**Research Program**

**Scientific background**

Sex hormones, including androgens, estrogens, and progestins, establish and maintain a specific neuroendocrine milieu influencing brain structure and function throughout life. In studies on rodents1, 2, 3 and 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 changes in brain morphology.9 In humans, effects of sex hormones have been documented in individual differences in childhood play patterns, aggression, cognitive abilities, socio-developmental or psychiatric conditions such as autism, depression, schizophrenia,2, 10-13 and brain structure.14, 15 The greatest effect of sex hormones on brain structure and function occurs during two sensitive periods in development: the first is during the *prenatal* and/or *neonatal* period, and the second during the *postnatal* period.16, 17

*Prenatal hormones and the corpus callosum.* Early exposure to sex hormones triggers a variety of cellular processes which, in turn, influence the sexually dimorphic developing brain.18 Previous studies have 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. Animal studies have provided evidence of the sexually dimorphic CC in terms of its size and structure.23, 24 Some 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 Much of the extensive research conducted among adults has shown that women generally have a larger CC than men.25 However, the role of prenatal sex hormones in determining CC size and structure has remained largely unexplored. Chura and colleagues14 investigated the effects of fetal testosterone on the CC of 8-11-year-old boys. They did not find an 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 association between prenatal hormones and the developing CC. Furthermore, given the findings on the role of ovarian hormones in the development of the 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 the development of individual differences in the size of the CC.

*Prenatal hormones and cognitive abilities.* It has been suggested that hormonal secretion during sensitive periods of development 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 hormone 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 hormone levels comes from three main areas of research: cognitive abilities of individuals with sex development disorders, the relations between somatic markers for prenatal hormone exposure and later cognitive abilities, and the relationship between prenatal amniotic hormones and later cognitive abilities.29

Cognitive abilities 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 domains 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 have presented mixed results. Research focusing on disorders of sex development have 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, impact 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 research method involves using somatic markers to estimate prenatal hormone levels. One of the most studied biomarkers is the ratio between the lengths of 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 thought 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 have also shown mixed results. Some revealed a significant positive association between fetal testosterone and higher performance on mental rotation among girls,52 while others failed to demonstrate this association.53 This inconsistency in research findings resulting from divergent methods of investigating prenatal hormonal exposure and various visuospatial measures, indicates a need for further investigation in order to deepen our understanding of the organizational effects of sex hormones on later cognitive development. Furthermore, these differences in cognitive performance may also have clinical significance, as they appear to also play a role in different psychopathological states. It has been suggested that these differences may contribute to the etiology 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 associated with increased recognition of facial expressions of emotion. Their findings also support earlier studies suggesting a potential mediating role in 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 region activation in response to social and affective stimuli. Furthermore, the stage in development (fetal, adolescent, adult) had a modulating effect on this association. The authors asserted that particularly *prenatal* exposure to sex hormones 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 findings supported by cross-cultural investigations.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 the cognitive profile of individuals with ASD is characterized by increased systemizing performance and 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 noted earlier, various studies have documented the effects of sex hormones on promoting individual differences in visuospatial and in emotional processing tasks. Therefore, greater understanding of the relationship between prenatal exposure to sex hormones and individual differences in these tasks may shed light on the origins of psychopathological states such as ASD.

*Prenatal hormones, CC, brain volume, and ASD*. Previous studies have demonstrated the relationship between fetal testosterone and autistic traits in the general population, with higher levels of testosterone related to a 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 have not been studied yet. Furthermore, an effect on CC size and brain volume has been demonstrated in previous studies showing a reduction in midsagittal CC size in individuals with ASD,67-70 and an increased brain volume.71 The primary candidate suggested to be responsible for these neuroanatomical findings is fetal testosterone.14

In conclusion, the role of sex hormones on brain structure development and function has been widely studied. Nevertheless, the role of prenatal exposure to sex hormones and its consequences on development is less documented. Given the far reaching influence of the organizational effects of sex hormones on later development of the brain and on behavior, the goal of the present study is to capture this critical period in development by measuring the hormonal milieu as well as measuring an important brain structure – the CC, and brain volume, involved in later cognitive and emotional development. Furthermore, this study extends the frame of investigation beyond testosterone and examines 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, and to consequently uncover the factors associated with psychopathological states. We hypothesize that prenatal sex hormones will correlate with 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 (see Figure 1).

### Research objectives and expected significance

 Our research will examine the role of prenatal hormone in the development of brain structure and function. Specifically, our research is a longitudinal study that will take place in three significant stages in development: the first, sex hormone measurement in the early prenatal period, second - measurement of CC size and brain volume later in the prenatal period, and third - measurement in childhood of cognitive and emotional processing. The specific aims of this study 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 effects of prenatal sex hormones on performance in emotional and visuospatial processing tasks.
3. To examine the effects of prenatal sex hormone exposure on development of autistic traits.
4. To examine the association between performance in 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 volumes 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 will exhibit a negative correlation with the width of the CC, whereas estrogen and progesterone will negative correlate with the length of the CC.
2. Testosterone will positively correlate with brain volume, whereas estrogen and progesterone will negatively correlate with brain volume.
3. Testosterone will be associated with improved performance in visuospatial processing, decreased performance in emotional processing. In contrast, estrogen and progesterone will be associated with improved performance in visuospatial processing, but decreased performance in emotional processing.
4. Testosterone will exhibit a positive association with the prevalence of autistic traits.
5. Performance on visuospatial and emotional processing will be 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 will be negatively correlated with the prevalence of autistic traits, whereas, brain volume will 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, will be associated with higher prevalence of autistic traits.

**Methodology**

**Participants**. 150 fetuses, males and females, with follow up at age 5 years, will participate in the present study. The number of participants was established by power analysis using data from our preliminary results, with an addition of 20% to the sample size to compensate for possible drop-outs during the study.

**Research procedure**. Pregnant women will be recruited prior to their scheduled amniotic fluid test at 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 agree to use 5ml of the amniotic fluid for measurement of sex hormones. The study design (Figure 2) includes 3 stages: In the first stage of the study, amniotic fluid tests will be performed between 14 and 16 weeks’ gestation. Levels of sex hormones - testosterone, estrogen, and progesterone, will be measured. In the second stage of the study, an obstetric ultrasonography will be performed in the third semester (28 weeks’ gestation) and will measure the CC (width, length, thickness) and brain volume (biparietal diameter -BPD; head circumference – HC). The third stage will include tests of cognitive performance, emotional processing and autistic traits, 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 the completion of cognitive and emotional tasks. The rationale for examining eye-movements in research is derived from evidence that eye movement patterns allow for more robust assessment of cognitive processing and allocation of visual attention.72 In addition, participants' parents will be asked to complete a broad spectrum of questionnaires, evaluating the report on their children's behaviors and traits.

**Research tools.**

Stage 1 -

1. Hormone level analyses in the amniotic fluid, including measurement of testosterone, estrogen, and progesterone levels, will be analyzed in the endocrine laboratory at the Rambam Health Care Campus.

Stage 2 -

1. CC and brain volume measurements: Obstetric ultrasonography will measure the CC (width, length, thickness) and brain volume (biparietal diameter [BPD], head circumference [HC])

Stage 3 -

1. Personal information questionnaire containing eleven questions will be adapted to the current research, including questions about demographic background and pregnancy history.
2. Judgement of Line Angle and Position (JLAP). 73 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 the target lines in the top of the page to a numbered line from the bottom of the page.
3. Mental Rotation test (MRT).74 This test involves rotating figures in three-dimensional space or in the picture plane. We measure two difficulty levels: with 3-D and 2-D models.75 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 (RMET). 76 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).77 The faces are taken from the NIMSTIM.78 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).79 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).80 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).

10) Eye movements. Fixation duration will be measured using a Tobii Pro X3-120 Eye-tracker at a sampling rate of 120 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).81 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 time of the slide.82

**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, and to research 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, measurement of the CC and brain volume in the third semester. Brain-volume measures and fetal sex hormones were obtained from sixty-nine fetuses, and CC size measurements were obtained from forty-six fetuses. 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 statistical significance. In contrast, testosterone was positively correlated with brain volume measures, however these correlations did not reach significance either, 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 was 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 relationship between the different classes of abilities will be explored. Lastly, the proposed longitudinal study will provide a chain of factors that are suggested to be involved in psychopathological states such as ASD, and to further expand our understanding of the neuroendocrine factors underlying the disorder.

**Expected results and pitfalls**

With longitudinal research study, we expect to deepen our understanding of the 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, estrogen and progesterone seem to also play a central role in determining individual differences in brain morphology and in later behavior. The inclusion of ovarian hormones will enable us to construct a comprehensive model of our understanding of the basis of individual differences in brain structure and function. In contrast to animal studies, which implement prenatal and perinatal hormonal interventions, the observational nature of the present study precludes determination of causality. However, conducting a longitudinal study provides the second-best paradigm investigating the study' hypotheses of the predictive role of sex hormones and brain structure in generating 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 to achieve statistical significance in the first two stages of this longitudinal study, 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 techniques83 to address this challenge.

**Resources available for the research**

The proposed research is based on an 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 his research focuses on early detection of fetal abnormalities. Dr. Maya Steinberg, MD, is a senior physician in the Ultrasound in Obstetrics and Gynecology unit (Rambam Health Care Campus) with extensive experience in prenatal diagnosis. 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 with diagnostic experience in the Educational Psychology Service with children aged 3–12. Furthermore, she has extensive background in research regarding cognitive and emotional development and its relation to hormonal status throughout the lifecycle.

The study will be conducted at the Rambam Health Care Campus. The laboratory staff of Rambam will carry out the measurement of hormone levels in the amniotic fluid. The Ultrasound in Obstetrics and Gynecology staff will perform the CC and brain volume measurements during fetal ultrasound tests. A graduate psychology student will take part in data collection of cognitive, emotion, and autistic trait measurements at childhood. Undergraduate students will also take part in recruitment of pregnant women that have not yet completed amniotic fluid testing. Statistical advisory and computer support are provided by the college. It is important to note that we intend to approach the families who took part in the preliminary study, thus enabling us to meet the time table. Parents of children in the preliminary phase showed willingness to continue and participate in later stages in development.

**Figures**

**Figure 1.** Theoretical model for the relations between fetal hormones, CC, brain volume, cognitive and emotional processing, and autistic traits.

childhood

prenatally

**Age 5.**

**Visuospatial tasks:**

**JLAP**

**2-D and 3-D MRT**

**Emotion recognition tests:**

**RMET**

**NIMSTIM**

**Autistic traits:**

**AQ-Child**

**CAST**

**14 to 16 weeks gestation.**

**Amniotic fluid tests for:**

**Fetal testosterone**

**Fetal estrogen**

**Fetal progesterone**

**28 weeks gestation.**

**Ultrasonographic examination for:**

**CC length and thickness**

**Brain volume**

**Figure 2.** Study design. Phase 1 (between 14 and 16 weeks’ gestation) includes tests for fetal sex hormones (testosterone, estrogen, progesterone). Phase 2 (28 weeks’ gestation) includes ultrasonographic examination for CC size and brain volume. Phase 3 (age 5) includes visuospatial tests (JLAP, judgement of line angle; MRT, mental rotation test); emotion recognition tests (RMET, reading the mind in the eyes test; NIMSTIM, emotion recognition task); and autistic traits questionnaires (AQ-Child, the autism spectrum quotient – children's version; CAST, childhood autism spectrum test).

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