Running Head: UMBILICAL CORD SEX STEROIDS AND DIGIT RATIO

Adult Digit Ratio (2D:4D) is Not Related to Umbilical Cord Androgen or Estrogen Concentrations, their Ratios or Net Bioactivity

Lauren P. Hollier[1,2], Jeffrey A. Keelan[3], Esha S.L. Jamnadass[1,2], Murray T. Maybery[2], Martha Hickey[4*], and Andrew J.O. Whitehouse[1*]

[1]Telethon Kids Institute, University of Western Australia

[2]Neurocognitive Development Unit, School of Psychology, University of Western Australia

[3]School of Women's and Infant's Health, University of Western Australia

[4] Department of Obstetrics and Gynaecology, University of Melbourne and the Royal

Women's Hospital, Victoria Australia

* Joint senior authors

Correspondence to:

Lauren Hollier

School of Psychology

University of Western Australia

35 Stirling Highway, Crawley

Western Australia, 6009

Phone: + 61 8 6488 1300

Fax : + 61 8 6488 1006

Email: lauren.hollier@graduate.uwa.edu.au

Abstract

Background

Ratio of second digit length to fourth digit length (2D:4D) has been extensively used in human and experimental research as a marker of fetal sex steroid exposure. However, very few human studies have measured the direct relationship between fetal androgen or estrogen concentrations and digit ratio.

Aims

We investigated the relationships between both androgen and estrogen concentrations in umbilical cord blood and digit ratio in young adulthood. In addition we calculated measures of total serum androgen and total estrogen bioactivity and investigated their relationship to digit ratio.

Study Design

Prospective cohort study.

Subjects

An unselected subset of the Western Australian Pregnancy Cohort (Raine) study (159 female; 182 male).

Outcome Measures

Cord serum samples were collected immediately after delivery. Samples were assayed for androgen (testosterone, Δ4-androstenedione, dehydroepiandrosterone) and estrogen (estrone, estradiol, estriol, estetrol) concentrations using liquid-chromatography mass-spectrometry. Digit ratio measurements were taken from hand photocopies at age 19-22 years.

Results

For both males and females, there were no significant correlations between digit ratio and any androgen or estrogen concentrations considered individually, the testosterone to estradiol ratio, total androgen bioactivity measure or ratio of androgen to estrogen bioactivity (all p

> .05). In males, but not females, total estrogen bioactivity was negatively correlated with left hand digit ratio (r = -.172, p = .02), but this relationship was no longer significant when adjusted for variables known to affect sex steroid concentrations in cord blood.

Conclusions

Our findings indicate that digit ratio is not related to fetal androgens or estrogens at late gestation.

Key words: androgens, estrogens, digit ratio, 2D:4D, cord blood, Raine study

Introduction

Prenatal exposure to sex steroids has long been posited to influence human development (Collaer & Hines, 1995; Finegan, Bartleman, & Wong, 1989). However, the direct examination of sex steroid exposure has been limited due to inherent difficulties in obtaining biological samples during prenatal life. Currently there is no 'gold standard' approach to the measurement of fetal sex steroid concentrations (van de Beek, Thijssen, Cohen-Kettenis, van Goozen, & Buitelaar, 2004). Obtaining samples of circulating human fetal sex steroids during early and mid gestation would require invasive fetal sampling (such as cordocentesis), which confers significant risks to the pregnancy. Amniotic fluid samples provide an approximation of circulating fetal hormones in mid-gestation by measuring the sex steroids that have entered the amniotic fluid via fetal urination or diffusion through fetal skin (Nagami, McDonough, Ellegood, & Mahesh, 1979). A significant limitation of this approach is that amniocenteses are performed only in high-risk pregnancies and therefore research samples are typically small and unlikely to be representative of the broader population. Alternatively, umbilical cord blood reflects fetal sex steroid concentrations at late gestation and can be easily collected from uncomplicated pregnancies following delivery. Studies have demonstrated consistent sex-differences in sex steroid concentrations in cord blood (Herruzo et al., 1993; Keelan et al., 2012; Maccoby, Doering, Jacklin, & Kraemer, 1979; Troisi, Potischman, Roberts, Harger, et al., 2003), suggesting that this approach can be used to examine the relationship between early-life sex steroid exposure and human development.

Previous studies by our group and others have demonstrated significant associations between umbilical cord testosterone concentrations and human development (Hollier, Keelan, Hickey, Maybery, & Whitehouse, 2014). Higher concentrations of cord serum testosterone were associated with reduced vocabulary in males at 2 and 5 years of age (Farrant, Mattes,

Keelan, Hickey, & Whitehouse, 2013; Hollier et al., 2013), increased risk of language delay in early childhood (Whitehouse et al., 2012), reduced spatial ability in 6 year old females (Jacklin, Wilcox, & Maccoby, 1988), and left hemisphere lateralisation of language in adult males (Hollier, Maybery, Keelan, Hickey, & Whitehouse, 2014). Sex differences in behaviour have also been associated with cord testosterone concentrations. Jacklin et al. (1983) reported that males with low cord serum testosterone concentrations were more timid. We have recently reported that higher cord testosterone concentrations are associated with behavioural problems in early childhood. Attention difficulties in males and withdrawal symptoms in females were both negatively related to cord blood testosterone levels (Robinson et al., 2013). Together, these data suggest that umbilical cord blood is a valid method for examining early-life sex steroid exposure.

Characteristically, males have a longer fourth digit relative to their second digit, while women have comparable second and fourth digit lengths (Manning, Stewart, Bundred, & Trivers, 2004). A recent meta-analysis illustrated that although the sex difference is small, it is consistent across the published literature (Hönekopp & Watson, 2010). Differences in digit ratios compared to controls have been partially supported in clinical populations exposed to atypical levels of prenatal hormones. There is some evidence individuals with congenital adrenal hyperplasia (CAH), where the fetus is exposed to supraphysiological levels of androgens, have a lower 2D:4D ratio (male pattern) than typically developing individuals (Brown, Hines, Fane, & Breedlove, 2002; Ökten, Kalyoncu, & Yaris, 2002). However, the relationship was not consistently observed in both hands. In addition, a study conducted by Buck, Williams, Hughes and Acerini (2003) using radiographs found no significant difference in digit ratio between females with CAH and typically developing females. These findings cast doubt as to whether the 2D:4D ratio is a reliable proxy measure of fetal testosterone levels (Putz, Gaulin, Sporter, & McBurney, 2004).

To date, only three relatively small prospective studies have investigated the relationship between fetal sex steroid exposure and digit ratio. Lutchmaya et al. (2004) examined the association between testosterone and estradiol levels in amniotic fluid collected mid-gestation with digit ratio recorded at 2 years of age in 33 children (18 males, 15 females). A low 2D:4D ratio in the right hand was associated with high testosterone relative to estradiol levels. No significant relationship was found between digit ratio and testosterone or estradiol concentrations individually. These observations suggest that the 2D:4D ratio reflects the relative levels of prenatal androgens and estrogens.

Ventura et al. (2013) further investigated the relationship between amniotic testosterone concentrations sampled during mid-gestation and digit ratio measured at birth in a sample of 106 children (54 females, 52 males). For females, but not males, amniotic testosterone levels were negatively related to the digit ratio of both hands. This finding provides further evidence that digit ratio may be related to sex steroid concentrations *in utero*. However, Ventura and colleagues did not measure estrogen levels, so it was not possible to examine whether the ratio of testosterone to estradiol levels was related to the digit ratio.

Hickey et al. (2010) provided an initial investigation into the relationship between cord testosterone concentrations and the 2D:4D ratio in a subset of females from the Western Australian Pregnancy Cohort (Raine) Study. No statistically significant relationship was found between umbilical cord blood testosterone concentrations and the 2D:4D ratio recorded for the females at 14 to 16 years of age (n = 82) or between maternal testosterone concentrations at 18 (n = 118) or 34 weeks (n = 114) of gestation and digit ratio. These findings suggest that variations in 2D:4D in females are not related to fetal testosterone concentrations late in gestation. However, the findings from Lutchmaya et al. (2004) suggest that it may be the ratio of androgen to estrogen concentrations that is related to digit ratio. Furthermore, both Hickey et al. (2010) and Lutchmaya et al. (2004) utilised

radioimmunoassay (RIA) to analyse sex steroid concentrations. Increasing awareness of the limitations of RIA for the measurement of umbilical cord sex steroids has led to the adoption of mass spectrometry as the preferred approach (Keelan et al., 2012). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) can be more sensitive than RIA (Stanczyk & Clarke, 2010). Further, sex steroid measurements with LC-MS/MS are consistently lower than those derived by RIA, reflecting the superior specificity of the LC-MS/MS technique (Demers, 2010; Hollier, Keelan, et al., 2014; Krogh et al., 2011; Soldin et al., 2005; Stanczyk & Clarke, 2010; Vicente, Smith, Sierra, & Wang, 2006). Although, Ventura et al. (2013) utilised LC-MS/MS to measure testosterone concentrations amniotic fluid, they examined only the relationship between testosterone and digit ratio.

Present Study

The aim of the current study was to investigate whether androgen and estrogen concentrations in cord blood, measured using LC-MS/MS, are related to digit ratios recorded in early adulthood for samples of both males and females from the Raine study. Most published studies of fetal sex steroid exposure have measured only the most biologically active sex steroids: testosterone, and estradiol, both of which are bound to sex hormone binding globulin (SHBG) which greatly attenuates their bioactivity. However, the human fetus is exposed to a number of other androgens and estrogens in the prenatal environment, some of which are not bound by SHBG and so exert significant bioactivity despite a relative lack of potency. Accordingly, in the present study we measured the adrenal androgens, Δ4-androstenedione and dehydroepiandrosterone, as well as estrone, estriol and estetrol. Using these data, combined with data on protein binding and relative potency, we derived total composite measures of bioavailable androgen and estrogen exposure. We were then able to test whether these variables or their ratio correlated with the 2D:4D ratios.

We predicted that testosterone concentrations and the testosterone to estradiol ratio would be negatively related to digit ratio, while estradiol levels would be positively related. Similarly, it was predicted that the androgen composite and the androgen to estrogen composite ratio would be negatively associated with digit ratio, while the estrogen composite would be positively related.

Method

Participants

Participants were from the Western Australian Pregnancy Cohort (Raine) Study (www.rainestudy.org.au). Between May 1989 and November 1991, 2900 unselected pregnant women were recruited from the public antenatal clinic at King Edward Memorial Hospital in Perth, Western Australia, to study the effects of repeated ultrasound on fetal and postnatal growth, development and pregnancy outcomes. Among the 2834 women with singleton pregnancies, 1415 were randomised to the intensive ultrasound arm of the study, which included umbilical cord blood sampling, and formed the population for the present analysis. Immediately after delivery, mixed umbilical arterial-venous (UA:UV) cord blood was collected, allowed to clot and serum was frozen at -80C and stored without thawing until the initial investigation by Keelan et al., (2012). Eight hundred and sixty blood samples had sufficient serum for steroid analysis. Of these samples, there were 820 participants with complete androgen data (400 female; 420 male), and 853 participants with complete estrogen data (425 female; 428 male). Among these participants, 341 (159 female; 182 male) had digit ratio measured between 19 and 22 years of age.

Steroid Analysis

Cord serum samples were thawed, aliquotted and shipped from Perth, Western

Australia to Adelaide, South Australia for LC-MS/MS analysis (CPR Pharma Services Pty Ltd, Thebarton, SA); in total, samples were thawed and frozen less than three times following collection. Ten randomly selected cord blood samples confirmed the absence of detectable maternal contamination (Whitehouse et al., 2012). Assay performance was determined to be unaffected by up to three freeze-thaw cycles or 24 h at room temperature. Steroid analysis was performed blind to sample identity or characteristics.

Total testosterone (TT), Δ4-androstenedione (A4), and dehydroepiandrosterone (DHEA) were measured by liquid chromatography-tandem mass spectrometry after solvent extraction as described in detail by Keelan et al. (2012). Estrone (E_1), Estradiol (E_2), Estriol (E_3) and Estetrol (E_4) were measured by liquid chromatography-tandem mass spectrometry after solvent extraction as described in detail by Hickey, Hart and Keelan (2014).

Sex hormone binding globulin (SHBG) was measured by ELISA using a commercial kit (IBL International, Hamburg, Germany) according to the manufacturer's instructions. All samples were measured in duplicate by a single operator using assay kits from the same batch. Samples with an initial replicate CV of > 10% were reanalysed. The inter-assay imprecision (CV) was 4.5% (n = 25); intra-assay CV was 5.2% (n = 861).

Calculation of Bioavailable Testosterone, Estradiol and Estrone

We employed an empirical method for the calculation of free testosterone, validated for use in samples with low total testosterone and SHBG concentrations, described in detail by Keelan, et al. (2012) and Sartorius, et al. (2009). Bioavailable testosterone (BioT), representing the fraction of total testosterone either free (unsequestered by SHBG) or bound to serum albumin, was calculated by summing the concentrations of free testosterone and albumin-bound testosterone (Keelan et al., 2012). Albumin levels were adjusted using

published reference values to account for the decrease in serum albumin concentrations with gestational age (Zlotkin & Casselman, 1987).

A significant proportion of both E_1 and E_2 are bound to SHBG (Anderson, 1974). Therefore, the free and bioavailable proportion of E_1 (BioE$_1$) and of E_2 (BioE$_2$) were calculated using the method described by Mazer (2009).

$$fE_1 = \frac{E_1}{1 + 3.60e^{+04} \times Albumin \times \frac{10}{69000} + 9.38e^{-02} \times SHBG}$$

$$fE_2 = \frac{E_2}{1 + 4.55e^{+04} \times Albumin \times \frac{10}{69000} + 4.95e^{-01} \times SHBG}$$

We could not account for binding to corticosteroid binding globulin (CBG) as it was not measured in our sample. In addition, since our fT and BioT levels were estimated using a slightly different method, we did not account for competitive binding effects. However, comparisons between our model and Mazer's calculation illustrated very high correlations ($r > .95$). The bioavailable proportions were calculated using the standard formula: BioE$_1$ = [fE$_1$] + [albumin-bound E$_1$] and BioE$_2$ = [fE$_2$] + [albumin-bound E$_2$] where K$_{ALB}$ was equal to 36,000 L/mol and 45,500 L/mol respectively.

Calculation of Composite Measures

Studies of sex steroids typically examine only the most biologically potent of the androgens and estrogens, testosterone and E_2, respectively. However, there are a number of other androgens and estrogens which, although they are not as biologically potent, are present in much higher concentrations and are not bound by SHBG and other binding proteins. To assess the potential significance of total sex hormone bioactivity, we calculated composite

measures of androgens and estrogens, taking into account the biological potency, binding affinity and unbound fraction of each hormone.

Each hormone was then weighted based on its biological potency. Since testosterone is the most biologically potent androgen, it was weighted as 1; A4 has 10% of the potency of testosterone, and DHEA has 1% (Anderson, 1974). Neither is bound to SHBG. The formula for the androgen composite is as below:

$$Androgen\ Composite = BioT + 0.1[A4] + 0.01[DHEA]$$

Similarly, for the estrogen composite calculation, E_2, being the most biologically potent estrogen was weighted at 1. E_1 has only 50% of the potency, E_3 has 10% and E_4 has 2% (Holinka, Diczfalusy, & Coelingh Bennink, 2008; Watkins, 2007). Neither E3 nor E4 are bound to SHBG. The composite calculation for estrogen is shown below:

$$Estrogen\ Composite = BioE_2 + 0.5[BioE_1] + 0.1[E_3] + 0.02[E_4]$$

Hormone Ratios

The ratio of testosterone to estradiol levels was calculated by dividing the BioT concentration by the $BioE_2$ concentration ($BioT:BioE_2$). Similarly, the androgen to estrogen ratio was calculated by dividing the androgen composite by the estrogen composite (A:E).

Digit Ratio

Digit ratio was measured in the participants when they were young adults aged 19-22 years. The lengths of the second and fourth fingers on the ventral surface of the left and right hand were measured from the basal crease of the digit to its tip using Vernier callipers,

working from purpose-collected hand photocopies (Lanier LD 122 photocopier) under standardized and supervised conditions (Caswell & Manning, 2009). All measurements were made by one observer and repeated by a second observer blinded to the findings of the first. Digit ratio was calculated by dividing the length of the 2nd digit by the length of the 4th digit. Separate ratios were calculated for the left and right hand. In addition, the directional asymmetry (Dr-l) digit ratio was calculated by subtracting the left-hand digit ratio from the right-hand digit ratio.

Covariates

The measurement of umbilical cord hormones may be affected by a number of obstetric and maternal factors. The presence and duration of labour and gestational age at delivery have been found to significantly impact the androgen and estrogen concentrations in cord blood (Hickey, et al., 2014; Keelan, et al., 2012). In addition, birth weight and the presence of ante-partum haemorrhage or preeclampsia significantly impact cord estrogen levels (Hickey, et al., 2014). These covariates were taken into account in the analysis.

Statistical Analysis

Independent samples t-tests were used to test differences between males and females for hormone concentrations and other sociodemographic, antenatal and obstetric variables. Bivariate correlations were initially used to investigate the relationships between digit ratios, hormone concentrations and hormone ratios, separately for males and females. Any significant relationships were investigated further using hierarchical multiple regression analysis to control for covariates.

Results

Sex Steroid Composite Analysis

Examining the full sample for which sex steroid composites were available, revealed that males had a significantly higher androgen composite compared to females (males: n = 420, mean ± SD = .448 ± .224; females: n = 400, mean ± SD = .415 ± .197; t (818) = 2.24, p = .026); while, there was no difference between males and females for the estrogen composite (males: n = 428, mean ± SD = 96.06 ± 2.25; females: n = 425, mean ± SD = 96.07 ± 49.94; t (851) = -.005, p = .996). The androgen and estrogen composites were positively correlated with one another, r = .288, p < .001.

Digit Ratio Analysis

Table 1 presents the characteristics of male and female participants who had both sex steroid and digit ratio data available (159 female; 182 male). Males had significantly lower left and right 2D:4D ratios compared to females. There were a number of significant sex differences in the hormone concentrations and ratios: females had higher levels of $BioE_1$ and E_4; while males had higher BioT levels, DHEA levels, androgen composites, $BioT:BioE_2$ ratios and A:E ratios. Although there was no significant difference in the estrogen composite, it was higher in females than males. Given these statistically significant differences, the remainder of the analyses were conducted separately for males and females.

We initially investigated the relationships of the BioT and $BioE_2$ measures with the digit ratios. For both males and females, there were no significant correlations between the left, right or Dr-l digit ratio and BioT or $BioE_2$ concentrations. Additionally, the $BioT:BioE_2$ ratio was not significantly correlated with the left, right or Dr-l digit ratio (see Table 2). None of the other individual androgen or estrogen measures significantly correlated with the 2D:4D

or Dr-l ratios. Data analysis then turned to the composite hormone measures. For females, neither the androgen nor estrogen composite was significantly correlated with the left, right or Dr-l digit ratio. For males, there was a significant negative correlation between the estrogen composite and the left 2D:4D ratio ($r = -.172$, $p = .02$), but not the right or Dr-l. Neither the androgen composite nor the A:E ratio were significantly correlated with the left, right or Dr-l digit ratio (Table 2).

To further examine the relationship between the left 2D:4D ratio and the estrogen composite in males, a hierarchical multiple regression analysis was conducted. The estrogen composite was added to a model where the covariates found to significantly influence estrogen levels in cord blood (Keelan et al., 2012), were entered first in predicting the left hand 2D:4D ratio. Although, the model was not significantly improved with the addition of the estrogen composite there was a trend towards significance (see Table 3).

Discussion

Our primary aim was to investigate the relationship between fetal sex steroid concentrations in umbilical cord blood at delivery and hand digit ratios in young adults. Based on the published literature on prenatal androgen and estrogen exposure and digit ratio, we hypothesized that testosterone concentrations and the testosterone to estradiol ratio would be negatively related to the 2D:4D and Dr-l ratio and estradiol concentrations would be positively related. However, we found no statistically significant relationship between bioavailable testosterone concentrations, bioavailable estradiol concentrations or the BioT:BioE$_2$ ratio and the 2D:4D ratio of either hand or the Dr-l. These findings differ from those using amniotic fluid collected at mid-gestation to measure sex steroids, where both total testosterone and the ratio of total testosterone to total estradiol concentrations have been negatively associated with digit ratio (Lutchmaya et al., 2004; Ventura et al., 2013). However,

evidence supporting a relationship between amniotic fluid sex steroids and digit ratio are very limited. Both studies have moderate sample sizes drawn from a selected population of high-risk pregnancies undergoing amniocentesis (Lutchmaya et al., 2004; Ventura et al., 2013). In addition, there are a number of methodological differences between the previous studies and the current study.

First, Lutchmaya and colleagues analysed males and females together, and although sex was controlled in the analyses, there is emerging evidence that males and females respond differently to circulating sex steroid concentrations (Vitale, Mendelsohn, & Rosano, 2009), as evidenced by different associations identified for males and females (Hollier et al., 2013; Knickmeyer, Baron-Cohen, Raggatt, & Taylor, 2005; Lutchmaya, Baron-Cohen, & Raggatt, 2002; Robinson et al., 2013; Whitehouse et al., 2012). Therefore, males and females should be separated in association analyses.

Secondly, Lutchmaya et al. (2004) used RIA to measure sex steroid concentrations in amniotic fluid, which consistently overestimates testosterone concentrations and may suffer from interference (Keelan et al., 2012). Thirdly, although Ventura et al. (2013) utilised the preferred LC-MS/MS technique to measure sex steroid concentrations, the authors from both studies were limited by the availability of total sex steroid concentrations uncorrected for protein binding. Only the free fractions (unbound) of sex steroids are biologically active. Our data are based on LC-MS/MS measurements which are more accurate than RIA and we have calculated bioavailable testosterone and estradiol which adds strength to the quality of the data.

Fourthly, amniotic fluid is collected during the second trimester and may reflect mid-gestation sex steroid concentrations, while cord blood collected at birth reflects late gestation sex steroid concentrations (Keelan et al., 2012). It is not known how circulating sex steroid concentrations change through prenatal life. One small study comparing mid-gestation fetal

cord testosterone and amniotic fluid and maternal plasma concentrations showed no significant associations (Rodeck, Gill, Rosenberg, & Collins, 1985). Further, it is uncertain when in gestation digit ratio is established. Experimental data has indicated that supraphysiological androgen exposure in early, but not late gestation, increased the length of the second digit and had no effect on the fourth digit in female rhesus monkeys, thereby resulting in a less male-like ratio (Abbott, Colman, Tiefenthaler, Dumesic, & Abbott, 2012). Conversely, in female mice, *in utero* exposure to supraphysiological concentrations of the potent androgen dihydrotestosterone or a blocking estrogen action increased the length of the fourth digit, but had no effect on the second digit, resulting in a more male-like digit ratio pattern. In male mice androgen antagonism or estrogen treatment *in utero* decreased the length of the fourth digit, but did not affect the second digit, creating a more female like digit ratio pattern (Zheng & Cohn, 2011). Further, Auger et al. (2013) found the exposure to low (i.e. environmental) levels of estrogenic and anti-androgenic endocrine disruptors *in utero* feminised digit ratios in male rats. The findings from these experimental studies demonstrate that prenatal sex steroid exposure can modulate fetal digit growth, and that digit ratio may reflect both androgen and estrogen exposure (Dean & Sharpe, 2013). Although it remains unclear when in gestation the digit ratio is established and the results from these experimental studies cannot necessarily be applied to human development.

Two studies examined digit ratio in deceased human fetuses and found that the sexual dimorphism of the 2D:4D ratio is present by the second trimester (Galis, Ten Broek, Van Dongen, & Wijnaendts, 2010; Malas, Dogan, Hilal Evcil, & Desdicioglu, 2006). Additionally, both studies noted that digit ratio averages were smaller than those found in children and adults and the strength of the digit ratio sex difference varied across gestational age. These findings imply that both pre- and postnatal developmental processes are involved in the determination of the digit ratio sex difference. To date only four studies, including the current

study, have prospectively examined the relationship between digit ratio and fetal sex steroids in humans, and only Lutchmaya et al. (2004) and Ventura et al. (2013) have observed significant relationships. If circulating sex steroid levels are involved in determining digit ratio, the available evidence to date suggests that this relationship is not evident by late gestation.

The second aim of the current study was to calculate composite measures of total androgen and total estrogen exposure, taking into account the bioavailable proportions and the potencies of the individual hormones, and investigate whether these measures were associated with digit ratio. Although testosterone and estradiol are the most biologically potent sex steroids, some of the less potent sex steroids (e.g. E_1, E_3, A4, DHEA) are present in much greater quantities and may also affect human development. Therefore, it is important to consider the effects of the less potent steroids as well as those of testosterone and estradiol. To the best of our knowledge the current study is the first to generate measures of total androgen and total estrogen exposure. We found a significant positive correlation between the androgen and estrogen composites, consistent with the well-established biosynthetic relationship between the two classes of steroids in pregnancy: androgens are converted to estrogens through the actions of placental aromatase, and both groups of steroids are strongly influenced by fetal steroid production. These novel composite measures will allow future researchers to examine the effects of total androgen and total estrogen exposure.

We found no statistically significant relationship between the androgen composite, estrogen composite or A:E ratio and any of the digit ratios after control of covariates. However, there was a trend towards a significant negative association between the estrogen composite and the left hand digit ratio in males. This trend is in the opposite direction to that predicted based on the findings of Lutchmaya et al. (2004). Very little research has focused on the effect of estrogen levels on the development of digit ratio. Although the theory

proposed by Manning (2002) posits that it is the relative concentrations of androgens to estrogens which regulates digit ratio, most published research focuses solely on androgen exposure (McIntyre, 2006), rather than the relative levels of androgens and estrogens. Few studies have examined the relationship between prenatal estrogen exposure and postnatal human development and most studies address the potential relationship with reproductive cancers in females (Troisi, Potischman, Roberts, Siiteri, et al., 2003; Troisi, Potischman, Roberts, Harger, et al., 2003; Troisi, Potischman, Roberts, Ness, et al., 2003). The implications of this trend towards a significant negative association between the estrogen composite and the left hand digit ratio in males is not yet known, but our data do not support the prediction that digit ratio is positively associated with measures of estrogen bioactivity.

It is important to note that the main limitation of the use of cord blood to examine the effect of prenatal sex steroid exposure on human development is that the periods of fetal neurodevelopment that are sensitive to sex steroid exposure may occur earlier in gestation (Cohen-Bendahan, van de Beek, & Berenbaum, 2005), and, as noted above, the relative concentrations of circulating bioactive sex steroids during gestation are not known. The current evidence shows a more marked sex difference in fetal testosterone concentrations in mid-gestation compared to levels measured at birth (Scott, Mason, & Sharpe, 2009). However, there is increasing recognition from experimental studies that the effect of prenatal sex steroid exposure on postnatal development is not restricted to early fetal development and that sex steroid levels in the final trimester also contribute to fetal development (Roselli, Estill, Stadelman, Meaker, & Stormshak, 2011; Zambrano, Guzmán, Rodríguez-González, Durand-Carbajal, & Nathanielsz, 2014). Furthermore, evidence from studies examining relationships between umbilical cord sex steroids and subsequent development (Farrant et al., 2013; Hollier et al., 2013; Jacklin et al., 1983; Jacklin et al., 1988; Robinson et al., 2013; Whitehouse et al., 2012), indicate that these sex steroid concentrations are informative

markers for childhood development. . Currently, umbilical cord blood is the only practical means of assessing fetal sex steroid concentrations during uncomplicated pregnancies (Hickey et al., 2009; Hollier, Keelan, et al., 2014; Sloboda, Hickey, & Hart, 2011).

A key consideration in the interpretation of umbilical cord blood sex steroid concentrations is the modulating effect of obstetric and perinatal factors. Examinations of large unselected birth cohorts have found that the presence and duration of labour and gestational age at delivery significantly impact on cord androgen and estrogen concentrations (Hickey, et al., 2014; Keelan, et al., 2012). Birth weight and the presence of ante-partum haemorrhage or preeclampsia also modify cord estrogen levels (Hickey, et al., 2014). However, the influences of these factors can be mitigated through detailed phenotyping and statistical controls on longitudinal regression models, and we believe that this limitation does not significantly diminish the usefulness of cord blood in providing indices of late gestation circulating fetal sex steroids (Hollier, Keelan, et al., 2014).

An easily accessible postnatal marker of prenatal sex steroid exposure is necessary to be able to examine the effect of prenatal sex steroid exposure on postnatal development. Digit ratio is easy to measure, but its validity as an indicator of prenatal sex steroids is primarily based on associations with behavioural and physical sexually dimorphic traits (Cohen-Bendahan et al., 2005; McIntyre, Cohn, & Ellison, 2006), which vary across cultures and are strongly linked to social and behavioural norms. The exact relationship between prenatal sex steroid exposure and digit ratio is unclear. The current study is the largest prospective study of prenatal sex steroids and digit ratio, and the first to use the combination of LC-MS/MS and bioavailable fractions of sex steroids and properly adjust for pregnancy-related covariables. Therefore, our findings present highly accurate measurements from normal pregnancy.

In conclusion, this population-based study of umbilical cord sex steroids and adult digit ratio found no statistically significant relationship between late gestation sex steroid

exposure and the adult hand digit ratio. The current study also provides a new method of

calculating and examining total androgen and estrogen exposure through composite

calculations.

Conflict of Interest

The authors declare no conflict of interest.

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Table 1. *Characteristics of male and female participants. Between-sample comparisons are also provided.*

		Female		Male	Comparison
Digit Ratios	n	Mean (SD)	n	Mean (SD)	
Left 2D:4D	159	.967 (.033)	182	.957 (.031)	$t(339) = 3.040$, $p = .003$
Right 2D:4D	159	.976 (.033)	182	.962 (.031)	$t(339) = 3.874$, $p < .001$
Dr-l	159	.008 (.030)	182	.005 (.026)	$t(339) = 1.004$, $p = .316$
Estrogens					
BioE1	159	73.35 (37.53)	182	65.04 (30.53)	$t(339) = 2.255$, $p = .025$
BioE2	159	19.13 (9.96)	182	18.25 (8.86)	$t(339) = .864$, $p = .388$
E3	159	419.92 (193.18)	182	397.96 (193.43)	$t(339) = 1.046$, $p = .296$
E4	159	20.54 (9.45)	182	16.76 (7.56)	$t(339) = 4.098$, $p < .001$
Estrogen Composite	159	98.21 (42.32)	182	90.90 (37.41)	$t(339) = 1.693$, $p = .091$
Androgens					
BioT	159	.078 (.040)	182	.133 (.064)	$t(339) = -9.308$, $p < .001$
A4	159	2.14 (.702)	182	2.14 (.760)	$t(339) = -.083$, $p = .934$
DHEA	159	8.39 (4.07)	182	6.95 (3.18)	$t(339) = 3.676$, $p < .001$
Androgen Composite	159	.376 (.124)	182	.417 (.118)	$t(339) = -3.110$, $p = .002$
Hormone Ratios					
Testosterone:Estradiol	159	.0050 (.0035)	182	.0091 (.0066)	$t(339) = -7.012$, $p < .001$
Androgens:Estrogens	159	.0044 (.0022)	182	.0053 (.0026)	$t(339) = -3.397$, $p = .001$
Continuous Covariates					
Maternal age at conception (years)	159	28.79 (5.70)	182	28.78 (5.68)	$t(339) = .021$, $p = .983$
Gestational age at birth (weeks)	155	39.15 (1.85)	171	39.40 (1.91)	$t(324) = -1.166$, $p = .245$
Apgar scores 5 minutes after birth	159	8.99 (.842)	182	8.99 (.685)	$t(339) = -.086$, $p = .932$
Birthweight (g)	159	3233 (528)	182	3320 (587)	$t(339) = -1.419$, $p = .157$
Categorical Covariates	N	n (%)	N	n (%)	
Maternal education at pregnancy	159		182		$\chi^2 = 3.44$, df = 1, $p = .064$
Completed secondary school		61 (38.4)		88 (48.4)	
Did not complete secondary school		98 (61.6)		94 (51.6)	
Smoking in pregnancy	156		177		$\chi^2 = 1.77$, df = 2, $p = .413$
None		123 (78.8)		146 (82.5)	
1 – 10 cigarettes daily		16 (10.3)		19 (10.7)	
11+ cigarettes daily		17 (10.9)		12 (6.8)	
Alcohol consumption during pregnancy	156		177		$\chi^2 = .596$, df = 2, $p = .742$
None		87 (55.8)		93 (52.5)	
Once a week or less		58 (37.2)		73 (41.2)	
Several times a week or more		11 (7.1)		11 (6.2)	
Family income during pregnancy	156		176		$\chi^2 = .086$, df = 1, $p = .769$
<$24,000		52 (33.3)		56 (31.8)	
≥$24,000		104 (66.7)		120 (68.2)	
Parity	159		182		$\chi^2 = .008$, df = 2, $p = .996$
0		71 (44.7)		81 (44.5)	
1		50 (31.4)		58 (31.9)	
>1		38 (23.9)		43 (23.6)	
Delivery Mode	159		182		$\chi^2 = .969$, df = 1, $p = .325$
Labour		145 (91.2)		160 (87.9)	
No Labour		14 (8.8)		22 (12.1)	

Table 2. *Correlations (Pearson r values) between the left and right 2D:4D ratios and hormone concentrations, split by sex.*

	Left 2D:4D		Right 2D:4D		Dr-l	
	Male	Female	Male	Female	Male	Female
BioE$_2$	-.108	-.129	-.045	-.077	.076	.052
BioT	.015	-.112	.058	-.047	.051	.068
BioT:BioE$_2$	.097	.033	.086	-.061	-.013	-.102
Androgen Composite	-.108	-.128	-.018	-.103	.107	.023
Estrogen Composite	-.172*	-.140	-.132	-.080	.048	.062
A:E	.040	.087	.097	-.039	.068	-.135

Note: *$p < .05$

Table 3. *Outcomes of hierarchical multiple regression analyses predicting the left 2D:4D ratio (males only).*

		B	*SE B*	*β*	R^2	R^2 change
Step 1	Constant	.979	.062			
	Gestational Age	-5.04e-4	1.76e-3	-.031		
	Delivery Mode	2.61e-3	8.97e-3	.025		
	Birth weight	-7.78e-7	5.49e-6	-.014		
	APH	7.20e-4	.011	.005		
	Preeclampsia	7.17e-3	.015	.039		
					.006	
Step 2	Constant	.992	.062			
	Gestational Age	-5.24e-4	1.75e-3	-.032		
	Delivery Mode	-8.96e-4	9.10e-3	-.009		
	Birth weight	-7.41e-7	4.45e-6	-.013		
	APH	5.21e-4	.011	.004		
	Preeclampsia	7.09e-3	.015	.038		
	Estrogen	-1.24e-4	6.69e-5	-.147		
					.026	.020, $p = .065$

Note: *N*=182