**Research Program**

**Scientific background**

Sex hormones, including androgens, estrogens, and progestins, establish and maintain a specific neuroendocrine milieu influencing brain structure and function throughout the life span. In rodents,1, 2, 3 and in nonhuman primates,3-6 various interventions including administration of sex hormones, castration, and ovariectomy resulted in behavioral changes such as in aggression, sexual behavior, grooming, and spatial ability,7, 8  as well as in brain morphology.9 In humans, the role for sex hormones has been documented in individual differences in childhood play patterns, aggression, cognitive abilities, and sociodevelopmental or psychiatric conditions such as autism, depression, schizophrenia,2, 10-13 and brain structure.14, 15 Sex hormones greatest effect on brain structure and function occurs during two sensitive periods in development: the first is during the *prenatal* and/or *neonatal* period, the second during the *postnatal* period.16, 17

*Prenatal hormones and the corpus callosum.* Early exposure to sex hormones exerting a variety of cellular processes which, in turn, influence sexually dimorphic developing brain.18 Previous studies demonstrated the association between early hormonal levels and sexually dimorphic brain structures such as the amygdala and the hypothalamus;19; gray matter volume of specific brain regions;15 and the corpus callosum.14 Within this body of research, the corpus callosum (CC) has been the focus of research interest. The CC is the largest white matter tract and main interhemispheric commissure,20, 21 and it is thought to contribute to the lateralization of brain function.22. Non human species studies provided evidence as for the sexually dimorphic CC in size and structure.23, 24 Human studies have also demonstrated sex differences in the CC among adults,25 children,14 and fetuses,26 whereas others failed to support these findings.20 The extensive research conducted among adults usually show that women have larger CC than men.25 The role of prenatal sex hormones on CC size and structure has been scarcely study. Chura and colleagues14 studied the effects of fetal testosterone on the CC of 8-11- year old boys. They did not find association between fetal testosterone and CC size, however they did find that increased levels of fetal testosterone were related with increased rightward asymmetry of the CC. To the best of our knowledge, no studies in humans have directly tested the effects of prenatal hormones on the developing CC. Furthermore, given the findings as for the role of ovarian hormones in sexually dimorphic CC among non human species,27 the present study will further examine the role of estrogen and progesterone as well as testosterone in explaining individual differences in the size of the CC.

*Prenatal hormones and Cognitive abilities.* It has been suggested that the sensitive periods in hormonal secretion are also associated with sex differences in cognitive abilities.28 Empirical work on the effects of hormones on cognitive abilities in the *postnatal* period has been widely documented, using hormones replacement, normal hormonal fluctuations, and individual differences in hormonal levels.28 The influence of hormones in the *prenatal/neonatal* period has also been widely studied. Evidence of cognitive differences resulting from *prenatal/neonatal* sex hormones levels comes from three main research directions: cognitive abilities of individuals with sex development disorders, the relations between somatic markers for prenatal hormone exposure and later cognitive abilities, and the relations between prenatal amniotic hormones and later cognitive abilities.29

Behaviors demonstrating clear sex differences are considered best candidates for studying the effects of prenatal hormones on later development.30-32 One of the most established domain where sex differences have been observed is visuospatial ability, with males outperforming females on a range of tasks.17,33 Mental rotation tasks, which involve rotating figures in depth or in the picture plane, produced the largest sex differences, followed by spatial perception tasks showing medium size sex differences.34 Studies investigating the role of prenatal hormones on later cognitive development presented mixed results. Research focusing on disorders of sex development mainly examined individuals with congenital adrenal hyperplasia (CAH). CAH is an autosomal recessive genetic disorder that causes excessive production of androgens in female and male fetuses.28 Whereas some studies demonstrated enhanced spatial ability in girls with CAH,35-38 others showed no effect.39 Turner Syndrome (TS) results from a random error involving an absent or imperfect second sex chromosome. These chromosomal abnormalities in turn, impacts the development of the ovaries, impairing their ability to produce hormones.40 Girls with TS lack early androgen exposure and show alteration in estrogen production and have been characterized by a specific neurocognitive profile of normal verbal abilities, and impaired visuospatial and visuoperceptual abilities.41 Another method examines the somatic markers for prenatal hormone level. One of the most studied biomarkers is the ratio between the second and the fourth finger (digit ratio, 2D:4D). Digit ratio differs by sex, with men having a lower 2D:4D than women.42-45 The 2D:4D ratio is assumed to be established prenatally around the 14th week of gestation,46,47 and controlled by the same genes (HOXA and HOXD in particular) responsible for gonad differentiation.48 2D:4D has been found to be correlated with prenatal testosterone and estrogen. Studies investigating the association between 2D:4D ratio and performance on mental rotation have shown mixed results.49-51 Studies investigating the effects of prenatal hormones using sampling of amniotic fluid surrounding the fetus on later cognitive development also showed mixed results. Some revealed a significant positive association between fetal testosterone and higher performance on mental rotation among girls,52 however others failed to demonstrate this association.53 This inconsistency in research findings resulting from divergent methods investigating prenatal hormonal exposure and various visuospatial measures, calls for further investigating in order to deepen our understanding as for the role of the organizational effects of sex hormones on later cognitive development. Furthermore, the influence on individual differences in cognitive domains are not only academic interest but appear to also play a role in different psychopathological states. It has been suggested that these differences have implications on our understanding of autistic spectrum disorders (ASD).54,55

*Prenatal hormones and emotional processing.* The effects of sex hormones on emotional processing have been widely studied. In their review, Osório and collegues56 (2018) found that hormonal changes influence facial emotional processing, with increased levels of estrogen and progesterone related to increased recognition of facial expressions of emotion. Their findings also supported former suggestions as for the potential mediating role of brain areas associated with emotional processing such as the amygdala, hippocampus, and the CC.57 Another recent review58 documented the role of endogenous and exogenous levels of testosterone in amygdalar and parahippocampal regions activation in response to social and affective stimuli. Furthermore, the stage in development (fetal, adolescence, adulthood) had a modulating effect on this association. The authors asserted that exposure to sex hormones prenatally is a key factor in determining their effects on behavior through the expression of hormone receptors, which in turn influence hormone sensitivity in adulthood.

*Visuospatial and emotional processing, and ASD*. ASD is characterized by an impairment in social interaction, communication, and imagination.59 Individuals with ASD experience difficulties processing and interpreting socio-emotional cues,60-62 and cross-cultural investigation supported these findings.63 However, ASD is also associated with non-social characteristics such as perceptual abnormalities.64 According to the extreme male brain theory for autism,65 it has been suggested that the cognitive profile of individuals with ASD is characterized by an increase systemizing and a decreased empathizing performance.55 Visuospatial tasks such as mental rotation require systemizing skills, whereas emotional processing tasks require empathizing skills. Therefore, it is assumed that a negative association in performance in these tasks will emerge. Furthermore, as been noted earlier, various studies documented the role of sex hormones in understanding individual differences in visuospatial and in emotional processing tasks. Therefore, the need to further establish the role of prenatal exposure to sex hormones in individual differences in these tasks and their relation, will shed light on the origins of psychopathological states such as ASD.

*Prenatal hormones, CC, brain volume, and ASD*. Previous studies have demonstrated the relation between fetal testosterone and autistic traits in the general population, with higher levels of testosterone related to higher prevalence of autistic traits.66 However, the role of other prenatal sex hormones as well as their association to non-social and social characteristics of ASD has not been studied yet. Furthermore, the role of the CC and brain volume have been demonstrated in previous studies showing a reduction in midsagittal CC size in individuals with ASD,67-70 and increased brain volume.71 The primary candidate suggested as responsible to these neuroanatomical findings is fetal testosterone.14

In sum, the role of sex hormones on brain structure and function had been widely studied. Nevertheless, the role of parental exposure to sex hormones and its consequences on development is less documented. Given the organizational effects of sex hormones on later development have far reaching influence on brain and behavior, the present study aimed at capturing this critical period in development by measuring the hormonal milieu as well as measuring an important brain structure – the CC, and brain volume, involving in later cognitive and emotional development. Furthermore, the present study asks to extend the frame of investigation beyond testosterone and examine other sex hormones as well: estrogen and progesterone, in order to reveal the complex interplay between neuroendocrinological factors in later performance on emotional and cognitive processing, as a means to uncover the factors associated with psychopathological states. We hypothesized that prenatal sex hormones will be related to measures of the CC (length, width, thickness) and brain volume, which in turn will be associated with individual differences in emotional and visuospatial processing, as well as autistic traits. Model …

### Research objectives and expected significance

Our research will examine the role of prenatal hormone exposure on brain structure and function. Specifically, our research is a longitudinal study that will take place in three significant stages in development: the first, prenatally – the measurement of prenatal sex hormones, the second, prenatally – the measurement of CC size, the third, childhood – the measurement of cognitive and emotional processing. The specific aims are as follows:

1. To examine the association between prenatal sex hormones (fetal testosterone, estrogen and progesterone) and the fetal CC size (length, width, thickness) and brain volume.
2. To examine the role of prenatal sex hormones and performance on emotional and visuospatial processing.
3. To examine the role of prenatal sex hormones and performance on autistic traits.
4. To examine the association between performance on emotional processing, visuospatial processing, and autistic traits.
5. To examine the association between CC size, brain volume and autistic traits.
6. To examine the potential mediating role of CC and brain volume on the relation between prenatal sex hormones and autistic traits.

**Detailed description of the proposed research**

**Our working hypotheses**

Based on the literature review as well as on the preliminary findings our hypotheses are as follows:

1. Testosterone is negatively correlated with the width of the CC, whereas estrogen and progesterone is negatively correlated with the length of the CC.
2. Testosterone is positively correlated with brain volume, whereas estrogen and progesterone is negatively correlated with brain volume.
3. Testosterone is positively associated with performance on visuospatial processing, whereas negatively correlated with performance on emotional processing. In contrast, estrogen and progesterone are negatively associated with performance on visuospatial processing, whereas positively correlated with performance on emotional processing.
4. Testosterone is positively associated with the prevalence of autistic traits.
5. Performance on visuospatial and emotional processing is correlated with the prevalence of autistic traits, showing positive association between visuospatial processing and autistic traits, and negative association between emotional processing and autistic traits.
6. CC width is negatively correlated with the prevalence of autistic traits, whereas, brain volume is positively correlated with the prevalence of autistic traits.
7. Higher levels of testosterone will be associated with lower CC width, and increased brain volume, which in turn, are associated with higher prevalence of autistic traits.

**Methodology**

**Participants**. 150 fetuses, and later on children aged 5, males and females, will participate in the present study. The number of participants was established by power analysis, based on our preliminary results, with addition of 20% to compensate for possible drop-outs during the study.

**Research procedure**. Pregnant women will be recruited prior their scheduled amniotic fluid test in the Obstetrics and Gynecology Division of the Rambam Health Care Campus. Healthy women with no history of genetic illness and healthy pregnancy will be invited to participate in the study. Women agreeing to participate will sign a consent form in which they will permit to use 5ml of the amniotic fluid for sex hormones measures. The first stage of the study - the amniotic fluid tests will be performed between 14 and 16 weeks gestation. Levels of sex hormones: testosterone, estrogen, and progesterone, will be measured. The second stage of the study – an obstetric ultrasonography will be performed in the third semester and will measure the CC (width, length, thickness) and brain volume (biparietal diameter -BPD; head circumference – HC). The third stage – cognitive, emotional processing and autistic traits battery – will be performed at age 5 either at the Rambam Health Care Campus or at the YVC Psychobiology Laboratory. in this stage participants will be asked to complete a cognitive battery including visuospatial tests (mental rotation, line orientation) and emotional test (reading the mind in the eyes; emotion recognition test). In order to examine the processing mechanism of cognitive as well as emotional abilities, the present study will include the use of an eye-tracker in order to monitor eye movements during completing cognitive tasks. The rationale for examining eye-movements in research rely on evidence that eye movement patterns permit stronger inference regarding cognitive processing ad allocation of visual attention.72 In addition, participants' parents will be asked to complete a broad spectrum of questionnaires, evaluating …..Participants will be asked to complete a broad spectrum of questionnaires, evaluating their level of anxiety, depression, quality of sleep, and smoking dependence (questionnaires are described in detail below).

**Research tools.**

Stage 1 -

1. Hormones level analyses in the amniotic fluid..

Stage 2 -

1. CC and brain volume measures…

Sage 3 -

1. Personal information questionnaire containing XX questions adapted to the current research, including questions about demographic background,
2. Judgement of Line Angle and Position (JLAP) (Collaer and Nelson, 2002). This test evaluates spatial attributes of lines. It contains 20 test items, each item presents two target line segments located directly above the 13 numbered lines on the bottom of the page. Participants are asked to match of the target lines in the top of the page with a numbered line from the bottom of the page.
3. Mental Rotation test (Shepard and Metzler, 1971). This test involves rotating figures in depth or in the picture plane. We measure two difficulty levels: with 3-D and 2-D models (Gordon, 1986). In each test, in 18 trials, three (3-D or 2-D) models are presented randomly on the screen. Pairs of photographs of each model were prepared, in which the models appeared nearly identical, except that they are rotated in space with respect to each other. Participants are presented three models at a time and are instructed to decide which two models were the same by mentally rotating them in their head.
4. Children's Version of the Reading the Mind in the eyes Test (Baron- Cohen, Wheelwright.., 2001). The test includes 28 photographs of the eye region of the face. Participants are asked to choose which of 4 words best describes what the person in the photo is thinking or feeling.
5. The emotion recognition task measures six basic emotional expressions: anger, fear, disgust, surprise, sadness, and happiness, together with two expressions: calmness and neutral (calculated as one score: Markovits, Trémolière, & Blanchette, 2018). The faces are taken from the NIMSTIM (Tottenham et al., 2009). The task includes 20 trials presented in random order (conducted through three different versions). On each trial participants are asked to select one of four possible answers.
6. The Child Autism Spectrum Quotient (AQ-Child; Auyeng et al, 2008 – Auyeng 2009). The 50-item parent-report questionnaire detects autistic traits in children aged 4-11. The items are answered in a Likert format (definitely agree to definitely disagree).
7. The childhood autism Spectrum Test (Cast; Scott et al., 2002b – Auyeung et al., 2009). The 37-item parent-report questionnaire detects autistics spectrum conditions in children aged 4-11. Parents are asked to answer the items with a binary response (yes/no).
8. Eye movements. Fixation duration will be measures using XXX eye-tracking software at a sampling rate of 250 Hz. We selected this measure for analysis because fixation time has been used as an index of attention and visual processing (Dalton et al., 2005 – Nielsen et al., 2011). Eye movements events (fixations, saccads, blinks) will be exported using the software XXX. Fixation time percentage will be calculated by adding the time of each fixation event within a slide and dividing the total fixation time by the total tim of the slide (Nielsen, 2011).

**Preliminary results**

Our preliminary results support our initial hypotheses regarding the involvement of estrogen and progesterone in CC size and brain volume. Previous studies have focused on the role of testosterone in CC and brain morphology, and later individual differences in cognitive and emotional processing. Our preliminary findings suggest that there is a need to expand the focus from testosterone to estrogen and progesterone as well examining long term consequences of prenatal hormonal exposure on brain structure and function. Specifically, we initiated a preliminary study beginning with the first two stages of the proposed study that is taking place prenatally: the first, sex hormones measured in the amniotic fluid, and the second, measures of the CC and brain volume measured at the third semester. The findings demonstrated that estrogen was negatively correlated with brain volume measures [BPD: r = -.30; HC: r = .21]. Progesterone was also negatively correlated with brain volume measures; however, these correlations did not reach significance. In contrast, testosterone was positively correlated with brain volume measures, however these correlations did not reach significance as well, probably due to low number of participants. With regard to CC measures, estrogen was positively correlated with thickness of the CC [r = .25]. In addition, progesterone was negatively correlated with length of the CC, however it did not reach significance. Another important finding demonstrated a negative correlation between thickness and length of the CC [r = .61].

With the funding provided by the ISF we will be able to further expand this study by adding participants to the existing participants of the first two stages. Next, we will be able to pursue the main aims of the proposed longitudinal study and uncover the role of prenatal neuroendocrine factors in the development of individual differences in emotional as well as cognitive abilities. That is, this study provides an opportunity to refine the early influence of sex hormones exposure and brain morphology on the course of emotional and cognitive development. Furthermore, the relation between these differentiated classes of abilities will be explored. Lastly, the proposed longitudinal study will provide a chain of factors that are suggested as involved in psychopathological states such as ASD, and to further extend our understanding as for the neuroendocrine factors embedded in its etiology.

**Expected results and pitfalls**

By conducting longitudinal research, we expect to deepen our understanding regarding organizational effects of sex hormones on brain structure and function. Only few studies in humans have directly tested organizational effects of fetal testosterone on brain structure.14,15 As seen in animal studies, and based on our preliminary findings, the role of estrogen and progesterone seems to also play a central role in explaining individual differences in brain morphology and in later behavior. The inclusion of ovarian hormones will enable us to provide a comprehensive model in understanding the basis of individual differences in brain structure and function. As opposed to animal studies implementing hormonal interventions pre- and -perinatal, given the nature of the present study, causality cannot be concluded. However, conducting a longitudinal study provides the second-best paradigm investigating the studies' hypotheses as for the predictive role of sex hormones and brain structure in explaining individual differences in emotional and cognitive development.

By including a larger number of participants, we expect the non-significant results obtained in our earlier small-sample pilot study measuring the first two stages of the longitudinal study will achieve significance, thus confirming our hypotheses.

A major pitfall in longitudinal studies is potential dropout of participants due to medical conditions, unwillingness to continue participation and so on. To address this possible challenge, the number of participants is 20% higher than the number that is estimated to be sufficient for adequate statistical power. Higher dropout rates will require the recruitment of additional participants and extend the time needed for completing the data collection. Alternatively, we will implement statistical techniques73 to address this challenge.

**Resources available for the research**

The proposed research is based on interdisciplinary collaboration of experts in the fields of ultrasound in obstetrics, hormones and human development. Prof. Zeev Weiner, MD (Director of the Ultrasound in Obstetrics and Gynecology, Rambam Health Care Campus), is one of the leading specialists in prenatal diagnosis… and .. researcher in…. Dr. Maya Steinberg, MD….. Dr. Efrat Barel, PhD, is a faculty member at the Department of Behavioral Sciences of the Emek Yezreel Academic College, and the member of the Psychobiology Laboratory at the Yezreel Valley Academic College. Dr. Barel is a developmental psychologist and has extensive background in cognitive and emotion development and its relation to hormonal status throughout the life span.

The study will be conducted at the Rambam Health Care Campus. The laboratory staff of Rambam will carry out the hormonal measures in the amniotic fluid. The laboratory is equipped with….. The Ultrasound in Obstetrics and Gynecology staff will carry out the CC and brain volume measures in the fetal ultrasound…the department is equipped with…. A psychology graduate student will take part in data collection of cognitive, emotion, and autistic trait measures at childhood. An undergraduate student will also take part in recruitment of pregnant women in prior of the amniotic fluid test. Statistical advisory and computer support are provided by the college.

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