on the existence of s-IgA response post-stress, particularly observed in adolescents. However, this reactivity pattern appears to be influenced by factors such as childhood maltreatment and current psychopathological status. Notably, childhood maltreatment appears to expedite s-IgA reactivity to acute stress before puberty (Marques-Feixa et al., 2022). Additionally, behavioral issues in youth have the capacity to downregulate s-IgA reactivity (Laurent et al., 2015). The robust findings from these high-quality studies underscore s-IgA as a potential noninvasive salivary biomarker for acute stress in pediatric and childhood-adolescent psychiatry research. For optimal study design, we recommend the utilization of standardized stress protocols, collecting various salivary samples throughout the test, with consideration for the fact that s-IgA peaks around 6 minutes. Conducting the study in the afternoon or evening could mitigate potential biases related to s-IgA circadian rhythm.

Overall limitations of the presented word

The different studies conducted in this thesis have certain limitations. Although they have already mentioned in the discussion, the most notable are outlined below.

A primary constraint in all the presented works is the moderate sample sizes. However, it is crucial to consider the challenges associated with sampling pregnant women, children, and adolescents from the general population. Additionally, the longitudinal nature of the projects involving mother-infant dyads has led to potential dropouts. Dropouts are very common in longitudinal psychiatry studies and should be consider, as their data could impact in the results (Mazumdar et al., 2007). Furthermore, the development of the projects was further hindered by the challenges posed by the COVID-19 pandemic and subsequent state of emergency, preventing the collection of biological samples from some of the recruited subjects in all the cohorts involved in this thesis.

Second, concerning the articles in the initial section of this thesis, discerning the specific outcomes of childbirth or a child's neurodevelopment attributable to maternal distress or infections proves challenging. Factors such as the child's genotypic background and other unconsidered environmental elements, including social support or substance use, make it difficult to isolate the sole impact of maternal distress. Nonetheless, it is important to note that the study sample comprised healthy women from the general population with access to healthcare. This demographic exhibited minimal alcohol or tobacco consumption, no drug use, and generally enjoyed strong support, often from at least one family member.

Finally, in the context of the last section and the last three articles, identifying child maltreatment proves highly complex. The methodological simplification involved in this process entails the omission of crucial information about the individual's unique experience of maltreatment. However, these aspects, such as the relationship with the perpetrator, coping style, and family support, are crucial for both clinicians and researchers. They can explain varied consequences of similar events in different individuals and play a fundamental role in expressed psychopathology. Additionally, further research is needed, encompassing a higher proportion of resilient youth—those exposed to child maltreatment without associated psychiatric disorders. This exploration would shed light on the effects of these two conditions on the various variables under investigation.

Final Remark

As a final remark, I would like to retrieve the importance of monitoring and support the children from the early stages, even before they're born (by caring for pregnant women). As explained along this dissertation, the trajectory of a child's life begins with the earliest stages of development, including the prenatal period. During pregnancy, the mother's well-being and the environmental factors she encounters play a profound role in shaping the child's future mental health. The significance of the mother's role cannot be overstated, as her emotional and physical health during pregnancy significantly influences the child's developmental path. A caring and positive environment during pregnancy sets the stage for good mental health in the future for both the mother and the child. Consequently, it is essential to extend our focus beyond the child alone and actively monitor and support the mother throughout her pregnancy. Recognizing the interdependence of maternal and child well-being highlights the need for a complete approach that address the broader family context. By investing in comprehensive prenatal care and fostering positive maternal environments, we can improve maternal health and, consequently, influence the trajectory of the child's health and development.

In this regard, after birth, family, school, and society play crucial roles in the ongoing development of the child. From childhood through adolescence, the environment in which they grow up has a significant impact on their mental health and overall well-being. It is disheartening to observe that children exposed to abusive environments often find themselves caught in a cycle of abuse, endangering their emotional development and mental health. However, this destructive cycle can be interrupted through timely and effective interventions, especially within family. The implementation of preventive measures, early identification of signs of abuse, and the provision of adequate support can make a difference in the life course of these children, offering them a protective environment and the tools needed to build a solid foundation for their mental health and well-being throughout their lives.

Even though this thesis represents a small contribution to the broader research on the significance of early stages of life experiences in mental health, I hope it helps underscore the importance of nurturing both pregnant women and childhood. Our children are not only the future but also a reflection of how well we are taking care of ourselves. By prioritizing the well-being of our youth, we are investing in a better future for both individuals and society as a whole

6. Conclusions

The results of the studies included in the present thesis provide new evidence on the biological and early developmental consequences of prenatal and early life psychosocial stress exposure. The studies comprised in the Section I highlight the importance of screening for depressive symptoms and possible infections during pregnancy, since they might have an impact on endocrine dysregulation of the mothers and on newborn neurodevelopment. The studies comprised in the Section II highlight the relevance that chronic stressors, and particularly childhood maltreatment, play on key periods of development, dysregulating the endocrine and immunological stress response, increasing the vulnerability for mental health disorders.

The specific conclusions derived from studies included in the present work are developed below:

1. Maternal depressive symptoms during pregnancy seem to be associated with blunted cortisol rhythmicity in mid-pregnancy, although there might be a physiological readjustment of cortisol levels in late pregnancy. This cortisol rhythmicity alteration seems to be associated with increased risk of prematurity and low gestational age at birth.
2. DNA methylation of genes related to cortisol pathways, *FKBP5* and *NR3C1*, in placental chorionic villi seems to play a role in regulating cortisol-maternal stress during pregnancy, particularly in the first weeks of gestation. Moreover, DNA methylation of *FKBP5* in the maternal decidua could be a good indicator of the time of delivery.
3. Prenatal exposure to SARS-CoV-2 infection could be related to alterations in different infant behavioral domains at 7 weeks old, particularly those related to interactive behavior and motor development. The timing of exposure to the infection could play an important role on the severity and type of these deficits.
4. Children and adolescents exposed to maltreatment showed disturbances on HPA axis functioning, with higher cortisol evening levels and hyporeactivity to acute psychosocial stress (TSST-C). Furthermore, there is dose-response relationship between the severity and the frequency of the suffered maltreatment and the HPA axis alterations.
5. Acute psychosocial stress stimulates the secretion of s-IgA, but only after puberty. However, children with history of maltreatment showed a similar response to the adolescents, suggesting an anticipated maturation of the immune system in children victims of trauma.

6. s-IgA seems to be a reliable biomarker of psychosocial stress. However, the age of the subject and the type of the psychosocial stressor seem to modulate s-IgA stress reactivity.

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8. Curriculum vitae

Águeda

Castro Quintas

+ 34 699 112 263

aguedacastro@ub.edu

Biotechnologist and Predoctoral Researcher
Department of Evolutionary Biology, Ecology and Environmental Sciences, Faculty of Biology
Universitat de Barcelona
Diagonal Avenue 643, 2A
08028, Barcelona

FORMAL EDUCATION

2018 – present **PhD in Biomedicine, Neurociencies** – Universitat de Barcelona

2017 – 2018 **Master in Neurociencies** – Universitat de Barcelona
Qualification: **8.7/10**
- Master Thesis (**TFM**) developed in the Center for Brain and Cognition (CBC) – University Pompeu Fabra: "Fit to your group! Relating social groups and the principle of rationality from infancy". Supervisor: Dr. Nuria Sebastián Gallés. Qualification: **9.0/10**

2013 – 2017 **Degree in Biotechnology** – Universitat de Valencia
Qualification: **7.9/10**
- Degree Thesis (**TFG**) developed in the Principe Felipe Research Center (CIPF): "Effect of infliximab on neuroinflammation and cognitive impairment in hiperamonemic rats".
  Supervisors: Dr. Tiziano Balzano & Dr. Marta Llansola Gil. Qualification: **8.5/10**
- ERASMUS **practicum** developed in the Hospital Charité Universitätsmedizi, Institute of Medical Psychology, Berlin, Germany: "Comparison of C-Reactive Protein levels in human plasma and saliva in response to acute psychosocial stress".
  Supervisor: Dr. Karin de Punder. Qualification: **9.0/10**

ACADEMIC GRANTS AND HONORS

Name:	**"End of PhD" Grant.**
Organization:	Biomedicine PhD program, Universitat de Barcelona
Location:	Faculty of Biology, Universitat de Barcelona
Period:	27th March 2023.
Activity:	Grant for completing the PhD.
Name:	**Training Activities Grant FI.**
Organization:	AGAUR, Generalitat de Catalunya
Location:	Department of translational research in Psychiatry, Max Planck Institute of Psychiatry
Period:	30th October 2022 – 17th December 2022.
Activity:	To develop training activities on R&D transverse and common knowledge during the PhD.

Name:	**Grant to carry out research stay in Spain or abroad.**
Organization:	Santander Bank
Location:	Department of translational research in Psychiatry, Max Planck Institute of Psychiatry
Period:	14th September 2022 – 15th December 2022.
Activity:	To perform the Targeted Bisulfite Sequencing (TBS) in placental genes of interest and to elucidate the possible effects of the methylation pattern of these genes on early life stress.

Name:	**Scientific Exchange Grant.**
Organization:	European Molecular Biology Organization (EMBO)
Location:	Department of translational research in Psychiatry, Max Planck Institute of Psychiatry
Period:	14th April 2022 – 15th July 2022.
Activity:	To determine genetic regions of interest for methylation analyses and to design primers to perform the Targeted Bisulfite Sequencing (TBS) method in placental tissue.

Name:	**PhD Grant.**
Organization:	AGAUR, Generalitat de Catalunya
Location:	Department of translational research in Psychiatry, Max Planck Institute of Psychiatry
Period:	1st June 2020 – 22nd May 2023.
Activity:	To develop a research project (PhD) in a center of excellence.

Name:	**Research Technician contract.**
Organization:	Bosch i Gimpera Fundation (assigned to Dr. Lourdes Fañanás' project)
Location:	Faculty of Biology, Universitat de Barcelona
Period:	1st April 2020 – 31st May 2020.
Activity:	To do research on early stress biomarkers and mental health.

Name:	**PhD Grant.**
Organization:	Mental Health Networking Biomedical Research Centre (CIBERSAM) (assigned to Dr. Lourdes Fañanás' project, G08)
Place:	Faculty of Biology, Universitat de Barcelona
Location:	15th October 2019 – 31st March 2020.
Activity:	To develop a research project (PhD) in a center of excellence.

Name:	**Research Technician contract.**
Organization:	Bosch i Gimpera Fundation (assigned to Dr. Lourdes Fañanás' project)
Location:	Faculty of Biology, Universitat de Barcelona
Period:	1st July 2019 – 14th October 2019.
Activity:	To measure salivary cortisol using the ELISA technique.

Name:	**PhD Grant.**
Organization:	Universitat de Barcelona (assigned to the 2017SGR1577 project)
Location:	Faculty of Biology, Universitat de Barcelona
Period:	20th November 2018 – 30th June 2019.
Activity:	To develop a research project (PhD) in a center of excellence.

PARTICIPATION IN FUNDED SCIENTIFIC PROJECTS

2023-2025 Research member in the project "**Análisis de marcadores genéticos y epigenéticos de vulnerabilidad para el trastorno mental en niños y adolescentes expuestos a maltrato infantil: estudio multicéntrico**". Funded by the Instituto de Salud Carlos III (FIS) Ministerio de Economía y Competitividad (Ref: PI22/01245. 87.120€). PI: Prof. Lourdes Fañanás. Universitat de Barcelona.

2023-2025 Research member in the project "**El suïcidi com a fenotip extrem per entendre el comportament suïcida: una aproximació des de la interacció del genoma amb l'epigenoma**". Funded by the Marató Foundation (Ref: FGB-600391. 376.384,29€). PI: Dr. Bárbara Arias. Barcelona University.

2022-2024 Research member in the project "**Genes, Ambiente y Fenotipos: etapas de desarrollo en el estudio de trastornos mentales y enfermedades complejas en poblaciones humanas**". Funded by the Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR) (Ref: 2021 SGR 00706. 40.000€). PI: Prof. Lourdes Fañanás. Universitat de Barcelona.

2022-2023 Research member in the project "**Estudio del neurodesarrollo temprano en niños nacidos de gestantes con infección por covid-19: follow-up basado en la cohorte de gestantes covid-19 + BCNatal (H. Clínic – H. SJDD)**". PI: Dr. Marta López Rojano. Clinic Hospital of Barcelona.

2021-2022 Research member in the project "**Sincronizando mundos: neurociencia para adolescentes (MENTESCOPIA)**". Funded by the Spanish Foundation for Science and Technology (FECYT), Grants for promoting scientific culture, technology and innovation (Ref: FCT-20-16227. 25.000€). PI: Dr. Benedicto Crespo. Sevilla University.

2017-2019 Collaborator researcher in the project "**Genes, Ambiente y Desarrollo: una visión longitudinal en la comprensión del origen de la enfermedad mental y de la diversidad de la conducta humana**". Funded by the Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR). (Ref: 2017 SGR-1577. 35.014€). PI: Prof. Lourdes Fañanás. Universitat de Barcelona. (amplified to 2021)

2016-2018 Research member in the project "**Estudio multicéntrico del maltrato infantil en niños y adolescentes con trastornos psiquiátricos: modificaciones epigenéticas y correlatos con marcadores periféricos de inmunidad innata**". Funded by the Instituto de Salud Carlos III (FIS) Ministerio de Economía y Competitividad. (Ref: PI15/00097. 135.520€). PI: Prof. Lourdes Fañanás. Universitat de Barcelona. (amplified to 2020)

2015-2017 Collaborator researcher in the project "**Maternal prenatal stress and HPA axis sensitization mediated by 11β-HSD2 gene epigenetic signatures and its interplay with childhood psychosocial stress in explaining risk for psychopathology in adolescence**". Funded by the Instituto de Salud Carlos III (FIS) Ministerio de Economía y Competitividad. (Ref: INT1512. 35.000€). PI: Prof. Lourdes Fañanás. Universitat de Barcelona. (amplified to 2018)

SCIENTIFIC FORMATIVE STAYS

1. Internship in the Group of Translational Research in Psychiatry, Max Planck Institute of Psychiatry, Munich, Germany.
   Period: 15th April – 17th December 2022.

   Activity: To develop a project in the context of my PhD (Study of placental and cord blood epigenetic signatures as biomarkers of early life stress exposure in a cohort of pregnant women and their offspring) and to obtain the Mention as International PhD.
   Person in charge: Prof. Elisabeth Binder.

2. Internship in de Group of Mental Illnesses, Valdecilla Sanitary Research Institute (IDIVAL), Santander, Spain.
   Period: 1st – 5th October 2021.

   Activity: to understand the different tools for exploring infant's neurodevelopment and to give a seminar to the group.
   Person in charge: Dr. Rosa Ayesa.

3. Internship in the Department of Pharmacology and Toxicology in the Complutense University of Madrid, Spain.
   Period: 1st – 31st July 2019.

   Activity: to learn different biomarkers and techniques used in the context of psychological stress and to give a seminar to the group.
   Person in charge: Prof. Juan Carlos Leza

SCIENTIFIC PUBLICATIONS

Articles published in scientific international journals

1. **Castro-Quintas A.**, Palma-Gudiel H., Eixarch E., San Martin González N., Röh S., Sauer S., Rex-Haffner M., Monteserin-Garcia J.L., Crispi F., Garcia Portilla M.P., Binder E.*, Fañanas L.* *Distress during pregnancy and epigenetic signatures at glucocorticoid related genes in placental chorionic villi and maternal decidua layers: a pilot study in Spanish primiparous women*. Submitted to Clinical Epigenetics. 2023.
2. San Martin-González N., Moya-Higueras J., **Castro-Quintas Á.**, Eixarch E., Crispi F., Daura-Corral M., de la Fuente-Tomás L., Monteserín-García J.L., García-Portilla M.P., Fañanás-Saura, L. *Intergenerational effects of maternal childhood maltreatment on newborn's stress regulation and the role of maternal depressive symptoms*. Submitted to Journal of Child Psychology and Psychiatry. 2023
3. Schowe A.M., Czamara D., Lahti-Pulkkinen M., PhD, Girchenko P., **Castro-Quintas A.**, MSc, Fañanas L., Binder E.B., Räikkönen K. *Diurnal Cortisol Profiles across 9,356 Salivary Samples during Pregnancy – Associations with Maternal Cardiometabolic Complication*. Under Review in The Journal of Clinical Endocrinology & Metabolism (JCEM). 2023.
4. **Castro-Quintas A.**, Eixarch E., San-Martin-Gonzalez N., Daura-Corral M., Marques Feixa L., Palma-Gudiel H., Rocavert-Barranco M., Miguel-Valero A., Monteserin-García J.L., de La Fuente Tomás L., Crispi F., Arias B., Garcia-Portilla M.P., Fañanas L. *Diurnal cortisol throughout pregnancy and its association with maternal depressive symptoms and birth outcomes*. Psychoneuroendocrinology, 161:106930 (2023), doi: 10.1016/j.psyneuen.2023.106930.
5. **Castro Quintas A.**, Palma-Gudiel H., San Martin-Gonzalez N., Caso JR, Leza JC, Fañanas L. *Salivary secretory immunoglobulin A as a putative biomarker of psychosocial stress response during the first stages of life: A systematic review*. Frontiers in neuroendocrinology, 19:101083 (2023), doi: 10.1016/j.yfrne.2023.101083
6. San-Martín-González N., **Castro Quintas A.**, Marqués-Feixa L., Ayesa-Arriola R., López M., Fañanas L. *Maternal respiratory viral infections during pregnancy and offspring's neurodevelopmental outcomes: a systematic review*. Neuroscience and Biobehavioral Reviews, 105178:0149-7634 (2023), doi: 10.1016/j.neubiorev.2023.105178
7. Marques-Feixa L*, Moya-Higueras J.*, Romero S, Santamarina-Perez P., San Martin-Gonzalez N., Mas A., Rapado-Castro M, Blasco Fontecilla H, Zorrilla I, Fornet-Puntonet M., Anglada E., Ramirez M, Mayoral M., Muñoz M.J., Fañanas L, EPI Young stress group (**Castro-Quintas, A.**). C*omplex post-traumatic stress disorder (CPTSD) of ICD-11 in youths with childhood maltreatment: Associations with age of exposure and clinical outcomes*. Journal of Affective Disorders, 332:92-104 (2023), doi: 10.1016/j.jad.2023.03.088

8. Ayesa-Arriola R.*, **Castro-Quintas A.***, Ortiz-Garcia de la Foz V., Miguel-Corredera M., San Martin-Gonzalez, N., Murillo-Garcia N., Neergaard K., Fañanás L., De las Cuevas-Terán I. *Exploring the impact of COVID-19 on newborn neurodevelopment: a pilot study*. Scientific Reports, 13, 2983 (2023), doi: 10.1038/s41598-023-29680-z.
9. Marques-Feixa L*, **Castro-Quintas A.***, Palma-Gudiel H., Romero S, Morer A, Rapado-Castro M, Martin M, Zorrilla I, Blasco Fontecilla H, Ramirez M, Mayoral M, Mendez I, San Martin-Gonzalez N, Rodrigo-Yanguas M, Monteserin-Garcia JL, Fañanas L, EPI Young stress group. *Secretory immunoglobulin A (s-IgA) reactivity to acute psychosocial stress in children and adolescents: the influence of pubertal development and history of maltreatment.* Brain Behavior and Immunity, 103:122-129 (2022), doi: 10.1016/j.bbi.2022.04.010.
10. Marques Feixa L., Moya-Higueras J., Romero S., Santamarina-Perez P., Rapado-Castro M., Zorrilla I., Martin M., Anglada E., Jose-Lobato M., Ramirez M., Mayoral M., Marin-Vila M., Arias B., Fananas L., EPI Young stress group (**Castro-Quintas A**.). *Risk of Suicidal Behavior in Children and Adolescents Exposed to Maltreatment: The Mediating Role of Borderline Personality Traits and Recent Stressful Life Events*. Journal of Clinical Medicine, 10(22):5293 (2021), doi: 10.3390/jcm10225293.
11. Marques Feixa L., Palma-Gudiel H., Romero S., Zorrilla I., Blasco-Fontecilla H., Rapado-Castro M., **Castro-Quintas A.**, Moya-Higueras J., March J., Fananas L. *Childhood maltreatment disrupts HPA-axis activity under basal and stress conditions in a dose-response relationship in children and adolescents*. Psychological Medicine, 16:1-14 (2021), doi: 10.1017/S003329172100249X
12. Balzano T., Dadsetan S., Forteza J., Cabrera-Pastor A., Taoro-Gonzalez L., Malaguarnera M., Gil-Perotin S., Cubas-Nuñez L., Casanova B., **Castro-Quintas A.**, Ponce-Mora A., Leone P., Llansola M., Felipo V. *Chronic hyperammonemia induces peripheral inflammation that mediates cognitive impairment in rats*. Journal of Hepatology 73 (3): 582-592 (2019), doi: 10.1016/j.jhep.2019.01.008.

Reviewer activity for international scientific journals

1. Clinical Epigenetics, February 2022
2. International Journal of Molecular Sciences, February 2021
3. Psychoneuroendocrinology, October 2020
4. Methods and Protocols, July 2020

Congresses communications published in international scientific journals

1. **Castro Quintas Á.**, Daura-Corral M., Eixarch E., Crispi F., De La Fuente Tomas L., Rocavert Barranco M., Miguel Valero A., Marques Feixa L., Palma Gudiel H., Garcia-Portilla M.P., Fañanas L. (2022) *The role of subclinical depressive symptomatology during the prenatal period in cortisol rhythm alterations and postpartum depression risk*. European Psychiatry abstracts, S102-S103. Virtual, 7th June 2022. Oral. doi: 10.1192/j.eurpsy.2022.293.

2. **Castro-Quintas Á.**, San Martín-González N., De Las Cuevas I., Eixarch E., Daura-Corral M., Lopez M., De La Fuente Tomas L., Garcia-Portilla M.P., Fañanas L., Ayesa-Arriola R. *The impact of maternal SARS-COV-2 infection in early stages of newborn neurodevelopment: preliminary results in a multicenter Spanish study*. European Psychiatry abstracts, S103. Virtual, 7th June 2022. Oral. doi: 10.1192/j.eurpsy.2022.294.
3. Palma-Gudiel H., Marques Feixa L., Romero S., Rapado-Castro M., Blasco-Fontecilla H., Zorrilla I., Martín M., **Castro Quintas Á**., Monteserin-Garcia J.L., Font E., Ramirez M., Moreno D., Marín-Vila M., Moreno N., Binder E., Fañanás L (2021) *Children and adolescents exposed to maltreatment already exhibit epigenetic patterns suggestive of heightened low-grade inflammation.* European Psychiatry abstracts, S71. Virtual, 7th September 2021. Poster. doi: 10.1192/j.eurpsy.2022.223.
4. San Martín-González N., **Castro Quintas Á.**, Daura-Corral M., Marques-Feixa L., Eixarch E., Crispi F., De La Fuente Tomas L., Garcia-Portilla M.P., Fañanas L. (2022) *Childhood and recent maternal adverse experiences and mother-infant attachment influence early newborns' neurobehavioural profiles.* European Psychiatry abstracts, S144. Virtual, 7th September 2021. Poster. doi: 10.1192/j.eurpsy.2022.389.
5. Marques-Feixa L, **Castro-Quintas Á**, Palma-Gudiel H, Monteserín-García JL, Romero S, Rapado-Castro M, Blasco-Fontecilla H, Zorrilla I, Fañanás L (2022). *Secretory IgA reactivity to acute psychosocial stress in children and adolescents: the influence of childhood maltreatment and psychopathology* Pshychoneuroendocrinology abstracts, 131: S11. Virtual, 10th September 2021. Oral. doi: 10.1016/j.psyneuen.2021.105493.
6. **Castro-Quintas A**, Daura-Corral M, Eixarch E, De la fuente-Tomás L, San Martín-González N, Rocavert-Barranco M, Miguel-Valero A, Crispi F, Marques-Feixa L, Palma-Gudiel H, García-Portilla M, Fananas L (2021). *Cortisol diurnal changes during pregnancy and subclinical anxious-depressive maternal symptomatology: A follow-up study of the EPI_maternal_project cohort.* Psychoneuroendocrinology abstracts, 131: S2. Virtual, 9th September 2021. Oral. doi: 10.1016/j.psyneuen.2021.105461.
7. Palma-Gudiel H, Marques-Feixa L, **Castro-Quintas A**, Fananas L (2019). *The impact of puberty in HPA axis deregulation after exposure to childhood maltreatment.* Psychoneuroendocrinology abstracts, 107(S): 47. Symposium 1: How early adversities get under the skin: a behavioral epigenetics perspective across human development. Milan, 31st August 2019. Oral. doi: 10.1016/j.psyneuen.2019.07.133.
8. **Castro-Quintas A**, Daura-Corral M, De la fuente-Tomás L, Palma-Gudiel H, Marques-Feixa L, García-Portilla M, Fananas L (2019). *Neuroticism modulates HPA axis reactivity during the first trimester of pregnancy.* Psychoneuroendocrinology abstracts, 107: S27. Milan, 31st August 2019. Poster. doi: 10.1016/j.psyneuen.2019.07.075.

ATENDANCE AND PARTICIPATION IN CONGRESSES

Oral communications

1. **Castro-Quintas Á.**; Marques-Feixa L.; Palma-Gudiel H.; Romero S.; Morer A.; Rapado-Castro M.; Martín M.; Zorrilla I.; Blasco-Fontecilla H.; Ramírez M.; Mayoral M.; Mendez I.; San Martín-Gonzalez N.; Rodrigo-Yanguas M.; Monteserin-García J.L., Fañanás L., EPI-Young Stress Group. *La inmunoglobulina A secretora (s-IgA) como posible biomarcador de estrés psicosocial agudo en niños y adolescentes: la influencia del desarrollo*

puberal y la historia de maltrato. X Laboratorio de ideas CIBERSAM. Reus, Spain, 20th April 2023.

2. **Castro-Quintas Á.**, Marques-Feixa L., Palma-Gudiel H., Romero S., Morer A., Rapado-Castro M., Martín M., Zorrilla I., Blasco-Fontecilla H., Ramírez M., Mayoral M., Mendez I., San Martín-Gonzalez N., Rodrigo-Yanguas M., Monteserín-García J.L., Fañanás L., EPI-Young Stress GROUP. *Reactividad de la inmunoglobulina A secretora (s-IgA) al estrés psicosocial agudo en niños y adolescentes: la influencia del desarrollo puberal y la historia de maltrato*. XXV Congreso Nacional de Psiquiatría. Santiago de Compostela, Spain, 17th November 2022.
3. **Castro-Quintas Á.**, San-Martin Gonzalez N., Lopez M., de las Cuevas Terán I., Eixarch E., Daura-Corral M., de la Fuente L., Garcia Portilla M.P., Fañanás L., Ayesa R. *The impact of maternal SARS-COV-2 infection in early stages of newborn neurodevelopment: preliminary results in a multicenter Spanish study*. 30th European congress of Psychiatry (EPA). Virtual, 7th June 2022.
4. **Castro-Quintas Á**, Daura-Corral M., Eixarch E., Crispi F., de la Fuente L., Rocavert-Barranco M., Miguel-Valero A., Marques-Feixa M., Palma-Gudiel H., Garcia Portilla P., Fañanás L. *The role of subclinical depressive symptomatology during the prenatal period in cortisol rhythm alterations and postpartum depression risk*. 30th European congress of Psychiatry (EPA). Virtual, 7th June 2022.
5. San Martin-Gonzalez N.*, **Castro-Quintas Á*.**, Eixarch E., Crispi F., Daura.Corral M., de la Fuente L., Garcia Portilla M.P., Fañanás L. *Experiencias adversas maternas y estrés psicosocial durante el embarazo: su influencia en el neurodesarrollo del recién nacido en las primeras semanas de vida*. IX Laboratorio de ideas CIBERSAM. Vitoria, Spain, 27th May 2022.
6. Pérez-Guerrero I., de las Cuevas Terán I., **Castro-Quintas Á.**, Ortiz-García de la Foz V., Miguel Corredera M., San Martín González N., Murillo-García N., Neergaard K., Fañanás L., Ayesa-Arriola R. *Neurodevelopmental impact on newborns exposed to SARS-Cov2 during pregnancy: COGESTCOV-19*. IX Laboratorio de ideas CIBERSAM. Vitoria, Spain, 27th May 2022.
7. Marques-Feixa L., Moya-Higueras J., Romero S., Santamarina-Pérez P., Rapado-Castro M., Zorrilla I., Martín M., Anglada E., Lobato M.J., Ramírez M., Moreno N., Mayoral M., Marín-Vila M., Arias B., Fañanás L., Epi-Young Stress Group (**Castro-Quintas Á**). *Maltrato infantil, acontecimientos vitales estresantes y riesgo de conductas suicidas y parasuicidas en niños/as y adolescentes.* XXIV Congreso Nacional de Psiquiatría. Valencia, Spain, 29th October 2021.
8. **Castro-Quintas Á.**, de las Cuevas Terán I., Miguel-Corredera M., Murillo-García N., Vacas-Revilla F., Hernández-Pinto P., Palma-Gudiel P., Daura-Corral M., Eixarch E., Crispi F., de la Fuente L., Garcia-Portilla M.P., Fañanás L., Ayesa R. *Estudio de cohortes de embarazadas de alto y bajo riesgo para COVID-19 y de sus recién nacidos como modelo etiopatogénico para trastornos del neurodesarrollo y de enfermedad mental.* VIII Laboratorio de ideas CIBERSAM. Virtual, 25th May 2021.
9. Marques-Feixa L**.,** Palma-Gudiel H., **Castro-Quintas Á.**, Anglada E., Muñoz M.J., Martin M., Rapado-Castro M., Rubio P., Romero S., Mas A., Mendez I.,

Santamarina-Perez P., Font E., Mayoral M., Moreno L., Moreno C., Vidal J., Carballo J., Ramos M., Blasco-Fontecilla H., Lobato M.J., Rodrigo M., Gayubo L., Zorrilla I., Laborde M., Ramirez M., Fañanas L.. *The effect of maltreatment on the HPA axis dysregulation in children and adolescents with psychopathology: the impact of severity and frequency of experiences.* XXIII Congreso Nacional de Psiquiatría. Virtual, 30th October 2020.

10. Marques-Feixa L., Palma-Gudiel H., **Castro-Quintas Á.**, Anglada E., Muñoz M.J., Martín M., Rapado-Castro M., Rubio P., Romero S., Mas A., Mendez I., Santamarina-Pérez P., Font E., Mayoral M., Moreno L., Moreno C., Vidal J., Carballo M., Ramos H., Blasco-Fontecilla H., Lobato M., Rodrigo M., Gayubo L., Zorrilla I., Laborde M., Ramírez M., Fañanás L. *The impact of childhood maltreatment on HPA axis responsitivity to Trier Social Stress Test (TSST): Paradoxical findings between exposed children and adolescents.* XXII Congreso Nacional de Psiquiatría. Bilbao, 27th October 2019.
11. Palma-Gudiel H., Marques-Feixa L., **Castro-Quintas Á.**, Fananas L. *The impact of puberty in HPA axis deregulation after exposure to childhood maltreatment*, 49th Congress of International Society of Psychoneuroendocrinology (ISPNE). Milán, Italy, 31st August 2019.
12. Palma-Gudiel H., Marques-Feixa L., **Castro-Quintas A.**, Fananas L. *Proximal Exposure to Severe Childhood Maltreatment and HPA Axis Deregulation in Young Individuals.* 27th European congress of Psychiatry (EPA). Varsow, Poland, 6th April 2019.

Poster presentations

1. **Castro-Quintas Á.**, Palma-Gudiel H., Elisenda E., San Martin-González N., Sauer S., Rex-Haffner M., Roeh S., Monteserin-Garcia J.L., Crispi F., Garcia Portilla M.P., Binder E., Fañanás L. *Distrés materno durante el embarazo y cambios epigenéticos placentarios de riesgo para el recién nacido: estudio longitudinal Intramural_Maternal_EPI_project*. XXVI Congreso Nacional de Psiquiatría. Salamanca, Spain. 23re-25th November 2023.
2. San Martín-González N., **Castro-Quintas Á.**, Eixarch E., Crispi F., Daura-Corral M., de la Fuente-Tomás L., Monteserín-García J.L., García-Portilla M.P., Fañanás L. *Historia de maltrato infantil en mujeres embarazadas y desregulación de la respuesta al estrés psicosocial en recién nacidos: estudio longitudinal de la cohorte Maternal_EPI_Project.* XXVI Congreso Nacional de Psiquiatría. Salamanca, Spain. 23re-25th November 2023.
3. **Castro-Quintas Á.**, Palma-Gudiel H., Elisenda E., San Martin-González N., Sauer S., Roeh S., Rex-Haffner M., Monteserin-Garcia J.L., Crispi F., Garcia Portilla M.P., Binder E., Fañanás L. *Placental epigenetic signatures as biomarkers of maternal distress during pregnancy and offspring's outcomes: analysis of NR3C1, FKBP5 and HSD11B2 genes.* 36th European College of Neuropsychopharmacology (ECNP). Barcelona, Spain, 10th October 2023
4. San Martín-González N., **Castro-Quintas Á.**, Eixarch E., Crispi F., Daura-Corral M., de la Fuente-Tomás L., Monteserín-García J.L., García-Portilla M.P., Fañanás L., *Maternal history of childhood maltreatment is associated with poorer newborns stress regulation: the mediating effect of postnatal maternal subclinical depression.* 36th European College of Neuropsychopharmacology (ECNP). Barcelona, Spain, 9th October 2023

5. San Martín González N., **Castro-Quintas Á.**, Eixarch E., Crispi F., Daura M., de la Fuente L., García-Portilla M.P., Fañanás L. *Desregulación de la respuesta al estrés en niños nacidos de madres con historia de maltrato infantil: estudio longitudinal de la Cohorte Maternal_Epi_Project*. I Jornada de Recerca i Divulgació de Doctorat de la UB. Barcelona, Spain, 19th May 2023.
6. San Martín González N., **Castro-Quintas Á.**, Eixarch E., Crispi F., Daura-Corral M., de la Fuente-Tomás L., García-Portilla M.P., Fañanás L. *Estudio del distrés materno durante el embarazo como factor sensibilizador del eje HHA fetal y del desarrollo temprano del recién nacido: seguimiento longitudinal de la cohorte Intramural Maternal_EPI_Project*. X Laboratorio de ideas CIBERSAM. Reus, Spain, 20th April 2023.
7. Moreno N., Marques-Feixa L., Romero S., Fañanás L., EPI-Young Stress GROUP (**Castro-Quintas Á.**). *Chronic stress related to child maltreatment experiences disrupts biological systems and increases mental disorder vulnerability: an approach based on Allostatic Load Index explored in the EPI_Young_Stress Project sample* X. Laboratorio de ideas CIBERSAM. Reus, Spain, 20th April 2023.
8. San Martín González N., **Castro-Quintas Á.**, Eixarch E., Daura-Corral M., de la Fuente L., Crispi F., García-Portilla M.P., Fañanás L. *Estrés percibido y rasgos depresivos en el embarazo y su influencia sobre el apego materno y las puntuaciones del recién nacido en la escala NBAS*. XXV Congreso Nacional de Psiquiatría. Santiago de Compostela, Spain. 17th-19th November 2022.
9. Palma Gudiel H., Marques Feixa L., Romero S., Rapado Castro M., Blasco Fontecilla H., Zorrilla I., Martín M., **Castro Quintas Á.**, Monteserín García J.L., Font E., Ramírez M., Moreno C., Marín Vila M., Moreno N., Binder E., Fañanás L, EPI Young Stress GROUP. *Children and adolescents exposed to maltreatment already exhibit epigenetic patterns suggestive of heightened low grade inflammation* 30th European congress of Psychiatry (EPA). Virtual, 4th-7th June 2022.
10. Marques-Feixa L., Romero S., Moya-Higueras J., March-Llanes J., Santamarina-Perez P., Muñoz M.J., Zorrilla I., Rapado-Castro M., Blasco-Fontecilla H., Anglada E., Ramírez M., Fañanas L, EPI-Young Stress GROUP (**Castro-Quintas A.**). *Reinforcing the new diagnosis of Complex Post-Traumatic Stress disorder (CPTSD) of ICD-11: exploring the clinical outcomes in youth exposed to complex trauma.* 30th European congress of Psychiatry (EPA). Virtual, 4th-7th June 2022.
11. San Martín-González N., Castro-Quintas Á., Daura-Corral M., Marqués-Feixa L., Eixarch E., Crispi F., de la Fuente-Tomas L., Garcia-Portilla M.P., Fañanas L. *Childhood and recent maternal adverse experiences and mother-infant attachment influence early newborns' neurobehavioral profiles.* 30th European congress of Psychiatry (EPA). Virtual, 4th-7th June 2022.
12. Moreno N., Marqués-Feixa L., Romero S., EPI-Young Stress GROUP (**Castro-Quintas Á**). *Child Maltreatment is associated to Allostatic Load & Psychiatric Conditions in exposed children and adolescents.* XIX Curso Intensivo CIBERSAM de Introducción a la Investigación en Neurociencias: The early Origin of adult mental health: the prenatal, perinatal and infant environmental risk factors in mental disorders. Barcelona, Spain, 8th September 2022.
13. San Martín-González N., **Castro-Quintas Á.**, Eixarch E., Daura-Corral M., Marques-Feixa L., Crispi F., de la Fuente L., García-Portilla M.P., Fañanás L. *Newborns' neurobehavioral deviances are associated with maternal gestational stress, perinatal*

depressive symptoms and history of childhood maltreatment. XIX Curso Intensivo CIBERSAM de Introducción a la Investigación en Neurociencias: The early Origin of adult mental health: the prenatal, perinatal and infant environmental risk factors in mental disorders. Barcelona, Spain, 8th September 2022.

14. **Castro-Quintas A.**, Daura-Corral M., Eixarch E., De la Fuente-Tomás L., San Martin-González N., Miguel-Valero A., Rocavert-Barranco M., Crispi F., Marques-Feixa L., Palma-Gudiel H., García-García-Portilla M.P., Fañanas L. *Cortisol diurnal changes during pregnancy and subclinical anxious-depressive maternal symptomatology: A follow-up study of the Epi_Maternal_Project Cohort.* 51th Congress of International Society of Psychoneuroendocrinology (ISPNE). Virtual, 7th-9th September 2021.
15. Marques-Feixa L., **Castro-Quintas Á.**, Palma-Gudiel H., Monteserín-Garcia J.L., Romero S., Rapado-Castro M., Blasco-Fontecilla H., Zorrilla I., Fañanas L. *Secretory sIgA reactivity to acute psychosocial stress in children and adolescents: the influence of childhood maltreatment and psychopathology*. 51th Congress of International Society of Psychoneuroendocrinology (ISPNE). Virtual, 7th-9th September 2021.
16. **Castro-Quintas Á.**, Daura-Corral M., de la Fuente-Tomás L., Miguel-Valero A., Rocavert-Barranco M., Marqués-Feixa L., Eixarch E., Crispi F., Palma-Gudiel H., García-Portilla M.P., Fananas L. *Maternal depressive symptoms and perceived stress modulate cortisol circadian rhythm during pregnancy*. 4th National Congress of Young Investigators in Biomedicine. Virtual, 4th-6th November 2020.
17. Marques-Feixa L.**,** Palma-Gudiel H., Romero S., Zorrilla I., Blasco-Fontecilla H., Rapado-Castro M., **Castro-Quintas Á.**, Moya-Higueras J., March-Llanes J., Fañanás L. *The enduring effect of childhood maltreatment on HPA axis reactivity during psychological stress in children and adolescents affected and non-affected by mental disorders: the impact of severity and frequency of adverse experiences.* 27th International symposium on Controversies in Psychiatry –Violence and aggression. Virtual, 28th September 2020.
18. **Castro-Quintas Á.**, Daura-Corral M., de la Fuente-Tomás L., Palma-Gudiel H., Marques-Feixa L., Garcia-Portilla M.P., Fananas L. *Anxious-depressive status during pregnancy modulate HPA axis reactivity during exposure to acute stress*. 6th CORE in mental Health seminar. Barcelona, Spain, 14th November 2019.
19. **Castro-Quintas Á.**, Daura-Corral M., de la Fuente-Tomás L., Palma-Gudiel H., Marques-Feixa L., Garcia-García-Portilla M.P., Fananas L. *Neuroticism modulates HPA axis reactivity during the first trimester of pregnancy.* 49th Congress of International Society of Psychoneuroendocrinology (ISPNE). Milan, Italy, 29th-31st August 2019.
20. **Castro-Quintas Á.**, Daura-Corral M., de la Fuente-Tomás L., Palma-Gudiel H., Marques-Feixa L., Soler J., García-Portilla M.P., Fananas L. *La ansiedad y el neuroticismo modulan la reactividad del eje HPA durante el embarazo.* VII Laboratorio de Ideas, CIBERSAM. Oviedo, Spain, 13th-14th June 2019.
21. Marques-Feixa L., Palma-Gudiel H., **Castro-Quintas A.**, Anglada E., Muñoz M.J., Martin M., Rapado-Castro M., Rubio P., Romero S., Mas A., Mendez I., Santamarina P., Font E., Mayoral M., Moreno L., Moreno C., Vidal J., Carballo J., Ramos M., Blasco H., Lobato M.J., Rodrigo M., Gayubo L., Zorrilla I., Laborde M., Ramirez M., Fananas L. Differential HPA axis response to stress according to pubertal stage in children and adolescents exposed to maltreatment. VII Laboratorio de Ideas, CIBERSAM. Oviedo, Spain, 13th-14th June 2019.

22. Marques-Feixa L., Palma-Gudiel H., **Castro-Quintas A.**, Martin M., Rapado-Castro M., Mayoral M., Zorrilla I., Ramirez M., Romero S., Mendez I., Blasco-Fontecilla H., Lobato M.J., Fañanas L. *Children and adolescents affected by psychiatric disorders exhibited lower HPA axis reactivity: the influence of sex and pubertal stage.* 27th European Congress of Psychiatry (EPA). Warsow, Poland, 6th-9th April 2019.

Congresses attendance

1. XXVI Congreso Nacional de Psiquiatría. Salamanca, Spain, 23rd-25th November 2023.
2. 36th European College of Neuropsychopharmacology (ECNP). Barcelona, Spain, 7th-10th October 2023
3. XI Forum in Psychiatric Research CIBERSAM. Barcelona, Spain, 8th-9th June 2023.
4. 66 congress Spanish Association of Psychiatry in the Infancy and the Adolescence (AEPNYA). Valencia, Spain, 1st-3rd June 2023.
5. X Laboratorio de ideas CIBERSAM. Reus, Spain, 20th-21st April 2023.
6. IV Jornada del grup de treball en Psiconeuroendocrinologia (PNECAT). Barcelona, Spain, 24th February 2023.
7. PhD Day Gen Mic Est VI Edition. Barcelona, Spain, 13th January 2023.
8. XXV Congreso Nacional de Psiquiatría. Santiago de Compostela, Spain, 17th-19th November 2022.
9. 30 European Congress of Psychiatry congress. Virtual congress, 4th-7th June 2022.
10. 5th annual Biomed PhD Day. Barcelona, Spain, 10th February 2022.
11. XXIV National Psychiatry Congress. Valencia, Spain, 28th-30th November 2021.
12. 51st Congress of International Society of Psychoneuroendocrinology (ISPNE)., Online, 7th-9th September 2021
13. VIII Laboratorio de ideas CIBERSAM. Online, 13th-14th June 2021
14. IV National Congress for Young Researchers in Biomedicine. Virtual, 4th-6th November 2020.
15. 6th Core Seminar. Barcelona (Spain), 14th November 2019.
16. 49th Congress of International Society of Psychoneuroendocrinology (ISPNE). Milan, Italy, 29th-31st August 2019.
17. VII Laboratorio de ideas CIBERSAM. Oviedo, Spain, 25th-27th May 2019.
18. VII International Schizophrenia Forum. Madrid, Spain, 15th-16th November 2018.

UNIVERSITY ACADEMIC ACTIVITIES

Research supervision

Academic year 2020-2021

Final Year Degree projects (TFG)

1. Title: *Changes on the cortisol diurnal patron during pregnancy as a predictor of delivery complication and developmental milestones in the newborn.*
   Degree: Biomedicine.
   Student: Mireia Rocavert Barranco
   Co-direction: Águeda Castro Quintas & Lourdes Fañanás.
   Date of reading: 11th February 2021
   Qualification: 9.1/10

2. Title: *Psychosocial maternal stress and Hypothalamic-Pituitary-Adrenal axis reactivity during pregnancy: analysis of salivary cortisol and IgA biomarkers.*
   Degree: Biomedicine
   Student: Alba Miguel Valero
   Co-direction: Águeda Castro Quintas & Lourdes Fañanás.
   Date of reading: 11th February 2021
   Qualification: 9.5/10

Lectures and seminars in official degrees and masters

Academic year 2023-2024

1. Lectures on the subject "Basis and foundations for a good practice in perinatal mental health" of the postgraduate course of Postnatal Mental Disorders (1 hour). Institut for continuing education IL-3 of de Universitat de Barcelona, 30th October 2023-30th May 2024

Academic year 2022-2023

2. Lectures on the subject "Introduction to Neuroscience Research" of the master of Introduction to Mental Health Research (4 hours). Universitat de Barcelona, 1st and 13th March 2023.

3. Lectures on the subject "Laboratory practices in Stay in Research Units" of the master of Introduction to Mental Health Research (10 hours). Universitat de Barcelona, 16th-20th January 2023.

4. Lectures on the subject "Biology I: Introduction to Biomedicine" of the Biomedicine degree (3 hours). Universitat de Barcelona, 10th January 2023.

5. Lectures on the subject "Laboratory practices in Human Biology" of the master of Biological Anthropology (16 hours). Universitat de Barcelona, 19th-21st October 2022.

Academic year 2021-2022

6. *Stress, Neurodevelopment and mental health.* Online seminar on the official master in General Health Psychology (2 hours). Lleida University, 12nd May 2022.

7. Lectures on the subject "Laboratory practices in Stay in Research Units" of the master of Introduction to Mental Health Research (32 hours). Universitat de Barcelona, 17th-21st January 2022.

8. *Stress, Neurodevelopment and Mental Health: Neurobiological and Immune System´s alterations.* Online seminar on the subject "Physiological basis in Neurologic and Psychiatric illnesses" of the master in Neurosciences. Universitat de Barcelona. 1 hour. 5th November 2021.

9. Lectures on the subject "Laboratory practices in Human Biology" of the master of Biological Anthropology (16 hours). Universitat de Barcelona, 22nd-26th October 2021.

Academic year 2020-2021

10. *Stress biology in the first stages of life.* Online Seminar on the official master in General Health Psychology (1 hour). Lleida University, 22nd March 2021.

11. *Stress biology in the first stages of life*. Online seminar on the subject "Physiological basis in Neurologic and Psychiatric illnesses" of the master in Neurosciences (1 hour). Universitat de Barcelona, 10th November 2020.

12. Lectures on the subject "Laboratory practices in Human Biology" of the master of Biological Anthropology (15 hours). Universitat de Barcelona, 23rd-27th October 2020.

Academic year 2019-2020

13. *Stress biology in the first stages of life.* Online Seminar on the official master in General Health Psychology (1 hour). Lleida University, 4th March 2020. Academic year 2019-2020.

SCIENTIFIC DIVULGATION TO GENERAL POPULATION

Lectures and oral presentations

1. Coordinator of the program "*Mente Maravillosa*" of the *Pint of Science Festival*. Spain, 2018-currently
2. Scientific Advisor in *MenteScopia*, Multimedia Project Improving Mental Health in adolescents (Ref. FCT-20-16227), Spain, 2018-currently.
3. Scientific Advisor in *Bioemprender*. Spain, 2018-currently
4. *Cuando el period prenatal deja huella: regulando la respuesta al estrés*. Participation in the 2nd Edition of the 3 minuts talk. Universitat de Barcelona, Spain. 20th December 2022
5. *Mental Health,* Fundació La marató de TV3, divulgative sessions in educative and civic centers. 14 hours. Barcelona, Spain, October-December 2021.
6. *The biology of stress.* Seminar in the "Nit Europea de la Recerca". 1 hour. Online. 24th September 2021
7. *Covid-19,* Fundació La marató de TV3, divulgative sessions in educative and civic centers. 4 hours. Barcelona, Spain, October-December 2020.
8. Participation in the "I call-for-entry videos #QueSigaLaCiencia", of the Consorcio Centro de Investigación Biomédica en Red (CIBER). Virtual, October 2020
9. *The biology in Mental Health.* Seminar in the MENTALFEST festival, Dominiques de l'Ensenyament Highschool. 2 hours. Barcelona, Spain, 19th June 2019.

Articles publication in newspapers and webpages

1. *Los bebés expuestos a la covid en el útero presentan cambios en el desarrollo neurológico.* La Razón Journal. 6th June 2022. https://www.larazon.es/salud/20220606/ysb7iwaeevh4dgff3dguolbn5q.html
2. *Los niños y niñas con antecedentes de maltrato podrían tener una maduración precoz del sistema inmunológico.* Cibersam ISCIII. 11th May 2022. https://www.cibersam.es/noticias/los-ninos-y-ninas-con-antecedentes-de-maltrato-podrian-tener-una-maduracion-precoz-del-sistema-inmunologico
3. *Los niños con antecedentes de maltrato podrían tener una maduración precoz del sistema inmunológico.* Universitat de Barcelona. 11th May 2022. https://web.ub.edu/es/web/actualitat/w/los-ninos-con-antecedentes-de-maltrato-podrian-tener-una-maduracion-precoz-del-sistema-inmunologico

COURSE ATTENDANCE

Neurosciences

1. *XX Curso Intensivo CIBERSAM de Introducción a la Investigación en Neurociencias: Redefiniendo la enfermedad mental: La esquizofrenia*. Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM). Universitat de Barcelona. Barcelona, Spain, 8th September, 2023.
2. *XIX Curso Intensivo CIBERSAM de Introducción a la Investigación en Neurociencias: The early Origin of adult mental health: the prenatal, perinatal and infant environmental risk factors in mental disorders*. Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM). Universitat de Barcelona. Barcelona, Spain, 9th September, 2022.
3. *XVIII Curso Intensivo de Introducción a la Investigación en Neurociencias. Actualización en la Evaluación, Intervención e Investigación del maltrato infantil al principio de la vida*. Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM). Virtual, 28th-19th June 2021.
4. *XV Curso Intensivo de Introducción a la Investigación en Neurociencias: Neuroendocrinología del trastorno mental infanto-juvenil*. Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM). Universitat de Barcelona. Barcelona, Spain, 10th May 2019.

Scientific and research skills

5. *Suport a la reflexió ètica: identificació i resolució de dilemes o altres preocupacions ètiques*. Programa presentació DialÈtic. Professional Development Institute (IDP-ICE). Universitat de Barcelona. Barcelona, Spain. 19th April 2023
6. *Module IV: What to do after the PhD*. Young investigators mentoring program Professional Development Institute (IDP-ICE). Universitat de Barcelona. Barcelona, Spain, 30th March 2023
7. *Research integrity, Second Edition* (Prog1064580090). Epigeum, Oxford University Press. Online course. 3rd May 2022.
8. *When and how to start to impulse creativity and entrepreneurship?* From the classroom to the enterprise. Professional Development Institute (IDP-ICE). Universitat de Barcelona. Barcelona, Spain. 29th March and 5th April 2022.
9. *From gender to feminism, passing through university*. Professional Development Institute (IDP-ICE). Universitat de Barcelona. Barcelona, Spain. March 2022.
10. *Stress Management*. Professional Development Institute (IDP-ICE). Universitat de Barcelona. Barcelona, Spain. February 2022.
11. *DATAETHICS Summer School: big data, big implications the ethics of artificial intelligence in biomedicine*. Universitat de Barcelona, Spain, 12th-16th July 2021.
12. *Two keys to incorporate gender perspective on teaching*. Professional Development Institute (IDP-ICE). Equality commission. Universitat de Barcelona. Barcelona, Spain. May 2021.

13. *Module III: R&D transfer: transference, innovation, entrepreneurship*. Young investigators mentoring program. Professional Development Institute (IDP-ICE). Universitat de Barcelona. Barcelona, Spain. April 2019.
14. *Module II: Techniques for the improvement of the investigation*. Young investigators mentoring program. Professional Development Institute (IDP-ICE). Universitat de Barcelona. Barcelona, Spain. March 2019.
15. *Module I. Introduction and resources in research*. Young investigators mentoring program. Professional Development Institute (IDP-ICE). Universitat de Barcelona. Barcelona, Spain. January 2019.
16. *XIII Scientific Meeting Alicia Koplowitz Foundation*, Madrid, Spain, 25th-26th October 2018.

Statistical Skills

17. *Introduction to R*. Datacamp. 4 hours. November 30th, 2022.
18. *Basic Course of R*. Professional Development Institute (IDP-ICE). Universitat de Barcelona. 24 hours. Barcelona, Spain, 10th-8th October 2022.
19. Biostatistics workshop for PhD Students. Universitat de Barcelona, Spain. 25th-26th October 2021.

LENGUAGES

Spanish (mother tongue)

Galician (mother tongue)

English – C1.2 level

Catalan – B2.1 level

French – B1.2 level

German –A1.2 level

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