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Elevated prenatal maternal sex hormones, but not placental aromatase, are associated with child neurodevelopment

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ABSTRACT

Fetal exposure to testosterone may contribute to vulnerability for autism spectrum disorder (ASD). It is hypothesized that placental aromatase prevents fetal exposure to maternal testosterone, however, this pathway and the implications for child neurodevelopment have not been fully explored. We examined the relationships between prenatal maternal testosterone and estradiol at 19.2 ± 1.3 weeks, cord blood testosterone and estradiol at birth, placental aromatase mRNA expression, and neurodevelopment using the Social Communication Questionnaire (SCQ), the Behavioral Assessment System for Children, 3rd Edition (BASC-3), and the Empathizing Quotient for Children (EQ-C) at 4.5–6.5 years of age in a sample of 270 Nulliparous-Mothers-to-be (nuMoM2b) study participants. Maternal testosterone levels were positively associated with SCQ scores, but the association was not significant after adjusting for maternal age at delivery, nor was there a significant interaction with sex. Maternal estradiol levels were negatively associated with BASC-3 Clinical Probability scores among males (n = 139). We report a significant interaction effect of cord blood testosterone and fetal sex on both total SCQ scores and t-scores on the Developmental Social Disorders subscale. Placental aromatase was not associated with any neurodevelopmental or hormone measure, but under conditions of low placental aromatase expression, high maternal testosterone was positively associated with SCQ scores in males (n = 46). No other associations between hormone levels and neurodevelopment were significant. Our findings provide a foundation for further investigation of the mechanisms through which maternal sex hormones and placental steroidogenesis may affect fetal hormone production and neurobehavior.

1. Introduction

Prenatal exposure to sex hormones such as testosterone has profound effects on mammalian brain development and function with lifelong implications for social behavior. Given robust sex differences in the prevalence and presentation of many psychiatric conditions, particularly autism spectrum disorder (ASD), studying how prenatal hormones influence the developing brain may offer insight into possible etiological

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mechanisms and early-emerging biological indicators of psychiatric outcomes (Gore et al., 2014). ASD is a pervasive neurodevelopmental disorder that is typically diagnosed during early childhood and is characterized by impairments in social functioning and restricted and repetitive behaviors and interests. Males are more likely to be diagnosed with ASD compared to their female counterparts (Gore et al., 2014; Lord et al., 2018), though it is becoming widely-recognized that biases in diagnostic measures and sex differences in ASD presentation may in part account for the reported sex bias in prevalence (Halladay et al., 2015; Hull et al., 2020).

Elevated steroidogenic activity in amniotic fluid has been associated with ASD-related behaviors in childhood, as well as sub-clinical, yet developmentally-relevant behavioral and physiological differences (Auyeung et al., 2009a; Auyeung et al., 2010; Baron-Cohen et al., 2015, 2019, 2020; Knickmeyer and Baron-Cohen, 2006; Lutchmaya et al., 2001, 2002, 2004), though two studies did not replicate this association (Kung et al., 2016; Long et al., 2019). By 12 months of age, the duration and number of instances of eye contact are predicted by lower testosterone in amniotic fluid during mid-pregnancy, especially in males (Lutchmaya et al., 2002) and toddlers with more restricted vocabularies tended to have higher levels of testosterone in their amniotic fluid, suggesting a prenatal influence (Lutchmaya et al., 2001). Also by 12 months of age, children have sex-typed play behavioral patterns with distinct preferences in toys (Servin et al., 1999; Snow et al., 1983) and playmates (Hines and Kaufman, 1994). Several studies have explored the relationship between prenatal testosterone levels and sex-typed play behavior and have yielded conflicting results (Auyeung et al., 2009b; van de Beek et al., 2007; Knickmeyer et al., 2005). At 6–9 years, the ability to accurately assign an emotion to images of the eye region of a face (Eyes-C) was negatively correlated with testosterone in amniotic fluid and performance on the child version of the Empathizing Quotient (EQ-C) was also negatively correlated with amniotic fluid testosterone levels, but only among boys (Chapman et al., 2006). Importantly, two studies report no associated between androgens measures in cord blood at birth and autism phenotypes or diagnoses in childhood (Park et al., 2017; Whitehouse et al., 2012), suggesting a possible time-dependent effect of androgen exposure on neurobehavioral outcomes. Clinically, a large case-control study of males with and without a diagnosis of ASD implemented a principal component analysis (PCA) of hormones along the Δ-4 steroid pathway and cortisol measurements collected from amniotic fluid samples (Baron-Cohen et al., 2015). Though this study did not find an androgen-specific association, it suggests that elevated steroidogenic activity may be associated with a diagnosis of ASD.

It has been suggested that the fetus is the primary contributor of in-utero sex hormones and thus amniotic fluid levels of testosterone are understood to be of fetal origin. This assumption is driven by the presumed role of the placenta in the conversion of testosterone to estradiol through the actions of aromatase, an enzyme that is highly expressed in syncytiotrophoblast cells of the human placenta. It has been suggested that placental aromatization of maternal testosterone may protect the fetus from exposure to high levels of maternal testosterone that would otherwise enter fetal circulation (Edman et al., 1981; Gore et al., 2014; Illingworth et al., 1992; Kallak et al., 2017; Kragie, 2002; Makieva et al., 2014; Sarachana et al., 2011; Siiteri and MacDonald, 1966). However, there is limited empirical evidence from which to conclude that maternal testosterone reaches the fetus via transplacental transmission, and the associations between reduced aromatase activity in the placenta, fetal hormone exposure, and neurodevelopment have not been previously evaluated. In fact, studies involving special clinical populations that are affected by endocrine conditions have provided a theoretic basis for our hypothesis that elevated maternal androgens during pregnancy may be associated with child neurobehavioral outcomes. Women with polycystic ovary syndrome (PCOS) often experience hyperandrogenism (Kosidou et al., 2015; Nisenblat and Norman, 2009) especially during pregnancy (Palomba et al., 2010; Sir-Petermann et al., 2002). Two large matched case-control analyses found that the odds of having a child with ASD were up to 60% higher among women with PCOS compared to the healthy controls (Cherskov et al., 2018; Kosidou et al., 2015), and additional studies have supported this association (Cesta et al., 2020; Chen et al., 2020; Palomba et al., 2012).

In the present study, we address current gaps in our understanding of the complex relationships between maternal and fetal sex hormones, the regulatory function of aromatase in the human placenta, and long-term child neurobehavioral developmental outcomes associated with ASD. Given the previously-reported associations between androgen levels in amniotic fluid sampled during mid-gestation and child neurobehavioral outcomes, we hypothesized that maternal testosterone levels collected at 19.2 ± 1.3 weeks would be associated with child neurobehavioral development. Additionally, we hypothesized that lower aromatase mRNA expression would be associated with child neurobehavior. Taking into account the reported sex difference in hormones measured in cord blood (Herruzo et al., 1993; Keelan et al., 2012; Maccoby et al., 1979) and in amniotic fluid (Auyeung et al., 2009b; Auyeung et al., 2010; Judd et al., 1976; Nagamani et al., 1979; Robinson et al., 1977; Rodeck et al., 1985), and the observed sex bias in the prevalence of neurodevelopmental disorders (Al-Zaid, 2017; Constantino and Charman, 2012; Werling and Geschwind, 2013), we hypothesized that male vulnerability (Bale, 2016) would be reflected in sex-specific associations. Data were collected from a highly diverse and well-characterized sample of 661 pregnant women who were enrolled as part of the large-scale, multicenter Nulliparous-Mothers-to-be (nuMoM2b) study. This study design allowed us to examine the effects of maternal sex hormones and placental aromatase on child neurodevelopmental outcomes.

2. Methods

2.1. Study participants

Between 2010 and 2013, 8 participating clinical centers across the United States enrolled 10,037 nulliparous women with singleton pregnancies into the Nulliparous Pregnancy Outcomes Study: Monitoring Mothers-to-be (nuMoM2b) study (ClinicalTrials.Gov; NCT01322529) (Haas et al., 2015). To participate in the nuMoM2b study, nulliparous pregnant women were screened for eligibility during the first trimester. Eligible women were between 6^0 and 13^6 weeks of gestation based on an ultrasound crown-rump length measurement. Pregnant women were excluded if they had a prior pregnancy lasting 20 weeks' gestation or more, were younger than 13 years of age, had a history of 3 or more spontaneous abortions, or if a likely fatal fetal malformation was identified during screening.

A total of 1005 of the nuMoM2b study participants were enrolled at Columbia University Medical Center. Of these 1005 participants, 661 met inclusion criteria to be included in this ancillary study. Women were eligible to participate in the secondary analyses if they participated in the original nuMoM2b study, delivered at the Columbia University Medical Center site, were fluent in English or Spanish, were ≥ 18 years old at the time of enrollment in the nuMoM2b study, had provided a sample of maternal blood during the second trimester, and had provided a sample of cord blood and placental tissue at delivery. Additionally, women had to have provided written consent to the future use of their biological samples and written consent to be re-contacted by study staff. Medical records were requested for the 661 eligible infants. Upon reviewing the data, one woman's data were not included in the present analysis because she had an intrauterine fetal demise.

In addition to providing biological samples, women whose children had reached the age of 4.5–6.5 years (mean = 61.75 months, range = 54.0–79.0 months) were re-contacted via telephone and email and were asked to complete a brief online questionnaire. The questionnaire was completed by $n = 270$ mothers and included items pertaining to any physical and psychiatric diagnoses that the child received since birth, maternal diagnoses of endocrine-related conditions (e.g., polycystic ovary syndrome), and maternal use of medications with the potential to

alter endocrine function (e.g., oral contraceptives). The online questionnaire included a subset of items from the Behavioral Assessment System for Children, 3rd Edition (BASC-3) (Altmann et al., 2017) Parent Rating Scales (Preschool), the Social Communication Questionnaire (SCQ) (Allen et al., 2007), and the Children's Empathy Quotient (EQ-C) (Auyeung et al., 2009c). All procedures were reviewed and approved by the Columbia University Medical Center Institutional Review Board and participants provided informed written consent prior to all study procedures. A complete consort diagram is presented in (Fig. 1).

2.2. Collection of biological samples

2.2.1. Maternal blood

Maternal plasma was collected between 15 and 23 weeks of gestation (mean: 19.2 ± 1.3 weeks). Whole blood was collected by venipuncture into an EDTA-coated collection tube. Collection tubes were repeatedly inverted to distribute the anticoagulant and were centrifuged at 1500*g* for 10 min at 4 °C. Plasma was extracted and transferred to 2 ml collection tubes and stored at −80 °C until further processing.

2.2.2. Umbilical cord blood

After delivery and umbilical cord clamping, study staff transported the whole placenta in a closed container to a laboratory for further processing. Whole umbilical cord blood was obtained by venipuncture of the umbilical cord or vessels on the surface of the chorionic plate using a sterile syringe and was collected into an EDTA-coated collection tube. The collection tubes were repeatedly inverted to distribute the anticoagulant and were centrifuged at 1500*g* for 10 min at 4 °C. Plasma was extracted and transferred to 2 ml collection tubes and stored at −80 °C until further processing.

2.2.3. Placenta tissue

After delivery, whole placentas were stored on ice and brought to the laboratory for further processing. Efforts were made to obtain placenta tissue within 30 min of delivery, but in some cases, placentas were stored on ice or in refrigerators at 4 °C for up to 72 h prior to processing. Placentas were rinsed in normal saline to remove blood clots and placed on a sterile hospital pad with the fetal side of the placenta facing up. Using a punch biopsy or a scalpel, a piece of tissue (0.7 × 0.7 × 1 cm or 8 mm × 1 cm punch biopsy) was dissected from the midpoint between the cord insertion and the edge of the placenta. The first 88 samples (33%) were flash frozen in liquid nitrogen and stored at −80 °C. The subsequent 182 samples (67%) were stabilized in RNA*later* (Invitrogen) for 48 h at 4 °C and then stored at −80 °C. Due to variability in placenta collection, we excluded 96 samples with RNA concentrations <100 ng/μl from the analysis.

2.3. Laboratory assays of biological samples

2.3.1. Radioimmunoassay of testosterone and estradiol in maternal and umbilical cord plasma

To measure total testosterone and estradiol, radioimmunoassays were conducted using commercially available kits and performed as indicated by the supplier (MP Biomedicals; 17β-estradiol cat# 07–138,105, testosterone cat# 07–189,105). For all assays, samples were run in duplicate and values were averaged. For the testosterone assays, the intra-assay coefficient of variations (CV) for maternal and umbilical cord testosterone were 11.15 and 16.48, respectively. The lower limits of detectability (LLD) for maternal and umbilical cord blood testosterone were 0.09 ng/ml and 0.10 ng/ml, respectively. Samples that were below detection (e.g. <0.09) were assigned the value of the lowest level of detectability.

Due to the relatively high levels of estradiol in maternal and umbilical cord plasma, all estradiol samples were diluted in assay buffer and the raw values multiplied by the dilution factor. For the estradiol assays, the intra-assay CVs for maternal and umbilical cord estradiol were 13.72 and 8.51, respectively. The LLDs for maternal and umbilical cord blood were 9.07 pg/ml and 8.12 pg/ml, respectively. Samples that were below detection were assigned the value of the lowest level of detectability.

2.3.2. Placenta RNA extraction

We prepared 30 mg sections of placental tissue containing chorionic villi for RNA extraction. Tissue was homogenized in 700 μL lysis buffer RLTplus (Qiagen) with 1% β-mercaptoethanol using a tissue homogenizer (Omni) for 15 s. RNA and DNA were simultaneously extracted using the Qiagen All Prep DNA/RNA Mini Kit and the Qiagen RNase-Free DNase I Kit from batches of up to 24 samples per extraction. RNA concentration yields were measured using a spectrophotometer (Nanodrop). When applicable, remaining RNA samples were normalized to a concentration of 100 ng/μL. 10uL of cDNA were generated from RNA and nuclease-free water using the SuperScript III Reverse Transcriptase Kit (ThermoFisher) in a thermocycler (Applied Biosystems).

2.3.3. Gene expression in placental tissue

Relative gene expression was measured using real-time quantitative PCR (RT-qPCR) on a 96-well 7500 Fast qPCR machine (Applied Biosystems) using Fast SYBR Green Master Mix. The $2^{-\Delta\Delta Ct}$ method is a widely used formula to calculate the relative gene expression in relation to a reference gene in samples based on data acquired through RT-qPCR. Using the $2^{-\Delta\Delta Ct}$ method, we calculated relative gene expression of the aromatase gene (*CYP19A1*; forward: GCGCAAAGCCTTAGAAGATG, reverse: CAAAGCCAAATGGCTGAAAG) using *YWHAZ* (forward: GACGTCCCTCAAACCTTGCT, reverse: GCCAGTTTGGCCTTCTGAAC) as a control gene with high expression stability in human placental tissue (de Sousa et al., 2016). As an additional quality control measure, we excluded samples with cycle threshold (Ct) values that were equal to or greater than 35. Samples that were identified as outliers using Grubbs' test for outliers were removed from analyses (Grubbs, 1969). We calculated the $2^{-\Delta\Delta Ct}$ using females as the reference group.

2.4. Child neurobehavioral measures

2.4.1. Social Communication Questionnaire (SCQ)

The SCQ lifetime version is a parent-report questionnaire to evaluate ASD-related behaviors in children 4 years (or mental age of 2 years) and older (Allen et al., 2007). The SCQ contains 40 yes-or-no items that are intended to determine whether the child has ever demonstrated behaviors associated with ASD spectrum disorder. The SCQ parallels the Autism Diagnostic Interview Revised (ADI-R) with robust agreement. The SCQ provides a continuous score (0–40) such that higher scores indicate more ASD-related behaviors.

2.4.2. Children's Empathizing Quotient (EQ-C)

The EQ-C is a parent-report questionnaire that was adapted from the adult version of the Empathizing Quotient questionnaire (EQ) and is designed to assess trends in sex-typical empathizing behavior in children (Auyeung et al., 2009c). It was combined with the Children's Systemizing Quotient (SQ-C) to include 55 items that pertain to the child's real-life situations, experiences, and interests. Of the 55 items, 27 measure the child's empathizing behaviors and 28 measure the child's systemizing behaviors. In an effort to reduce participant fatigue and increase completion of the entire survey, we asked parents to only complete the 27 empathizing items from the EQ-C scale. Parents can respond to each item by selecting one of the following choices: 'slightly agree,' 'definitely agree,' 'slightly disagree,' or 'definitely disagree.' Responses to each item were reverse scored when appropriate and summated to generate a continuous total score ranging from 0 to 54.

2.4.3. Behavioral Assessment System for Children (3rd Edition) (BASC-3)

The BASC-3 Parent Rating Scales (Preschool), is a validated parent-report questionnaire that is designed to assess a child's

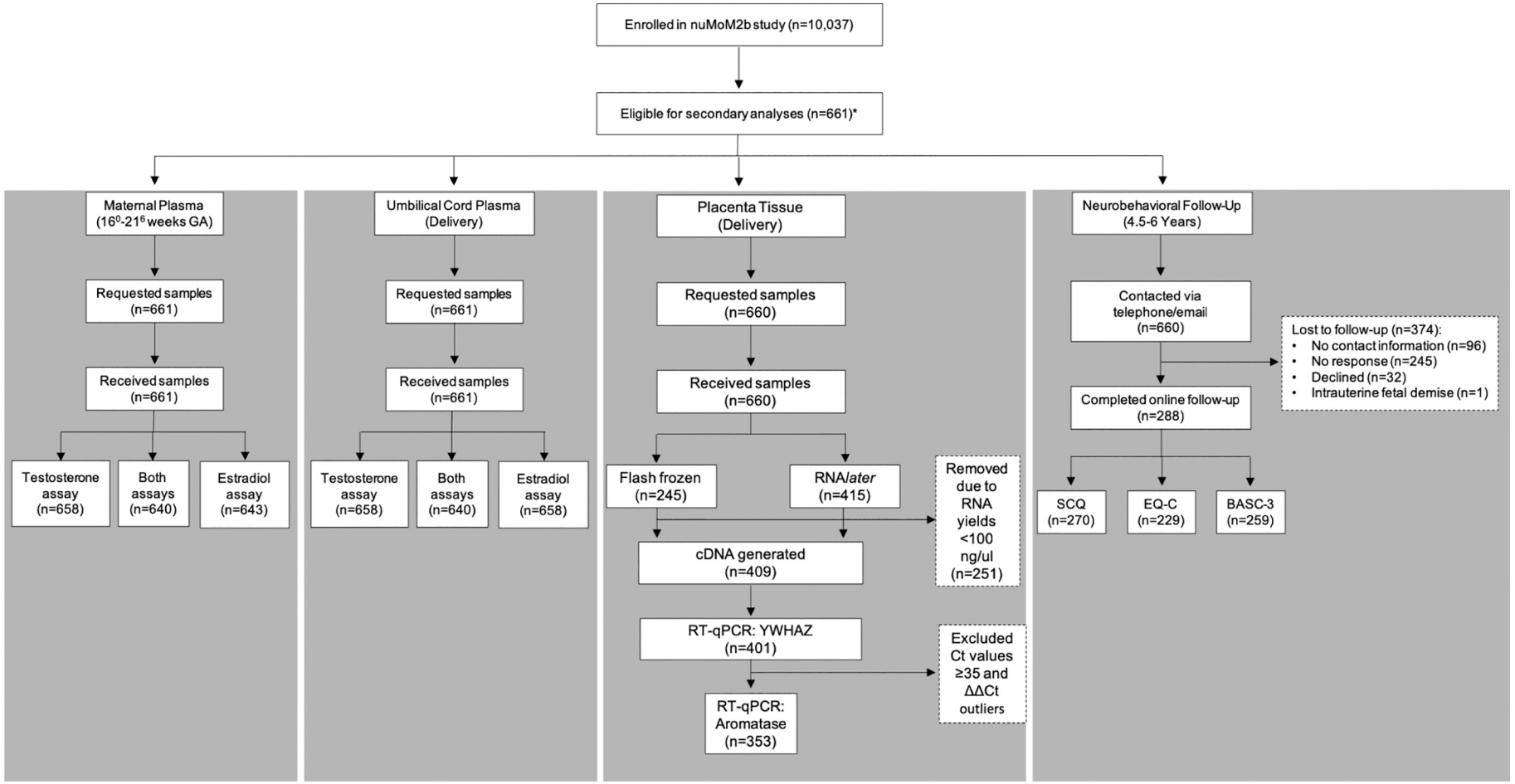

Fig. 1. Consort diagram. *Eligible mothers participated in nuMoM2b study, delivered at Columbia University Medical Center site, were fluent in English or Spanish, were $\geq$ 18 years old at the time of enrollment in the nuMoM2b study, provided maternal plasma during the second trimester, provided cord blood plasma at birth, provided placenta tissue at birth, provided consent to the future use of their biological samples and written consent to be re-contacted.

neurobehavioral development between the ages of 2 years 0 months and 21 years 11 months (Altmann et al., 2017). The BASC-3 provides cutoffs that address diagnostic criteria from the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) and can be used to identify children who should be evaluated further. The BASC-3 provides clinical, adaptive, content, composite, and probability index scales. We analyzed participant responses to the Hyperactivity, Developmental Social Disorders, and General Clinical Probability subscales. The Hyperactivity scale measures the child's tendency to be overly active, rush through tasks, and act without thinking. The Developmental Social Disorders subscale measures the child's tendency to demonstrate behaviors associated with deficits in and restricted expression of social skills, communication, interests, and activities. Finally, the General Clinical Probability index can be used to identify children who are at increased likelihood for presenting with a clinical disorder. Parent responses to each item were entered using the online Q-Global scoring system. For each child, Q-Global generated t-scores such that higher t-scores on each subscale indicated more problem behaviors.

2.5. Statistical analyses

Statistical analyses were conducted using R and SPSS software. Maternal testosterone, cord blood testosterone, aromatase mRNA expression, and total SCQ scores were positively skewed and 1-min Apgar scores and infant gestational age were negatively skewed. All skewed variables were square-root transformed prior to statistical analysis. We examined several covariates including mode of delivery, maternal and paternal age at delivery, maternal educational attainment, maternal ethnicity, infant gestational age at birth, infant birth weight, 1-min Apgar scores, and the child's age at the time of follow-up. Specifically, analyses of bivariate Pearson correlations between continuous covariates and the primary outcome and predictor variables including the maternal hormone variables, were conducted. Linear modeling including simple linear regression and multiple linear regression was used to determine the associations between continuous predictor and outcome variables with and without covariates. A two-way analysis of variance (ANOVA) was conducted. We also performed Welch's two sample two-tailed *t*-tests to determine whether any of our variables of interest differed by mode of delivery, type of labor onset, and sex of the infant. Candidate covariates were included in the adjusted linear models if they were associated with both the predictor and outcome variables at a significance level of 0.1. In the event of co-linearity between covariates meeting this criteria, one covariate was selected and included in the model.

3. Results

3.1. Sample characteristics

Demographic and medical information for infants and their mothers were provided by the NuMoM2b data coordinating and analysis center and are shown in Table 1. The average maternal age at delivery was 34.5 years (range 24.0–49.0 years) and the average paternal age at delivery was 31.6 years (range 18.0–64.0 years). 59.8% of the mothers self-identified as being Hispanic. The primary reasons for delivery, in order of prevalence, were labor onset ($n = 163$), rupture of membranes ($n = 29$), elective ($n = 28$), a maternal medical condition ($n = 19$), oligohydramnios ($n = 18$), abnormal fetal testing ($n = 7$), fetal growth restriction ($n = 3$), an unspecified fetal condition (n = 1), abruptio placenta (n = 1), and unknown reasons (n = 1). 35.5% of the infants were delivered by cesarean delivery. The majority of cesarean deliveries were unscheduled and urgent ($n = 57$), followed by unscheduled and non-urgent ($n = 20$), and scheduled ($n = 17$). In two cases, details regarding the cesarean delivery were unavailable. The average gestational age at delivery was 39.3 weeks (range 28.0–42.1 weeks, median 39.3 weeks), with an average birth weight of 3279 g (range 1405–3564 g, median 3315 g). In this study sample, there were 121 female infants and 149 male infants.

Table 1
Study sample characteristics.

Characteristics		Total $n = 270$	
		Mean	SD
Gestational age at delivery (weeks)		39.37	1.48
Birth weight (grams)		3279.47	457.59
Infant head circumference (cm)		34.09	1.49
Maternal age (years)		29.46	5.88
Paternal age (years)		31.16	6.76
		%	*n*
Infant sex (male)		55.2	149
Cesarean section		29.6	80
Reason for delivery	Labor onset	52.2	141
	Rupture of membranes	11.1	30
	Convenience	8.9	24
	Late-term	8.1	22
	Maternal health condition	7.8	21
	Oligohydramnios	6.3	17
	Abnormal fetal testing	2.6	7
	Fetal health condition	1.1	3
	Intrauterine growth restriction	1.1	3
	Placental abruption	0.37	1
	Unknown	0.37	1
Maternal education	No high school degree	1.5	4
	High school degree	7.4	20
	Some college, no degree	17.0	46
	College degree	27.8	75
	Graduate/professional degree	46.3	125
Maternal ethnicity (Hispanic)		51.1	138
Paternal ethnicity (Hispanic)		50.4	136

*Infant, parental, and pregnancy-related information was not available on all participants.

3.2. Sex differences in maternal and cord blood hormone levels

There were no statistically significant differences in maternal testosterone levels during the second trimester between pregnancies with a male (0.58 ng/ml ± 0.41, non-transformed) versus female (0.58 ng/ml ± 0.53, non-transformed) fetus ($t(267) = 0.15$, $p = 0.88$, $d = 0.02$). There were also no significant differences in maternal estradiol levels between women carrying a male (3961.87 pg/ml ± 1546.81) versus female (4010.2 pg/ml ± 1405.52) fetus ($t(257) = -0.26$, $p = 0.80$, $d = -0.03$). At birth, cord blood testosterone levels did not differ between male (1.59 ng/ml ± 1.05, non-transformed) and female (1.75 ng/ml ± 1.34, non-transformed) neonates ($t(267) = -1.04$, $p = 0.30$, $d = -0.13$). Male (6191.32 pg/ml ± 3370.25) and female (6559.86 pg/ml ± 3464.79) neonates did not differ significantly with regard to cord blood estradiol levels ($t(267) = -0.88$, $p = 0.40$, $d = -0.11$).

3.3. Sex differences in neurobehavioral outcome measures

No sex differences were observed in total SCQ scores (males, 5.19 ± 4.0; females, 4.75 ± 4.0, non-transformed; $t(268) = 0.62$, $p = 0.54$, $d = 0.94$). Total EQ-C scores were significantly higher in female children (41.56 ± 8.23) compared to male children (37.54 ± 10.23) ($t(226) = 3.29$, $p = 0.001$, $d = -0.43$). Males had significantly higher scores on the Hyperactivity (males, 49.30 ± 7.12; females, 47.32 ± 6.60; $t(257) = -2.29$, $p = 0.02$, $d = 0.29$), Developmental Social Disorders (males, 47.40 ± 7.73; females, 45.60 ± 6.60; $t(256) = -2.02$, $p = 0.04$, $d = 0.25$), and Clinical Probability subscales of the BASC-3 (males, 48.14 + 7.51; females, 45.43 ± 6.14; $t(257) = -3.13$, $p = 0.002$, $d = 0.39$).

3.4. Maternal testosterone levels in the second trimester and child neurodevelopmental outcome

Bivariate Pearson correlations were assessed between maternal testosterone measured during the second trimester and child neurobehavioral measures. Maternal testosterone was significantly positively correlated with total SCQ scores in the overall sample ($r = 0.18$, $p = 0.003$, Table 2). Within each sex, maternal testosterone was positively correlated with total SCQ scores, though this association only reached statistical significance among females (females: $r = 0.21$, $p = 0.02$; males: $r = 0.15$, $p = 0.07$, Table 2, Fig. 2). We performed a linear regression model to adjust for covariates that were associated with both the predictor and outcome variables. The model revealed that the association between maternal testosterone and SCQ scores was no longer significant in the overall sample or in each sex after controlling for maternal age at delivery (overall: $\beta = 0.09$, $p = 0.16$; males: $\beta = 0.10$, $p = 0.28$; females: $\beta = 0.08$, $p = 0.35$, Table 3). As a secondary analysis, we performed a linear regression to evaluate a testosterone-by-sex interaction and found that there was not a significant interaction effect of sex while controlling for maternal age at delivery ($\beta = -0.21$ $p = 0.59$). Maternal testosterone was not associated with any of the other outcome measures in the overall sample or within each sex (Tables 2, 3).

3.5. Maternal estradiol levels in the second trimester and child neurodevelopmental outcome

Overall, maternal estradiol was not associated with total SCQ scores, total EQ-C scores, or scores on the Hyperactivity, Developmental Social Disorders, and Clinical Probability subscales of the BASC-3 (Tables 2, 3). After stratifying the sample by sex, a weak, but significant correlation between maternal estradiol and t-scores on the Clinical Probability subscale of the BASC-3 was observed among male children ($r = -0.20$, $p = 0.02$, Table 2) such that increasing levels of maternal estradiol were associated with lower scores. We performed a linear regression model to adjust for covariates that were associated with both the predictor and outcome variables to further evaluate the association of maternal estradiol with Clinical Probability subscale scores among males. The model revealed that the association between maternal estradiol and Clinical Probability t-scores remained significant when controlling for maternal age at birth ($\beta = -0.19$, $p = 0.03$, Table 3). As a secondary analysis, a linear regression that included a testosterone-by-sex interaction term revealed a significant interaction between maternal estradiol levels and sex while controlling for maternal age at delivery such that elevated maternal estradiol levels were associated with lower scores among males ($\beta = -0.001$, $p = 0.03$). Maternal estradiol was not associated with any measure of child neurobehavior among female children (Tables 2, 3).

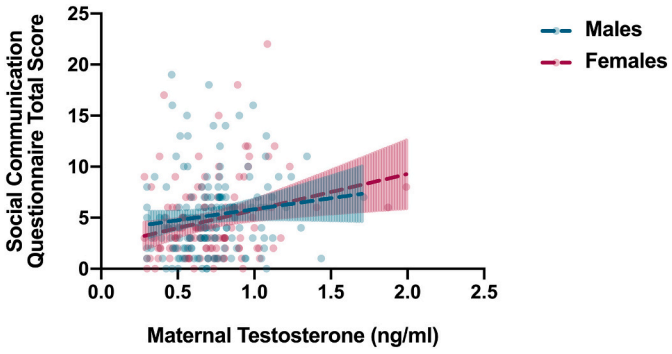

Fig. 2. The association between maternal testosterone and SCQ total scores. No significant interaction with sex was observed. Females are represented in pink and males are represented in green. Solid lines represent the regression line and shading represents the 95% confidence bands of the best-fit line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. Cord blood testosterone levels and child neurodevelopmental outcome

Bivariate Pearson correlations were assessed between cord blood testosterone and child neurobehavioral measures. Cord blood testosterone levels were not associated with total SCQ scores, total EQ-C scores, or t-scores on any of the BASC-3 subscales in the overall sample (Tables 2, 3). Multiple linear regression demonstrated a significant interaction effect between cord blood testosterone and fetal sex on total SCQ scores ($\beta = 0.83$, $p = 0.003$). Simple main effects analysis stratified by sex showed that cord blood testosterone was statistically significantly associated with total SCQ scores in males ($\beta = 0.47$, $p = 0.02$), but not in females ($\beta = -0.36$, $p = 0.08$). Similarly, a multiple linear regression revealed a significant interaction effect between cord blood testosterone levels and fetal sex on t-scores of the Developmental Social Disorders subscale ($\beta = 4.51$, $p = 0.04$). Simple main effects analysis stratified by sex showed that cord blood testosterone was not statistically significantly associated with t-scores of the Developmental Social Disorders subscale in males ($\beta = 2.44$, $p = 0.15$) or in females ($\beta = -2.01$, $p = 0.16$), suggestive of a crossover interaction.

3.7. Neonatal cord blood estradiol and child neurodevelopmental outcome

No significant correlations between cord blood estradiol and total

Table 2
Pearson correlations between maternal and cord blood hormones and child neurobehavioral measures within each sex.

Predictor Variable	Infant Sex	SCQ Total Score			EQ-C Total Score			BASC-3 Hyperactivity			BASC-3 Social Disorder			BASC-3 Clinical		
		n	*r*	*p*	*n*	*r*	*p*	*n*	*r*	*p*	*n*	*r*	*p*	*n*	*r*	*p*
	Combined	269	0.18	**<0.01**[a]	227	−0.06	0.34	258	0.03	0.60	258	0.04	0.56	258	0.02	0.72
Maternal Testosterone	Male	148	0.15	0.07	125	0.01	0.94	142	−0.01	0.89	142	−0.03	0.68	142	−0.05	0.58
	Female	121	0.21	**0.02**	102	−0.16	0.11	116	0.08	0.40	116	0.12	0.19	116	0.11	0.26
	Combined	259	−0.00	0.99	220	0.02	00.82	249	−0.08	0.20	249	−0.09	0.16	249	−0.10	0.12
Maternal Estradiol	Male	141	−0.01	0.91	121	0.00	0.97	136	−0.16	0.06	136	−0.16	0.06	136	−0.20	**0.02**
	Female	118	0.01	0.91	99	0.01	0.94	113	0.04	0.65	113	0.03	0.78	113	0.07	0.43
	Combined	269	0.02	0.73	227	−0.05	0.42	258	−0.04	0.52	258	0.01	0.92	258	−0.00	0.96
Cord Blood Testosterone	Male	149	0.20	**0.02**	126	−0.14	0.12	143	0.03	0.75	143	0.12	0.15	143	0.09	0.28
	Female	120	−0.16	0.08	101	0.02	0.84	115	−0.11	0.24	115	−0.13	0.16	115	−0.11	0.23
	Combined	269	0.04	0.47	227	0.001	0.91	258	−0.00	0.97	258	−0.04	0.54	258	−0.06	0.32
Cord Blood Estradiol	Male	148	0.08	0.31	125	−0.03	0.74	142	−0.01	0.94	142	−0.01	0.91	142	−0.06	0.49
	Female	121	0.00	0.96	102	0.02	0.85	116	0.02	0.84	116	−0.07	0.48	116	−0.05	0.60
	Combined	149	−0.08	0.35	124	−0.04	0.63	147	−0.02	0.79	147	−0.10	0.25	147	−0.07	0.41
Placental Aromatase	Male	84	−0.10	0.38	70	0.02	0.89	83	−0.00	0.99	83	−0.20	0.16	83	−0.11	0.33
	Female	65	−0.05	0.71	54	−0.17	0.23	64	−0.03	0.81	64	0.03	0.82	64	0.01	0.93

[a] Bold text indicates a *p*-value <0.05.

Table 3
Adjusted regression models of maternal hormones, cord blood hormones, and placental aromatase and child neurobehavioral measures within each sex.

Predictor Variable	Infant Sex	SCQ Total Score				EQ-C Total Score				BASC-3 Hyperactivity				BASC-3 Social Disorder				BASC-3 Clinical			
		n	β	95% CI	*p*	*n*	β	95% CI	*p*	*n*	β	95% CI	*p*	*n*	β	95% CI	*p*	*n*	β	95% CI	*p*
Maternal Testosterone	Combined	269	0.09[a]	−0.13, 0.80	0.16	227	−0.06	−7.04, 2.44	0.34	258	0.03	−2.40, 4.13	0.60	258	−0.00[c]	−3.67, 3.51	0.97	258	0.09[a]	−1.09, 6.16	0.70
	Male	148	0.10[a]	−0.31, 1.05	0.28	125	0.01	−7.11, 7.64	0.94	142	−0.01	−5.17, 4.49	0.89	142	−0.07[c]	−7.60, 3.37	0.45	142	−0.03[a]	−6.49, 4.92	0.79
	Female	121	0.08[a]	−0.34, 0.94	0.35	102	−0.16	−10.11, 1.11	0.12	116	0.08	−2.50, 6.21	0.40	116	0.07[c]	−2.98, 6.17	0.49	116	0.23[a]	0.82, 9.32	**0.02**
Maternal Estradiol	Combined	259	−0.09[a]	0.00, 0.00	0.18	220	0.02	−0.00, 0.00	0.82	249	−0.08	−0.00, 0.00	0.20	249	−0.12[c]	−0.00, 0.00	0.07	249	−0.10[a]	−0.00, 0.00	0.41
	Male	141	−0.06[a]	−0.00, 0.00	0.50	121	0.00	−0.00, 0.00	0.98	136	−0.16	−0.00, 0.00	0.06	136	−0.18[c]	−0.00, 0.00	**0.04**[d]	136	−0.19[a]	−0.00, 0.00	**0.03**
	Female	118	−0.01[a]	−0.00, 0.00	0.25	99	0.01	−0.00, 0.00	0.94	113	0.04	−0.00, 0.00	0.65	113	0.03[c]	−0.00, 0.00	0.79	113	0.16[a]	−0.00, 0.00	0.09
Cord Blood Testosterone	Combined	269	0.02	−0.23, 0.33	0.73	227	−0.05	−4.31, 1.80	0.42	258	−0.04	−2.79, 1.41	0.52	258	0.01	−2.11, 2.34	0.92	258	−0.00	−2.20, 2.10	0.96
	Male	149	0.20	0.08, 0.86	**0.02**	126	−0.14	−8.23, 0.98	0.12	143	0.03	−2.55, 3.54	0.75	143	0.12	−0.85, 5.73	0.15	143	0.09	−1.444, 4.98	0.28
	Female	120	−0.16	−0.76, 0.04	0.08	101	0.02	−3.40, 4.70	0.84	115	−0.11	−4.54, 1.17	0.24	115	−0.13	−4.96, 0.81	0.16	115	−0.11	−4.30, 1.05	0.23
Cord Blood Estradiol	Combined	269	−0.01[a]	0.00, 0.00	0.91	227	0.01	000, 0.00	0.91	258	−0.00	0.00, 0.00	0.97	258	−0.04	0.00, 0.00	0.54	258	−0.06	0.00, 0.00	0.32
	Male	148	0.06[a]	0.00, 0.00	0.50	125	−0.03	−0.00, 0.00	0.74	124	−0.01	0.00, 0.00	0.95	142	−0.01	0.00, 0.00	0.91	142	−0.06	0.00, 0.00	0.49
	Female	121	−0.08[a]	0.00, 0.00	0.37	102	0.02	0.00, 0.00	0.85	116	0.02	0.00, 0.00	0.84	116	−0.07	0.00, 0.00	0.49	116	−0.05	0.00, 0.00	0.60
Placental Aromatase	Combined	149	−0.07[b]	−0.31, 0.12	0.38	124	−0.04	−2.86, 1.74	0.63	147	−0.02	−1.86, 1.43	0.79	147	−0.10	−2.57, 0.67	0.25	147	−0.07	−2.27, 0.93	0.41
	Male	84	−0.14[b]	−0.41, 0.01	0.34	70	0.02	−3.11, 3.57	0.89	83	−0.00	−2.19, 2.16	0.99	83	−0.16	−3.97, 0.67	0.16	83	−0.11	−3.23, 1.10	0.33
	Female	65	0.02[b]	−0.32, 0.38	0.86	54	−0.17	−4.78, 1.18	0.23	64	−0.03	−2.85, 2.23	0.81	64	0.03	−1.94, 2.43	0.82	64	0.01	−2.27, 2.49	0.93

[a] Adjusted for maternal age at birth.
[b] Adjusted for child age at testing.
[c] Adjusted for maternal education.
[d] Bold text indicates a *p*-value <0.05.

SCQ scores were observed in the overall sample or within each sex (Tables 2, 3). Cord blood estradiol was not associated with t-scores on any of the three BASC-3 subscales in the overall sample and within each sex (Tables 2, 3), nor was it associated with scores on the EQ-C (Tables 2, 3).

3.8. Placental aromatase mRNA expression and child neurodevelopmental outcome

Placental aromatase mRNA expression was not significantly correlated with scores on the SCQ, EQ-C, or t-scores on the Hyperactivity, Developmental Social Disorders, and Clinical Probability subscales of the BASC-3 in the overall sample and within each sex (Tables 2, 3).

As an exploratory analysis, we were interested in the combined effects of increased maternal testosterone and decreased placental aromatase mRNA expression. A linear regression demonstrated a main effect of maternal testosterone levels ($\beta = 1.43$, $p = 0.01$), but supported neither a main effect of placental aromatase ($\beta = 0.20$, $p = 0.47$) nor an interaction effect of maternal testosterone and placental aromatase on total SCQ scores ($\beta = -0.41$, $p = 0.23$). Additionally, we performed a median split of the maternal testosterone and aromatase mRNA expression variables and assessed the interactive effect of the two variables on total SCQ scores by performing an ANOVA. Using this method, we found a nearly significant interaction effect between maternal testosterone and placental aromatase on total SCQ scores across the full sample, though the effect size was small ($F(2, 147) = 3.28$, $p = 0.07$, $\eta^2 = 0.02$). In the context of low aromatase mRNA expression, total SCQ scores were significantly higher in children born to mothers with high maternal testosterone compared to those with low maternal testosterone ($t(72) = -2.94$, $p = 0.004$, $d = -0.71$). We stratified the data by both sex and aromatase mRNA expression level (low versus high) and found that male children born to mothers with high testosterone scored significantly higher on the SCQ if the placenta was low in aromatase mRNA expression ($t(44) = -2.47$, $p = 0.02$, $d = -0.76$), whereas there was no difference among males born to women with high versus low testosterone if the placenta as high in aromatase mRNA expression ($t(35) = -0.43$, $p = 0.33$, $d = 0.73$). Among females, maternal testosterone (high versus low) was not associated with SCQ scores in those with high ($t(35) = -0.38$, $p = 0.36$, $d = 0.95$) or low ($t(26) = -1.62$, $p = 0.06$, $d = 0.91$) placental aromatase mRNA expression. It should be noted that this sex-specific finding may be due to sample size differences rather than underlying biological differences.

3.9. Relationship between maternal and cord blood sex hormones

Bivariate Pearson correlations revealed that maternal testosterone was positively, though weakly, correlated with cord blood testosterone across the entire sample ($r = 0.13$, $p = 0.03$, Table 4). Stratifying by sex, this relationship was significant in males, but not in females (Table 4). Maternal testosterone was also significantly positively associated with cord blood estradiol in the overall sample ($r = 0.23$, $p < 0.001$), among males ($r = 0.22$, $p = 0.01$) and among females ($r = 0.23$, $p = 0.01$, Table 4).

Maternal estradiol was significantly positively correlated with cord blood estradiol across the entire sample ($r = 0.23$, $p < 0.001$), among males ($r = 0.25$, $p = 0.003$), and among females ($r = 0.21$, $p = 0.02$, Table 4). Maternal estradiol was less strongly correlated with cord blood testosterone, and this association was present among males, but not females (Table 4).

3.10. Relationship between placental aromatase mRNA expression and maternal and cord blood sex hormones

We performed several bivariate correlations to determine whether placental aromatase mRNA expression was associated with any of the maternal or fetal sex hormone measures. We found no significant correlations between placental aromatase mRNA expression and maternal testosterone, maternal estradiol, cord blood testosterone, or cord blood estradiol overall and after stratifying by sex (Table 4).

As an exploratory examination of the effects of placental aromatase on the transfer of maternal testosterone to the fetus, we performed multiple bivariate correlations across the full sample and within each sex. We calculated a maternal testosterone:cord blood testosterone ratio ("MT:CT") and a maternal testosterone:cord blood estradiol ratio ("MT:CE2"). Across the entire sample and within each sex, placental aromatase mRNA expression was not associated with either the MT:CT (overall, $r = 0.08$, $p = 0.33$); males, $r = 0.01$, $p = 0.31$; females, $r = 0.05$, $p = 0.71$) or MT:CE2 (overall, $r = 0.01$, $p = 0.91$; males, $r = 0.01$, $p = 0.91$; females, $r = 0.02$, $p = 0.91$) ratios.

4. Discussion

We investigated the relationships between sex hormones measured in maternal blood during the second trimester of pregnancy, sex hormones in cord blood at birth, and child neurobehavioral outcomes. Additionally, we sought to determine whether neurobehavioral development was associated with placental aromatase mRNA expression either directly or indirectly through regulating fetal exposure to maternal sex hormones. Consistent with our hypothesis, maternal testosterone levels were positively associated with SCQ scores, but the association was not significant after adjusting for maternal age at delivery. Further, we did not observe the expected sex-specific association,

Table 4
Pearson correlations between maternal hormones, cord blood hormones, and placental aromatase mRNA expression.

Predictor Variable	Infant Sex	Maternal Estradiol			Cord Blood Testosterone			Cord Blood Estradiol			Placental Aromatase		
		n	*r*	*p*	*n*	*r*	*p*	*n*	*r*	*p*	*n*	*r*	*p*
Maternal Testosterone	Combined	258	0.39	**<0.0[a]01**	268	0.13	**0.03**	268	0.23	**<0.001**	148	0.03	0.70
	Male	140	0.46	**<0.001**	148	0.26	**0.001**	147	0.22	**0.01**	83	0.07	0.51
	Female	118	0.32	**<0.001**	120	0.01	0.94	121	0.23	**0.01**	65	−0.01	0.94
Maternal Estradiol	Combined	–	–	–	258	0.12	**0.05**	258	0.23	**<0.001**	141	−0.00	0.99
	Male	–	–	–	141	0.17	**0.04**	140	0.25	**0.003**	79	−0.07	0.55
	Female	–	–	–	117	0.06	0.49	118	0.21	**0.02**	62	0.09	0.48
Cord Blood Testosterone	Combined	–	–	–	–	–	–	268	0.055	**<0.001**	148	−0.04	0.60
	Male	–	–	–	–	–	–	148	0.59	**<0.001**	84	−0.16	0.15
	Female	–	–	–	–	–	–	120	0.50	**<0.001**	64	0.09	0.47
Cord Blood Estradiol	Combined	–	–	–	–	–	–	–	–	–	148	−0.10	0.25
	Male	–	–	–	–	–	–	–	–	–	83	−0.14	0.21
	Female	–	–	–	–	–	–	–	–	–	65	−0.03	0.82
Placental Aromatase	Combined	–	–	–	–	–	–	–	–	–	–	–	–
	Male	–	–	–	–	–	–	–	–	–	–	–	–
	Female	–	–	–	–	–	–	–	–	–	–	–	–

[a] Bold text indicates a *p*-value <0.05.

but rather, we found that the relationship between maternal testosterone and SCQ scores was comparable across males and females. In contrast, we found a significant interaction effect of maternal estradiol levels and sex on BASC-3 Clinical Probability scores such that increasing estradiol levels were associated with lower scores in males. With regard to cord blood hormone levels, we found significant interaction effects of testosterone and sex on two measures of neurobehavioral development: SCQ total scores and t-scores on the Developmental Social Disorders subscale of the BASC-3. Finally, we found that placental aromatase expression was not directly associated with any of the maternal or cord blood hormone measures, including ratios of maternal-to-fetal hormone levels, nor was it associated with neurodevelopmental outcomes. As an exploratory analysis, we found that high maternal testosterone levels in the context of low placental aromatase expression were associated with higher SCQ scores in males, though notably, this group had the largest sample size, which may have contributed to the sex-specific finding.

This is the first study to report a significant association between maternal testosterone during pregnancy and ASD-related behaviors in childhood. Two very recent papers investigated this association, but neither report a significant relationship between maternal testosterone levels and ASD-related outcomes (Bilder et al., 2019; Tsompanidis et al., 2021). Bilder et al. (2019) conducted a pilot study that included 53 ASD cases and 19 controls, approximately 50% of whom were exposed to a maternal prenatal metabolic syndrome (e.g., hypertension, diabetes). Maternal testosterone in mid-pregnancy was not associated with increased odds of an ASD diagnosis in the offspring. In a study by Tsompanidis et al. (2021), which is more comparable in design to our study, the authors also reported a non-significant association between maternal testosterone and child ASD-related behaviors as measured by the Quantitative Checklist for Autism in Toddlers (Q-CHAT). Despite many similarities in study design, three key differences may account for our inconsistent findings: 1) maternal blood sampling occurred at 12.7 weeks on average, which is considerably earlier in gestation than when our samples were collected (19.2 $\pm$ 1.3 weeks), 2) child ASD traits were assessed between 18 and 20 months of age, whereas we evaluated neurodevelopmental outcomes between 4.5 and 6 years of age, and 3) the sample size was limited to 100 subjects with maternal blood samples and Q-CHAT scores, suggesting the possibility that the study was underpowered to detect a statistically significant association between maternal testosterone and ASD traits. Thus, further research is needed to comprehensively evaluate this potential biological mechanism across multiple gestational timepoints, postnatal developmental stages, and study cohorts.

Additionally, our study contributes new information to the field as we directly evaluated the association between placental aromatase expression and child neurodevelopment as well as the combined impact of low placental aromatase and high maternal testosterone on neurodevelopmental outcome. Many prior studies that investigated the neurodevelopmental consequences of fetal hormone exposure have not addressed androgens of maternal origin as a contributor to ASD etiology because of the placental aromatase barrier (Baron-Cohen et al., 2015; James, 2014; Lutchmaya et al., 2004). Thus, prior research has placed emphasis on testosterone produced by the fetal adrenal gland and, in the case of males, by the fetal testes (Auyeung et al., 2010; Auyeung et al., 2009c; Baron-Cohen et al., 2015; Lutchmaya et al., 2001, 2002; Wakabayashi and Nakazawa, 2010). Though a previous study reported that mothers of children with ASD have elevated testosterone levels, these hormone measures were collected many years after the pregnancy and thus, many postnatal factors (e.g., maternal stress) could not be accounted for (Xu et al., 2013). In addition, women with pregnancy-related hypertensive disorders including preeclampsia and women with polycystic ovary syndrome exhibit elevated circulating testosterone during pregnancy and their offspring have a higher likelihood of receiving a diagnosis of ASD and other developmental delays (Kelley et al., 2019; Kosidou et al., 2015; Maher et al., 2018, 2020, p. 202; Palomba et al., 2012; Troisi, 2003). Many of these empirical studies have measured the effects of maternal hypertension and polycystic ovary syndrome on child neurodevelopmental outcomes as a proxy for elevated maternal testosterone during pregnancy.

Our findings suggest that the relationship between maternal testosterone during pregnancy and child neurodevelopment may be confounded by maternal age at delivery. A meta-analysis of maternal age and child ASD reported a relative risk ratio of 1.52 in offspring born to mothers who were 35 years or older at the time of delivery compared to those who were 25–29 years old (Sandin et al., 2012). Conversely, it has also been shown that younger maternal age at delivery is associated with increased likelihood for receiving a diagnosis of ASD (Sandin et al., 2016). Sandin et al. (2016) purport that younger maternal age at delivery may be both a biological contributor to ASD etiology and a marker of socioeconomic factors that in turn can impact child neurobehavioral development. For example, women who have a child at a younger age may experience disruptions to their education or employment. Similarly, biological predispositions may indirectly impact both academic achievement and child neurobehavioral outcomes. The complex interplay between biological and psychosocial factors will need to be investigated further to fully understand the mechanism(s) underlying the relationship between maternal testosterone and ASD-related behaviors in offspring.

We hypothesized that low levels of placental aromatase mRNA expression would be associated with neurobehavioral outcomes in childhood. Proxy measures for aromatase activity have been measured previously by calculating a testosterone-to-estradiol ratio (Baron-Cohen et al., 2019), but our study allowed for direct evaluation of the relationship between placental aromatase and neurodevelopment outcome measures. Further, several candidate gene studies of aromatase protein and expression levels in saliva and brain tissue have been associated with ASD, but until now, the role of aromatase in predicting neurodevelopmental outcomes has not been expanded to placental tissue (Chakrabarti et al., 2009; Crider et al., 2014; Sarachana et al., 2011). Contrary to our hypothesis, we did not find a main effect of placental aromatase on child neurobehavioral development, however, the degree to which maternal testosterone impacted SCQ scores differed marginally depending on placental aromatase mRNA expression. Children who were exposed to high maternal testosterone had higher SCQ scores than children exposed to low maternal testosterone, but only if their placentas expressed low levels of aromatase. This finding suggests that presence of a robust placental barrier that aromatizes maternal testosterone may influence the neurodevelopmental consequences of elevated maternal testosterone, though further investigation is necessary as our sample size was limited. Aromatase is exclusively expressed in the cytoplasm of the syncytiotrophoblast cells of the placenta (Fournet-Dulguerov et al., 1987; Inkster and Brodie, 1989). Syncytiotrophoblast cells form as the result of fusion of the more primitive cytotrophoblast cells (Rejniak et al., 2004). Thus, aromatase mRNA expression by syncytiotrophoblast cells is dependent on cellular and structural maturation of the placenta. Indeed, trophoblast inclusions, which are hypothesized to reflect abnormal folding of the trophoblast bilayer, are significantly more prevalent in placentas of children who are diagnosed with ASD (Walker et al., 2013). Low placental weight, another indicator of atypical placental maturation and structure, has been associated with hypospadias in male infants, which has been attributed to abnormal fetal exposure to sex hormones (Arendt et al., 2016). The lack of a direct relationship between placental aromatase and SCQ scores may be partially attributed to the timing of sample collection. Fetal testosterone production is highest during the second trimester and testosterone is markedly higher in males than in females during this window (Auyeung et al., 2013; Finegan et al., 1989; Hines, 2010). Therefore, steroidogenesis and placental hormone regulation during the mid-gestational period may be more dynamic than what we were able to capture by sampling at birth (van de Beek et al., 2004).

Androgens and estrogens play a critical role in organizing the brain during fetal development and these organizational effects may

contribute to the robust sex bias observed in ASD. Sex steroids are unique in their ability to program brain development both directly through receptor binding and indirectly through transcriptional and epigenetic regulation of DNA (Nugent and McCarthy, 2011). Prior to including maternal age as a covariate, we found an association between maternal testosterone and total SCQ scores in the overall sample and failed to detect an interaction effect of sex. On the other hand, cord blood testosterone appeared to have a much more robust association with SCQ scores in males. In this study, we cannot determine whether the sex-dependent relationship between cord blood testosterone and SCQ scores is due to increased male susceptibility to the effects of elevated in-utero androgen exposure or whether males have a smaller window of tolerance for variability in androgen exposure, perhaps because they endogenously produce higher levels of testosterone. Given that we did not observe a sex difference in cord blood testosterone levels, our results more strongly support the notion that males may be more susceptible to the neurodevelopmental effects of fetal exposure to androgens. Of course, it is also likely that other confounding variables were not fully accounted for in our analyses. These causal mechanisms should be further investigated, but our results suggest that elevated maternal testosterone in mid-gestation and higher cord blood testosterone at birth could be biological factors that contribute to the sex bias in ASD.

We performed several bivariate correlations both between and within maternal blood and cord blood hormone measures. Maternal testosterone was significantly correlated with cord blood testosterone, but only among males. There are at least two pathways that could explain the relationship between maternal and fetal testosterone levels. First, it is possible that maternal testosterone is, in fact, able to cross the placenta and reach the fetal circulatory system, especially in male fetuses. In light of our finding that placental aromatase transcription is not the primary predictor of cord blood testosterone, a possible second explanation for the correlation between maternal and cord blood testosterone is a heritable genetic predisposition for expression of CYP17A1, which encodes for the P450c17 cytochrome. The fetal zone of the adrenal cortex begins to express P450c17 by the 10th week of gestation and is responsible for the synthesis of dehydroepiandrosterone (DHEA) and DHEA-S from cholesterol and other androgen precursors (Kaludjerovic and Ward, 2012). The fetal adrenal gland also expresses the 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme that converts DHEA into androstenedione, the precursor for testosterone. Therefore, maternal circulating testosterone may be indicative of genetic regulation of androgen synthesis that is shared by both mother and fetus.

Several relationships between maternal and fetal hormones were found within our sample, highlighting the critical endocrine interplay ongoing with and between the mother and fetus. Maternal estradiol was positively correlated with cord blood estradiol, most likely due to the direct transfer of maternal estradiol across the placenta or due to estradiol synthesis by the placenta. Maternal estradiol was also correlated with cord blood testosterone. Since we cannot determine the direction of this association, two possible pathways can be considered. First, it is possible that a higher level of testosterone in cord blood is a reflection of elevated fetal adrenal activity, which could result in increased placental aromatization of testosterone into estrogen, thus leading to increased flow of estrogen into the maternal circulation. Second, signals of high maternal estradiol levels may trigger the fetus to down-regulate aromatase, leading to higher cord blood testosterone. We found a highly significant positive correlation between cord blood testosterone and estradiol. The conversion of radiolabeled testosterone to estrogen has been demonstrated in fetal ovaries, liver, and brain and placental aromatase is highly expressed by early to mid-gestation, so this relationship likely reflects fetal and/or placental aromatase activity (Doody and Carr, 1989). Interestingly, we did not find sex differences in testosterone or estradiol in the maternal blood or cord blood samples. Though the cause is not well-understood, there is significant inconsistency in the literature regarding sex differences in sex hormones measured in umbilical cord blood at birth (Hollier et al., 2014). Consistent with our finding, several prior studies have reported no sex difference in testosterone (Dawood and Saxena, 1977; McIntyre, 2006; Nabi et al., 2014; van de Beek et al., 2004). However, other reports indicate that cord blood testosterone levels are significantly higher in male neonates (Herruzo et al., 1993; Keelan et al., 2012; Maccoby et al., 1979). While some studies have reported higher cord blood estradiol levels in males (Simmons et al., 1994; van de Beek et al., 2004), the predominant finding is that there is not a sex difference in cord blood estradiol levels (Herruzo et al., 1993; Hill et al., 2014; Troisi et al., 2003a; Troisi et al., 2003b). All of the women in our study gave birth for the first time, an obstetric variable that is often not addressed in the literature. Though not well-studied, pregnancy-induced hormonal changes may result in long-term changes to the maternal hormonal milieu and may carry over to subsequent pregnancies (Blanchard, 2018; Maccoby et al., 1979; Panagiotopoulou et al., 1990).

4.1. Limitations and considerations

There are several important limitations to the present study. All of our measures of child neurobehavior were developed to detect subclinical behaviors and traits in children and this sample was not enriched for individuals with increased likelihood of receiving a diagnosis of ASD or other neurodevelopmental conditions. Therefore, the weak effects reported here may be more robust in a study population that includes participants with clinically-relevant diagnoses and behaviors. In addition, our outcome measures were dependent on parent reports of child behavior, which may not be as sensitive to atypical child behavior as clinician-reported observations. It is also possible that our outcome measures are subject to inherent sex biases that may capture "male-typical" neurobehavioral profiles rather than profiles of neurodevelopmental conditions (Evans et al., 2019). Finally, we acknowledge that important covariates, including experiences and environmental factors between birth and the time of assessment, may not have been measured as part of our study. This limitation, in particular, restricts our ability to draw conclusions of causality.

When interpreting results from the nuMoM2b study sample, it is important to note the significant time lapse between when the maternal blood samples were collected and when the cord blood and placenta samples were collected. Typically, maternal blood samples were collected between 19 and 24 weeks before the cord blood and placenta samples were collected. It is well documented that maternal hormones fluctuate and change dramatically over the course of pregnancy (Kuijper et al., 2013). By the second trimester, the fetus is also independently able to produce and secrete endogenous testosterone with peak production occurring between the 16th and 20th weeks of gestation (Abramovich and Rowe, 1973; Finegan et al., 1989; Reyes et al., 1973; Shigeo et al., 1977). The placenta also remains a source of endogenous estradiol throughout the pregnancy and aromatase activity in the placenta increases across gestation, reflected by a rise in relevant steroid hormone ratios (e.g. estradiol:testosterone, estrone:androstenedione) (Hill et al., 2010, 2014). Taken together, it is clear that the hormonal milieu during pregnancy is complex and highly-dependent on timing. Given that we compared maternal hormone levels during the second trimester to neonatal hormone levels at term-age, it is difficult to draw definitive conclusions about the role of the placenta in regulating fetal exposure to maternal hormones at a specific time point during pregnancy. Further, the small correlation coefficients likely reflect the influence of numerous prenatal and postnatal factors that we were unable to capture in this study and with the existing sample size.

It is also important to carefully consider the source of the hormone measurement. In this study, umbilical cord blood was taken from both the umbilical vein and the umbilical arteries. Numerous studies have collapsed across venous and arterial blood samples and strong correlations between steroid hormones, including estradiol and testosterone, collected separately from the umbilical vein and arteries have been demonstrated (Hill et al., 2014). However, higher levels of estrogens are

measured in blood from the umbilical vein compared to the umbilical arteries in Rhesus monkeys (Resko et al., 1975) and in humans (Laatikainen et al., 1982). Since cord blood was not sampled equally across the umbilical vein and arteries, it may have introduced bias, although it is unlikely to be systematic. Also, due to methodological restrictions, we were unable to control for diurnal fluctuations in maternal testosterone and estradiol, which have been shown to decrease from morning to evening (Panico et al., 1990).

In this study, we exclusively measured total testosterone and total estradiol. This is an important limitation because steroid hormones are only able to exert classical or genomic actions in the unconjugated form. In humans, sex hormone-binding globulin (SHBG), a plasma glycoprotein, binds to androgens and estrogens with very high affinity, acting as a transporter and thus, rendering the steroid hormone biologically inactive. SHBG levels are detectable in fetal blood and amniotic fluid as early as the 13th week of gestation and correlate closely with testosterone levels (Hammond, 2011). SHBG levels continue to increase during mid to late gestation across both sexes. It has been purported that SHBG during fetal development may protect females from exposure to high levels of testosterone. In rodents, alpha-fetoprotein (AFP) binds to estradiol and is believed to serve a similar protective function. The role of AFP in protecting females from high exposure to androgens has not been demonstrated in humans. Therefore, the function of SHBG may be redundant in non-human mammals, but it likely carries out critical neuroprotective effects in humans. The radioimmunoassay used in our study disregards the bioavailability of the measured testosterone and may have prevented us from detecting differences (e.g. between males and females) in the potential for steroid-mediated cellular and genomic changes and downstream effects.

Finally, we acknowledge that a large number of tests were performed, of which only a few yielded a statistically significant finding. While these significant findings were derived from planned analyses to evaluate our a priori hypotheses, are biologically plausible, and are in many cases consistent with the prior literature, we caution against over-interpretations of the results. Our findings warrant further replication and investigation as they have the potential to greatly contribute to our understanding of the etiology of ASD and related neurodevelopmental conditions.

4.2. Conclusions

The results of the current study suggest that elevated maternal testosterone during pregnancy in the context of reduced placental capacity to convert testosterone to estradiol is associated with child neurodevelopmental outcomes related to ASD. This study presents data from a large sample of demographically and medically diverse nulliparous women. Few studies have prospectively recruited nulliparous women, making this population underrepresented in the literature. The study sample is also unique demographically; maternal ethnicity is highly diverse and the average maternal age is significantly higher than that of many other study samples. Our findings both support and contrast previously reported relationships between demographic and health characteristics, maternal and infant hormonal profiles, and placental expression of sex steroid-related genes. Further investigation into placental regulation of fetal hormone exposure and the implications for long-term child neurodevelopmental outcomes will be critical to determining the pathways linking maternal and fetal endocrine function to psychiatric outcome.

Data availability

Restrictions apply to the availability of some or all data generated or analyzed during this study to preserve patient confidentiality or because they were used under license. The corresponding author will on request detail the restrictions and any conditions under which access to some data may be provided.

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