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Maternal salivary testosterone in pregnancy and fetal neuromaturation

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Abstract

Testosterone exposure during pregnancy has been hypothesized as a mechanism for sex differences in brain and behavioral development observed in the postnatal period. The current study documents the natural history of maternal salivary testosterone from 18 weeks gestation of pregnancy to 6 months postpartum, and investigates associations with fetal heart rate, motor activity, and their integration. Findings indicate maternal salivary testosterone increases with advancing gestation though no differences by fetal sex were detected. High intra-individual stability in prenatal testosterone levels extend into the postnatal period, particularly for pregnancies with male fetuses. With respect to fetal development, by 36 weeks gestation higher maternal prenatal salivary testosterone was significantly associated with faster fetal heart rate and less optimal somatic-cardiac integration. Measurement of testosterone in saliva is a useful tool for repeated-measures studies of hormonal concomitants of pregnancy. Moreover, higher maternal testosterone levels are associated with modest interference to fetal neurobehavioral development.

Keywords

prenatal development; testosterone; fetal heart rate; fetal movement; neuromaturation

Introduction

Investigation into the role of prenatal exposure to sex steroids on subsequent postnatal development flourished in the 1970's and 1980's following the seminal 'organizational hypothesis' proposed by Young and colleagues in 1959 suggesting essential function of steroid hormones in sexual differentiation (Phoenix, Goy, Gerall, & Young, 1959). At the time, there was a burgeoning interest in the potential biological origins of sex-typed behaviors (Maccoby, Doering, Jacklin, & Kramer, 1979; Quadagno, Briscoe, & Quadagno,

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1977; Reinisch, 1974). Recently, and notably marking the 50th anniversary of the organizational hypothesis (see *Hormones and Behavior*, Vol. 55), interest in how the maternal endocrine milieu may affect neural development of the human fetus and generate lasting influences on postnatal development has renewed focus on early sex steroid exposure. The prenatal period constitutes the most rapid phase of development as the architecture and neural scaffolding of the human brain is erected in the brief 266 days of human gestation and structural development occurs in tandem with functional gains. As such, fetal neural systems are particularly vulnerable to organizing influences of the intrauterine environment which includes maternal physiology (Andersen, 2003; Barker, 1998; Gluckman, Hanson, Cooper, & Thornburg, 2008; Peper & Koolschijn, 2012). Dramatic alterations in the maternal endocrine state serve to maintain the pregnancy and facilitate maturation of the fetal nervous system (Dorner, 2010), directly reaching the fetus via the placenta and indirectly effecting changes to placental function.

Quantification of prenatal exposure to sex steroids, including testosterone, presents methodological and interpretative challenges. Circulating fetal testosterone can only be directly measured in the human fetus by fetal blood sampling (Gitau, Adams, Fisk, & Glover, 2005), an invasive procedure done only with significant clinical indication. Another approach is through assay of testosterone in amniotic fluid (Finegan, Bartleman, & Wong, 1989); also a procedure conducted for diagnostic purposes and not without risk. Although the source of fetal testosterone in amniotic fluid is primarily from fetal urine, particularly in the second and third trimester, other sources include transplacental passage from maternal plasma in addition to transfer from fetal skin and lung liquid production (Sarkar, Bergman, Risk, O'Connor, & Glover, 2007; Underwood, Gilbert, & Sherman, 2005). At least one older study found no association between testosterone levels sampled from fetal plasma and amniotic fluid between 16 and 22 weeks gestation (Rodeck, Gill, Rosenberg, & Collins, 1985). Umbilical cord blood, primarily drawn from the umbilical vein at the time of delivery, has also been used as a downstream proxy for earlier fetal levels (Maccoby et al., 1979; Keelan et al., 2012; Whitehouse et al., 2012); although no association between amniotic fluid testosterone, measured between 15 and 23 weeks gestation, and umbilical cord testosterone has been found (van de Beek, Thijssen, Cohen-Kettenis, van Goozen, & Buitelaar, 2004). It is unclear whether this is the result of the intervening gestational interval, the stress of labor, or the relative mix of the maternal-fetal contribution to each source. Thus, there is little to establish whether either method provides accurate reflection of circulating fetal testosterone. Moreover, all three sources of assays (i.e., fetal and umbilical serum, and amniotic fluid) are further limited by an inability to sample more than once, thus allowing only cross-sectional analyses often conducted with fairly broad gestational tolerances.

As a result of the challenges inherent in efforts to more directly evaluate the amount of testosterone from the fetus, there are obvious advantages to measuring testosterone in the pregnant woman as an indicator of the pregnancy milieu and, perhaps, fetal exposure (Constantinescu & Hines, 2012). Maternal testosterone is converted to estradiol by aromatizing enzymes of the placenta (Ryan, 1959; Simpson & MacDonald, 1981) conferring an initial impression that the fetus was isolated from the effects of increasing maternal testosterone with advancing gestation. More recent evidence from animal studies indicates that exogenous testosterone, administered to the mother, crosses the placental barrier to

reach the fetal compartment (Veiga-Lopez, Steckler, Abbott, Welch, MohanKumar, Phillips, Refsal, & Padmanabhan, 2011). In human research, the feasibility of endogenous testosterone to operate similarly has been supported by significant correspondence of testosterone between contemporaneous maternal and fetal blood sampling ($r = .41$) in an analysis of 44 paired samples including both sexes (Gitau et al., 2005). This result was counter to an earlier report which found no association between testosterone levels in fetal and maternal plasma using a sample of comparable size ($n = 55$) (Rodeck et al., 1985). This disparity may be attributable to the time of sampling, which was considerably later in gestation in the more recent study. With respect to amniotic fluid, no significant associations were found between amniotic fluid and maternal serum testosterone in two studies when both were collected contemporaneously (Rodeck et al., 1985; van de Beek et al., 2004), including for total or unbound levels (van de Beek et al., 2004).

Despite the ambiguity in these findings, and the modest correspondence between testosterone in maternal and fetal serum, measurement of testosterone in maternal samples confers many methodological benefits. Maternal serum testosterone levels show high within-individual stability from the second to third trimesters (i.e., $r = .90$ for free testosterone; (van de Beek et al., 2004), $r = .75$ for total testosterone, (Carlsen, Jacobsen, & Romundstad, 2006)), thus demonstrating normal variation in the maternal neurohormonal milieu. Testosterone is also detectable in saliva, which alleviates the need for repeated maternal venipuncture. Saliva offers the additional advantage of being comprised primarily of the unbound, bioavailable component of testosterone (Groschl, 2008). Measurement of fluctuating maternal endocrines in saliva has become a common feature of longitudinal studies of human prenatal development and most frequently applied to cortisol (e.g., (Buss, Davis, Shahbaba, Pruessner, Head, & Sandman, 2012; DiPietro, Costigan, Kivlighan, Chen, & Laudenslager, 2011)). Although information on salivary testosterone in pregnancy is scant, a small study ($n = 28$) reports significant correspondence between testosterone measured in maternal blood and saliva ($r = .48$) in late gestation (Hampson, Phillips, Soares, & Steiner, 2013).

Male fetuses appear to be more vulnerable than female fetuses to prenatal exposures (DiPietro & Voegtline, 2017). Androgen exposure during the prenatal period is of interest as sex steroids target the fetal brain (McEwen, Davis, Parsons, & Pfaff, 1979). Specifically, it has been suggested that organizational influences of testosterone during prenatal development contribute to later sex differences in behavior (see Hines, 2008 for a review), and that testosterone may slow neural development (Morris, Jordan, & Breedlove, 2004), particularly for males (Martel, Klump, Nigg, Breedlove, & Sisk, 2009; Martel & Roberts, 2014). Animal research has provided a foundation for these theoretical suppositions. For example, male rodents implanted with testosterone on postnatal day 10, which may be similar to late term in human gestation (Dobbing & Sands, 1979; Clancy, Finlay, Darlington, & Anad, 2007), showed reduced neuronal development and density (King, Barkley, Delville, & Ferris, 2000). A multitude of additional animal studies demonstrate testosterone treatment induces functional brain changes (e.g., Roselli, Ellinwood, & Resko, 1984). As such, maturation of the fetal brain, and specifically autonomic nervous system, may mediate links between testosterone exposure and perinatal vulnerability.

No studies to date have evaluated the contemporaneous maternal testosterone environment in relation to neurobehavior of the developing fetus in situ. It is well accepted that expression and maturation of features of fetal function, principally involving patterns of heart rate and motor activity, and their interrelation, are reflective of the developing nervous system. This perspective has been established through data collected on fetuses of normally developing pregnancies, at risk pregnancies, those compromised by anomalous congenital conditions of the nervous system (Amiel-Tison, Gosselin, & Kurjak, 2006; DiPietro, Costigan, & Voegtline, 2015; DiPietro, Kivlighan, Costigan, Rubin, Shiffler, Henderson, & Pillion, 2010; Krasnegor, Fifer, Maulik, McNellis, Romero, & Smotherman, 1998; Morokuma, Fukushima, Otera, Yumoto, Tsukimori, Ochiai, Hara, & Wake, 2013; Nijhuis & ten Hof, 1999).

The goals of the current study are twofold. First, we seek to describe the trajectory of maternal salivary testosterone during the second half of gestation into the postpartum in a sample of healthy women with normal pregnancies. The second goal is to evaluate the correspondence between prenatal maternal testosterone and indicators of fetal neurodevelopment. We expect that higher maternal testosterone will be associated with indicators of reduced fetal neuromaturation, especially for male fetuses.

Material and Methods

Participants

Participants were 176 volunteer, non-smoking pregnant women with normally progressing, singleton pregnancies. Of these, 9 women enrolled but did not complete the prenatal protocol. Of the remaining 167, one fetus presented with an exclusionary congenital malformation (i.e., agenesis of the corpus callosum) and 8 delivered preterm, resulting in a final analytic sample of 158.

Accurate dating of the pregnancy was required for enrollment (M gestational age at pregnancy detection = 4.7 weeks; $SD = 1.6$). As seen in Table 1, women were well-educated, mature, and married; the majority was primiparous. The study was approved by the university's Institutional Review Board and women provided written consent.

Procedure

Participants were drawn from two prenatal cohorts (n s = 50 and 108). Both cohorts received the same fetal monitoring protocol described in this analysis at 24, 30, and 36 weeks gestation. Maternal testosterone was also collected from the larger cohort at 18 weeks gestation; fetal monitoring was not done at this time due to technical limitations in the ability to monitor the fetus continuously at this gestational age. To control for time of day and prandial effects, visits were scheduled at the same time of day (13:30) and women were instructed to eat 1.5 h prior to the start of data collection and not thereafter. Each visit began with a brief real-time ultrasound scan conducted to determine fetal position as a consideration in placement of the Doppler transducer and provide photographs to parents. Following the ultrasound, unstimulated whole saliva was collected via oral hydrocellulose swab. A 50 min undisturbed period of fetal monitoring followed during which women were lying in a semi-recumbent position. Fetal data were collected with a Toitu (MT320) fetal

actocardiograph utilizing a transabdominal Doppler transducer. Data were digitized and analyzed off-line (GESTATE; James Long Company, Caroga Lake NY).

Six months after birth, as part of a postnatal follow-up visit, unstimulated whole saliva was collected from the larger cohort of women via oral hydrocellulose swab. The sample was collected near the beginning of the visit which was typically scheduled at 10:00 (85% occurred between 10:00 and 12:00).

Measures

Maternal salivary testosterone—Oral swabs were stored frozen at -70°C until assay. Samples were assayed at the Center for Interdisciplinary Salivary Bioscience Research using commercially available enzyme immunoassays (Salimetrics, PA) without modification to the manufacturer's recommended protocols. The assay has a 25 μL test volume, calibrators that range from 6.1 to 600 pg/mL , and a lower limit of sensitivity of 1 pg/mL . Performance metrics for this protocol reveal that, on average, spike recovery is 104.3% and intra- and inter-assay coefficients of variation are 4.6 and 9.8%, respectively; in addition, the serum-saliva correlation in women is $r = .61$. All project samples were assayed in duplicate and the mean of duplicate testing was used in the statistical analysis.

Fetal neurodevelopment

Fetal heart rate: Fetal heart rate (FHR) underwent error rejection procedures based on moving averages of acceptable values as needed.

Fetal movement(FM): Fetal movement data represent raw voltage values generated from the actograph calibrated by multiplying by a conversion factor and scaling from 0 to 100 in arbitrary units (a.u.s). Movement bouts were identified each time the signal attained amplitude of 15 units until there was a cessation of signal for at least 10 seconds.

FM-FHR integration: The integration between fetal movement and fetal heart rate was characterized in two dimensions using previously developed criteria (DiPietro, Hodgson, Costigan, Hilton, & Johnson, 1996; DiPietro, Irizarry, Hawkins, Costigan, & Pressman, 2001; DiPietro, Caulfield, Irizarry, Chen, Merialdi, & Zavaleta, 2006). These are: the proportion of discrete fetal movement bouts that were associated with excursions in fetal heart rate ≥ 5 bpm over baseline within 5 s before the start of a movement or within 15 s after the start of a movement; and the latency, in seconds, between the start of the FM and the change in FHR. These characteristics were compiled into a single composite score, computed by taking the inverse of the latency component (i.e., shorter/faster latencies suggest greater integration), Z-scoring both since they rely on different metrics, and summing them.

Data analysis plan

Longitudinal analysis characterizing the trajectory of maternal prenatal salivary testosterone was conducted utilizing hierarchical linear modeling (HLM) (Raudenbush & Bryk, 2002; Singer, 1998) via the Mixed procedure in SAS Version 9.4. A HLM modeling approach is advantageous for longitudinal data analysis as it accounts for the dependency in repeated

measures data and supports an evaluation of trajectory will all available data, utilizing restricted maximum likelihood estimation (REML) to handle missing data. Comparisons of the rate of change in maternal testosterone levels from sequential gestational intervals were evaluated by paired *t*-tests utilizing individual-specific growth parameters generated by a SAS macro procedure.

Of the total analytic sample of 158 pregnant women, the majority of women provided salivary testosterone data at all of the latter three prenatal visits common to both cohorts (24, 30 and 36 weeks gestation, $n = 137$; 86.7%). Missing data due to an incomplete visit or insufficient sample volume resulted in the loss of 10 participants at 24 weeks (6.3%), 6 participants at 30 weeks (3.8%), and 7 participants at 36 weeks (4.4%). Exclusive to the second cohort of 108 pregnant women, 80 participants had 18 week prenatal salivary testosterone data at 18 weeks (18.5% or $n = 20$ missing) and 73 participants had 6 month postnatal salivary testosterone data (32.4% or $n = 35$ missing). Examination of salivary testosterone distributions at each assessment period revealed one top-bound testosterone outlier at 24 weeks gestation. The outlier value was winsorized to three standard deviations above the mean testosterone level, specific to distribution within the 24 week gestational period.

Differences in maternal testosterone level by fetal sex and parity were examined at each gestational period and in the slope from 18 to 36 weeks gestation. Levine's test for variance was employed to test increase in individual variability in testosterone levels by gestational period. Stability of maternal testosterone levels across gestational periods and at 6 months post-term was examined by Pearson correlation coefficient.

Associations of maternal prenatal salivary testosterone and fetal neurobehavior from 24 to 36 weeks gestation were examined in a cross-sectional approach at each gestational period. Separate models were constructed for outcomes of fetal heart rate, fetal movement, and FM-FHR integration and confidence intervals were evaluated in determination of association strength.

Fetal heart rate data, $n = 151$ at 24 weeks, $n = 153$ at 30 weeks, and $n = 151$ at 36 weeks, were excluded for 2 cases with congenital septal defects and inadequate signal quality or transient arrhythmia resulted in the additional loss of heart rate data at 24 ($n = 2$), 30 ($n = 1$), and 36 weeks ($n = 2$). Fetal movement data were excluded for $n = 1$ at 24 and 36 weeks gestation due to poor signal quality. Exclusions applied to both fetal heart rate and movement resulted in an analytic $n = 147$ at 24 weeks, $n = 150$ at 30 weeks, and $n = 144$ at 36 weeks gestation for the somatic-cardiac integration measure.

To further probe the relation of maternal prenatal salivary testosterone and fetal neuromaturation a median split was performed on the FM-FHR integration composite at 36 weeks gestation and pairwise *t*-tests within a general linear model were used to compare maternal prenatal salivary testosterone levels among fetuses presenting with varying degrees of neurological maturity. Sex differences were examined in all maternal testosterone-fetal neurobehavior associations.

Results

Developmental trends of maternal salivary testosterone during pregnancy

Figure 1 depicts a significant increase in maternal salivary testosterone levels in the prenatal period commencing at 18 weeks gestation, $\beta = 13.90$, $SE = 0.54$, $t = 25.62$, $p < .001$; mean values are presented in Table 2. There was more pronounced gain in maternal salivary testosterone from 30 to 36 weeks gestation as compared to the prior gestational interval, $t = 43.18$, $p < .001$. On average, maternal salivary testosterone levels increased 41% in the final gestational interval from 30 to 36 weeks gestation (see Table 2).

Stability in maternal salivary testosterone during the prenatal and early postnatal periods

As evident in the Figure 1 scatter of individual data points, the degree of variability in maternal salivary testosterone within each gestational period (SD range = 14.6 to 21.7 pg/mL) was similar from 18 to 30 weeks gestation, F s range from 1.06 to 1.17, $ps = .41$ to .74. However, by 36 weeks gestation variability of maternal salivary testosterone levels was significantly greater than prior gestational periods, F s range from 1.87 to 2.19, $ps < .001$.

Intercorrelations across gestational periods (see Table 3) revealed relatively high stability in maternal salivary testosterone in adjacent periods and across the full span from 18 to 36 weeks gestation, $r(84) = .62$, $p < .01$.

Postnatal maternal salivary testosterone sampled when infants were 6 months of age was substantially lower than the latter three prenatal values, yet comparable to that observed at the first prenatal assessment of 18 weeks gestation (see Figure 1 and Table 2). To evaluate prenatal to postnatal consistency, gestation-specific prenatal to postnatal 6 month correlations are shown in Table 3. Pre to postnatal associations in maternal salivary testosterone reveal a significant positive association between 18 week prenatal and postnatal testosterone levels, $r(60) = .36$, $p < .01$ and a trend association between 24 week prenatal and postnatal testosterone levels, $r(67) = .24$, $p = .05$. The pattern of reduction in the strength of the association (see Table 3) continued with advancing gestation; associations of 30 and 36 week prenatal and postnatal testosterone were non-significant.

Moderation by fetal sex and maternal parity

As shown in Table 2, maternal salivary testosterone concentrations did not differ by fetal sex at any gestational age. Fetal sex did not affect the overall trajectory of testosterone increase from 18 to 36 weeks, $\beta = 0.76$, $SE = 1.10$, $t = 0.70$, $p = .49$ nor incremental gains between adjacent gestational periods, ps range = .26 to .75. In the postnatal period, maternal testosterone levels also did not differ by sex $t(71) = 0.50$, $p = .62$.

An evaluation of sex differences in the stability of maternal salivary testosterone, via associations stratified by sex, revealed similarly high stability of prenatal salivary testosterone for both women carrying female and male fetuses (rs female = .56 to .76, rs male = .57 to .79). However, the association of prenatal to postnatal testosterone was significant for women with male fetuses at 18 and 24 weeks gestation (18 weeks: $r(32) = .$

51, $p < .01$, 24 weeks: $r(38) = .38$, $p < .05$, 30 weeks), and non-significant for women with female fetuses (18 weeks: $r(28) = .12$, $p = .55$, 24 weeks: $r(29) = .04$, $p = .84$).

Maternal parity was unrelated to maternal salivary testosterone concentrations, except for a trend for women with first pregnancies ($n = 89$) to have higher levels at 36 weeks gestation as compared to women who had previously given birth ($n = 69$) (M primiparous = 88.6 pg/mL, M multiparous = 82.7 pg/mL; $\beta = -5.90$, $SE = 3.48$, $t = -1.69$, $p = .09$). Among multiparous women, there was no association between a greater number of prior births and prenatal testosterone level at 36 weeks gestation, $p = .14$. No parity differences were found earlier in gestation, $ps = .37$ to $.64$. Maternal parity did not affect the overall trajectory of testosterone increase from 18 to 36 weeks, $\beta = 1.51$, $SE = 1.10$, $t = 1.37$, $p = .17$, nor were there parity differences in the incremental gains between adjacent gestational periods, ps range = $.20$ to $.70$.

Parity differences in the stability of maternal salivary testosterone revealed no differences in stability among maternal salivary testosterone across prenatal periods (rs primiparous = $.60$ to $.78$, rs multiparous = $.47$ to $.71$), and in continuity from the prenatal to postnatal periods (rs primiparous = $.14$ to $.41$, rs multiparous = $.16$ to $.33$).

Maternal prenatal salivary testosterone associations with fetal neurobehaviors

To examine associations of maternal prenatal salivary testosterone and fetal neurobehavior, analyses were restricted to the latter three gestational periods in which fetal data was collected.

Fetal heart rate—As expected, fetal heart rate declined over gestational periods, $\beta = -3.13$, $SE = 0.27$, $t = -11.66$, $p < .001$, with a greater rate of change observed for male fetuses relative to female fetuses, $\beta = -1.17$, $SE = 0.53$, $t = -2.20$, $p < .05$ such that females had higher heart rate by 36 weeks gestation. As depicted in Figure 2a which shows correlation estimates with confidence intervals, fetal heart rate was unrelated to maternal salivary testosterone at 24, $r(144) = .02$, $p = .81$, and 30 weeks, $r(149) = .04$, $p = .64$, for the full sample. At 36 weeks, higher maternal testosterone was associated with faster FHR, $r(147) = .17$, $p < .05$. To explore the contribution of sex to observed associations, in a second step, general linear models were constructed to evaluate the prediction of fetal heart rate by concurrent maternal salivary testosterone, fetal sex, and the interaction of maternal testosterone and sex at each gestational period. No main or interactive sex effects were detected, ps range = $.12$ to $.72$.

Fetal movement bouts—Fetal movement bouts also declined over gestational periods, $\beta = -4.68$, $SE = 0.73$, $t = -6.39$, $p < .001$, with no difference in the rate of change by fetal sex, $p = .31$. Figure 2b presents associations between maternal salivary testosterone and the number of fetal movement bouts; there was a trend association at 36 weeks gestation such that higher maternal testosterone was related to more fetal movement bouts, $r(148) = .15$, $p = .07$; no associations were observed earlier in gestation. Subsequent general linear models examining the contribution of sex revealed no effects, ps range = $.14$ to $.96$.

Fetal somatic-cardiac integration—The individual components of the somatic-cardiac integration variable displayed changes over gestation such that coupling increased, $\beta = 0.06$, $SE = 0.01$, $t = 10.36$, $p < .001$, and latency quickened (i.e., declined), $\beta = -0.64$, $SE = 0.10$, $t = -6.43$, $p < .001$, confirming expectations. FM-FHR integration was significantly and negatively associated with maternal salivary testosterone at 36 weeks gestation, $r(144) = -.19$, $p < .05$ (see Figure 3). Associations at 24 weeks, $r(144) = -.10$, $p = .21$, and 30 weeks, $r(149) = -.13$, $p = .11$, were non-significant. No sex effects were detected in follow up general linear models predicting the somatic-cardiac integration composite, ps range = .31 to .97.

Figure 4 presents post hoc evaluation probing the detected association between maternal salivary testosterone and the somatic-cardiac integration composite at 36 weeks gestation using median splits on the individual components (i.e., coupling and latency) to create clusters of fetal integration. As shown, in a general linear model including sex as a covariate, a significant difference in maternal testosterone levels was found between the group of fetuses with the most optimal integration (i.e., high coupling/fast latency, $n = 39$) versus those with the poorest integration (i.e., low coupling, slow latency, $n = 39$), $t = -2.58$, $p < .05$. Groups of mixed optimal/non-optimal integration patterns did not differentiate, ps range = .12 to .66.

Discussion

Results presented here are the first to show the natural history of maternal salivary testosterone spanning from 18 weeks gestation in the prenatal period to 6 months postpartum in a prospective longitudinal design; we are unaware of a similar investigation. Maternal salivary testosterone, indexing the free concentrations of biologically active testosterone, significantly increased across the second half of gestation. On average, levels increased 94% from 18 to 36 weeks gestation, nearly doubling across the 16 week gestational span. The greatest increase (42%) in maternal salivary testosterone was observed in the final gestational interval, from 30 to 36 weeks gestation approaching term. Earlier in pregnancy stepwise increases of 20% and 14% were observed between 18 to 24 weeks and 24 to 30 weeks, respectively. One prior study measured salivary testosterone in human pregnancy in 28 women on a single occasion between 32 and 38 weeks gestation and in a second group of 43 women in the first 6 months postpartum (Hampson et al., 2013). Prenatal salivary testosterone levels, though reported in a different metric, are comparable to that observed at the latter two gestational periods in the current study, as is the finding that testosterone peaked in the last month of pregnancy. Postpartum maternal salivary testosterone in the current study declined from prenatal values, consistent with another report (Hampson et al., 2013), and by 6 months postpartum salivary testosterone levels were commensurate with those in the 18th week of pregnancy. This aligns with an older report that the free testosterone level in maternal serum remained within range of non-pregnant women until 28 weeks gestation (Bammann, Coulam, & Jiang, 1980).

Maternal salivary testosterone levels at each gestational period did not differ by fetal sex, consistent with reports based on maternal serum testosterone in pregnancy (Carlsen et al., 2006; Tuutti, Hamalainen, Sainio, Hiilesmaa, Turpeinen, Alfthan & Stenman, 2011; van de

Beek et al., 2004). There was also no difference in postpartum maternal salivary testosterone for women who gave birth to boys versus girls. The consistent lack of sex difference in maternal concentrations indicates that in the maternal compartment, heterogeneity in testosterone levels is greater within than between fetal sexes. It may also indicate that in the prenatal period, there is a greater influence of upregulated maternal adrenal output and peripheral conversion of androgens in calibrating maternal salivary testosterone levels in comparison to any source of fetal contribution. The single detected association with fetal sex was the relatively strong association between prenatal, particularly at the earliest gestational age measured, and postnatal levels of maternal salivary testosterone in women who carried male fetuses and the absence of comparable levels of pre-post stability for female pregnancies. This may be on account of a differential hormone adjustment following birth or varying biochemistry associated with mothering behavior of a male versus a female infant.

Although there were striking changes in mean maternal testosterone concentrations for the sample during the second half of pregnancy, associations across the four prenatal assessments indicates that rank ordering of salivary testosterone levels is relatively stable within individual women. This finding corroborates prior study based on serum testosterone (van de Beek et al., 2004) although correlations coefficients detected in the current study were somewhat lower in magnitude. Given this, maternal testosterone should be considered as an individual difference characteristic of the maternal pregnancy milieu with the potential to shape fetal development.

Results of the current study support modest association between individual variation in maternal testosterone levels and fetal neurobehavioral development, but these were constrained to the final gestational period. Higher maternal salivary testosterone at 36 weeks gestation was significantly associated with faster fetal heart rate and there was a trend for a greater number of fetal movement bouts. Prior work has shown testosterone binds optimally to receptors in the limbic cortices and amygdala (Van Wingen, Ossewaarde, Backstrom, Hermans, & Fernandez, 2011) as well as the hypothalamus (Fernandez-Guasti, Kruijver, Fodor, & Swaab, 2000), which drives initiation of autonomic responses (Critchley, 2005), and as such may exert resultant impact on fetal cardiac parameters. It is plausible that the somatic nervous system and motor cortex associated with both reflexive and volitional fetal movements is a less sensitive target for testosterone exposure.

Higher maternal salivary testosterone was also associated with decreased somatic-cardiac integration at 36 weeks gestation measured both along a continuous dimension and also categorically, by comparing fetuses exhibiting high coupling and fast latencies to those with low coupling and slow latencies. Gains in somatic-cardiac integration have previously been shown to be valid indicators of maturation of the fetal nervous system (DiPietro et al., 1996; DiPietro et al., 2001; DiPietro et al., 2006). Greater integration of fetal heart rate and motor activity has been previously shown to be predictive of state regulation, including decreased irritability, increased alertness and orientation, and better regulatory capacity (DiPietro, Costigan, & Pressman, 2002), as well as, shorter neural conduction times as assessed by brainstem auditory evoked potentials in the neonatal period (DiPietro et al., 2010). To a degree, this supports empirical work in animal models demonstrating neural changes with exposure of the developing brain to testosterone (McCarthy, Arnold, Ball, Blaustein, & de

Vries, 2012) and theoretical impressions that high prenatal testosterone exposure slows neural maturation in humans (Martel et al., 2009; Morris et al., 2004).

The failure to detect associations between maternal salivary testosterone and fetal neurobehaviors earlier in gestation may reflect either the nature of the developmental trajectory of these measures or the underlying physiology of the influence of maternal testosterone on the fetal compartment, or both. Fetal heart rate and FM-FHR integration develop in predictable ways during prenatal development and continue to mature throughout; stability in the latter is not typically observed until later in gestation (DiPietro et al., 2015). Since FHR declines over time and FM-FHR integration increases, the observation that higher levels of testosterone are associated with faster heart rate and lower FM-FHR integration suggests a cumulative influence over time that perhaps is not fully expressed until the end of gestation. Near term, maternal bioavailable testosterone levels have peaked and increased individual variation in levels are observed among pregnant women; this, in combination with the biological plausibility of greater rate of placental transfer to the fetus may contribute to associations constrained to 36 weeks gestation.

The current findings provide preliminary empirical support that maternal testosterone is associated with interference of fetal neuromaturation development in late gestation. The modest size of these detected associations may be attributable to fact that measurement of maternal testosterone, whether in serum or saliva, provides only a proxy for actual fetal exposure which is directly measurable only within clinically indicated procedures that are infrequently administered and confer risk. The assumption that these are suitable proxies is bolstered by the significant associations found between circulating maternal and fetal serum (Gitau et al., 2005) as well as maternal and umbilical cord serum (van de Beek et al., 2004). Further, the free concentration (Johnson, Joplin, & Burrin, 1987) indexes the proportion of testosterone bioavailable to act on target fetal brain tissues. Thus, the non-invasiveness nature of saliva collection procedures makes salivary assay of maternal testosterone an appealing measurement tool as an analogue to serum, particularly for studies of human gestation that benefit from repeated measurement (Voegtline & Granger, 2015). The use of repeated measurements in this study allowed documentation of normative trends and intra-individual stability. More importantly, conclusions regarding prenatal to postnatal stability and associations with fetal neurodevelopment would have been different without sampling a broad gestational span as the most compelling findings for the former were detected at 18 weeks and the only findings for the latter at 36 weeks.

There are limitations of the current study to be addressed. First, maternal salivary testosterone was the sole sex steroid assayed in our salivary data. Given normative increases in other sex steroids, including but not limited to progesterone and estradiol, we may expect co-action of unmeasured sex steroids. Future studies would benefit from concurrent measurement of multiple endogenous sex steroids in pregnancy, which would be feasible with both saliva or serum collection. This would support an examination of the relative impact of maternal testosterone, specifically, versus other sex steroids on fetal neurodevelopment. Second, replicability of these findings is necessary given the relatively modest effects constrained to one gestational period. In replication designs, it would be of interest to not only examine if similar associations were detected, but also to determine if

associations would be stronger in magnitude in a sample of women with known excess testosterone such as cases of polycystic ovarian syndrome or congenital adrenal hyperplasia. This would provide stronger empirical support of the associations detected in the current study.

Conclusions

Animal models have provided evidence that sex steroids, including testosterone, provide an organizing effect on the developing fetal brain (McCarthy et al., 2009). Comparable research in human development has been relatively stymied by inaccessibility of the human fetus and associated methodological limitations in assessing fetal testosterone exposure. The current study provides support for the use of maternal testosterone in saliva as a proxy for fetal exposure. In a prospective longitudinal design, these data document normative upregulation of maternal salivary testosterone in the second half of gestation and ensuing postpartum decline. The consistency demonstrated in maternal salivary testosterone levels within women with healthy pregnancies provides support for persistent variation in fetal exposure. Findings also provide modest support that testosterone measured in maternal saliva may exert an inhibitory influence on fetal neuromaturation. The effect of prenatal sex steroids on non-sexual aspects of the developing fetal brain with potential ramifications for human postnatal development is a promising but under-investigated area of inquiry.

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Figure 1.

Maternal salivary testosterone. Lowess curve estimates depict significantly increasing testosterone in the second half of gestation, with more pronounced gain from 30 to 36 weeks gestation. Postnatal levels fall to approximate observed at 18 weeks gestation. Scatter points represent individual data at each gestational period.

Figure 2.

a) Pearson correlation coefficient and 95% confidence interval from Fisher's r-to-z estimating the relationship between maternal salivary testosterone and fetal heart rate by gestational period. At 36 weeks gestation, higher maternal salivary testosterone is significantly associated with higher fetal heart rate. b) Pearson correlation coefficient and 95% confidence interval from Fisher's r-to-z estimating the relationship between maternal salivary testosterone and number of fetal movements by gestational period. At 36 weeks gestation, a trend association was detected between higher maternal salivary testosterone and a greater number of fetal movements.

Figure 3.

Pearson correlation coefficient and 95% confidence interval from Fisher's r-to-z estimating the relationship between maternal salivary testosterone and fetal movement-fetal heart rate coupling and latency by gestational period. At 36 weeks gestation, higher maternal salivary testosterone is significantly associated with less integrated FM-FHR coupling/latency.

Figure 4.

A median split of coupling and latency components of fetal somatic-cardiac integration at 36 weeks gestation yields four maturation groups. Fetuses in the most mature group (high coupling/fast latency) had mothers with the significantly lower testosterone levels relative to fetuses in the most immature group (low coupling/slow latency).

Table 1Sample characteristics, $N = 158$

Characteristic	Mean (SD)	N (%)
Maternal age, y	32.1 (4.3)	
Education, y	17.1 (1.8)	
Race		
Non-Hispanic white		118 (74.7)
African-American		13 (8.2)
Asian		27 (17.1)
Nulliparous		89 (56.3)
Infant sex, male		89 (56.3)
Gestational age at delivery (wks)	39.5 (1.1)	
Apgar, 5 min	8.9 (0.5)	

Table 2

Mean maternal salivary testosterone level (pg/mL) in prenatal and early postnatal periods by fetal sex

Gestational period	All ^a	Male	Female	<i>t</i> value ^b
18 weeks	44.41 (14.6)	44.95 (14.5)	43.76 (15.0)	0.38
24 weeks	53.12 (15.4)	53.96 (16.3)	51.98 (14.2)	0.77
30 weeks	60.56 (15.9)	60.99 (16.2)	60.01 (15.6)	0.38
36 weeks	86.02 (21.7)	87.66 (21.4)	84.02 (22.1)	1.02
<hr/>				
Postnatal period				
6 months	43.29 (15.5)	42.45 (17.2)	44.30 (13.3)	-0.50

^aNote. *N*s vary by gestational period: 18 weeks *n* = 88, 24 weeks *n* = 148, 30 weeks *n* = 152, 36 weeks *n* = 151, 6 months *n* = 73.

^bTest of group difference in maternal salivary testosterone by fetal sex; no differences detected.

Table 3

Interassociations of maternal salivary testosterone level (pg/mL) across prenatal and early postnatal periods

	1	2	3	4
1. 18 weeks gestation	--			
2. 24 weeks gestation	0.76***	--		
3. 30 weeks gestation	0.74***	0.69***	--	
4. 36 weeks gestation	0.62***	0.56***	0.71***	--
5. 6 months postnatal	0.36**	0.24†	0.19	0.16

Note. *N*s vary by pairwise comparison, range = 60 to 145.***
p < .001,**
p < .01,†
p = .05