ASSESSING PRENATAL AND NEONATAL GONADAL STEROID EXPOSURE FOR STUDIES OF HUMAN DEVELOPMENT: METHODOLOGICAL AND THEORETICAL CHALLENGES

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ASSESSING PRENATAL AND NEONATAL GONADAL STEROID EXPOSURE FOR STUDIES OF HUMAN DEVELOPMENT: METHODOLOGICAL AND THEORETICAL CHALLENGES

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There is extensive evidence from animal models that gonadal steroids, produced in fetal and neonatal life, act on the developing organism to produce sex differences far beyond the reproductive system. That early gonadal steroid exposure also plays an important role in human development is supported by studies of individuals with disorders of sex determination and differentiation. It is much less clear whether normal variation in gonadal steroid exposure predicts sexually dimorphic health outcomes or within-sex variation. This is largely due to challenges related to the assessment of gonadal steroid exposure in the developing fetus and neonate.

Regarding the prenatal period, serial measurements of serum hormone levels in the fetus, for use in studies of later development, are not possible for ethical reasons. Researchers have measured hormones in maternal blood, umbilical cord blood, and amniotic fluid; used putative anthropometric indices such as the relative lengths of the 2nd and 4th digits (2D:4D); evaluated common variants in genes related to hormone production, transport, and metabolism; and examined development in opposite sex twins and the offspring of mothers with hyperandrogeny. Each of these approaches has particular strengths and notable weaknesses.

Regarding the neonatal period, serial measurements in serum are often impractical for studies of typical development. Salivary hormone assays, frequently used in studies of older children and adults, have not been extensively investigated in neonates. The most appropriate timing for testing is also open to debate. Early work suggested that testosterone levels in males begin to rise after the first postnatal week, peak around the 3rd to 4th months of life, and then drop back to very low levels by 1 year. However a more recent study of 138 infants did not

demonstrate this pattern. Testosterone was highest on the day of birth and gradually dropped over the first 6 months. Even less is known about patterns of early estrogen exposure, though highly sensitive bioassays indicate that sex differences are present in early childhood.

In addition, the design and interpretation of studies may be impacted by widespread acceptance of conceptual frameworks that are not well-supported empirically. For example, many researchers presume that the free hormone hypothesis, which states that unbound hormone is more readily diffusible into tissues and thus a better measure of actual exposure, is true. However this hypothesis has been challenged on multiple grounds. A second example: it is generally accepted that masculinization of the human brain is primarily mediated by the androgen receptor (in contrast to rodents where the estrogen receptor plays a major role), in part because chromosomal males with complete androgen insensitivity generally espouse a female gender identity. However this is not always the case, and other sexually dimorphic outcomes have not been carefully assessed in CAIS.

The aim of this research topic is to gather together experimental and review papers which address the diverse challenges in assessing prenatal and neonatal gonadal steroid exposure for studies of human development with the expectation that this will allow more critical appraisal of existing studies, identify critical research gaps, and improve the design of future studies.

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Assessing prenatal and neonatal gonadal steroid exposure for studies of human development: methodological and theoretical challenges

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Keywords: testosterone, gonadal hormones, hypothalamic–pituitary–gonadal axis, hypogonadism, sexual differentiation, prenatal development, minipuberty

Animal models provide compelling evidence that gonadal hormones, in particular testosterone, produced in the fetal and neonatal period, have life-long effects on physical characteristics, physiological functioning, and behavior (1–3). Studies of individuals with disorders of sex determination or sexual differentiation, largely congenital adrenal hyperplasia (4), Turner syndrome (5), and Klinefelter syndrome (6) strongly suggest that early gonadal steroid exposure is important in human development as well, and effects are not limited to the reproductive system alone. However, extending this work to the broader human population has proven challenging due to inherent difficulties in measuring testosterone exposure in developing fetuses and neonates.

In addition, the design and interpretation of studies may be impacted by widespread acceptance of conceptual frameworks that are not well supported empirically. For example, many researchers presume that the free hormone hypothesis, which states that unbound hormones are more readily diffusible into tissues and thus a better measure of actual exposure, is true. However, this hypothesis has not been rigorously validated and, indeed, there is evidence for active cellular uptake of SHBG-bound testosterone and for SHBG-bound testosterone mediating steroid hormone signal transduction at the plasma membrane (7). A second example: it is generally accepted that masculinization of the human brain is primarily mediated by the androgen receptor [in contrast to rodents where the estrogen receptor plays a major role (8)], in part because chromosomal males with complete androgen insensitivity (CAIS) generally espouse a female gender identity (9). However, this is not always the case (10), and other sexually dimorphic outcomes have not been carefully assessed in CAIS.

The aim of this research topic is to gather together experimental and review papers, which address the diverse challenges in assessing prenatal and neonatal gonadal steroid exposure for studies of human development with the expectation that this will allow more critical appraisal of existing studies, identify critical research gaps, and improve the design of future studies.

In terms of matrices used for the determination of testosterone exposure, Hollier et al. (11) review umbilical cord blood and Voegtline and Granger (12) review saliva. A theme running through both articles is that pre-analytic factors (collection, transport, storage, and processing) are absolutely critical in measuring testosterone exposure. Assay types and confounding factors also require careful attention. Also in the realm of measurement, Manning et al. (13) and Honekopp (14) focus on a widely used anthropometric index of prenatal testosterone exposure, the relative lengths of the second and fourth digits (2D:4D ratio). Manning et al. (13) review the evidence in support of 2D:4D and argue that this index is particularly relevant to "challenging" conditions such as aggressive and sexual encounters, which involve both organizational and activational hormone effects. Honekopp (14) carried out a meta-analysis of the relationship between 2D:4D and a functional polymorphism in the androgen receptor gene, the number of CAG repeats. He reports no evidence for a relationship and discusses the implications of this finding. Korsoff et al. (15) discuss whether prenatal testosterone transfer occurs in females from opposite sex twin pregnancies and report that anthropometric, metabolic, and reproductive characteristics relevant to polycystic ovarian syndrome (PCOS) do not differ between females from same sex and opposite sex twin pairs. Grinspon et al. (16) discuss the advantages and limitations of old and new markers used for the functional assessment of the hypothalamic–pituitary–testicular axis in boys suspected of fetal-onset hypogonadism.

It is clear that all current means of assessing early gonadal steroid exposure have unique strengths and notable weaknesses. We would argue that any results in this field should be treated with caution until converging evidence is available from multiple methods and replication. New approaches are also urgently needed. O'Connor and Barrett (17) highlight one promising area: placental gene expression.

Several papers address conceptual issues in the field. Alexander (18) highlights the potential role of the neonatal testosterone surge or "minipuberty" in male social behavior. The minipuberty has been relatively ignored by the field following early research on non-human primates, which suggested that suppression of the postnatal surge had minimal effects on a limited range of male behavioral phenotypes (19, 20). Alexander encourages us

to re-examine the potential importance of the minipuberty in sexual differentiation of the brain. Xia et al. (21) also focus on the minipuberty in an experimental article probing genetic and environmental contributors to individual variation in salivary testosterone during this period. O'Connor and Barrett (17) discuss the need to consider cross-talk between the hypothalamic–pituitary–gonadal (HPG) and the hypothalamic–pituitary–adrenal (HPA) axes. Finally, Grinspon et al. (16) provide a comprehensive review of fetal-onset hypogonadism. Because these conditions vary with regard to the level of the HPA axis affected, the testicular cell population initially impaired, and the developmental period when the condition is established, studying these disorders could produce a more detailed understanding of the role of the HPG axis in developmental programing. They also make the important point that male hypogonadism cannot be limited to hypoandrogenism. They draw attention to several other testicular secretions including insulin-like-3 (INSL3), inhibin B, and anti-Müllerian hormone (AMH). Relatively little research has investigated whether these hormones impact brain development and other phenotypes beyond the reproductive system. AMH represents a particularly interesting case in this regard as it has been observed to support the survival and differentiation of embryonic motor neurons *in vitro* (22) and may regulate the development of sexually dimorphic brain areas in male mice (23, 24). There is also one report of lowered AMH and inhibin B in boys with autism, a condition with a marked male bias (25).

In conclusion, we hope that this research topic will serve as a point of reference and source of inspiration for researchers interested in the role of prenatal and neonatal gonadal steroids in human development. Ultimately, a better understanding of how individual variation in the functioning of the HPG axis impacts later health will help us explain and treat sex-biased medical conditions.

AUTHOR CONTRIBUTIONS

Rebecca C. Knickmeyer drafted the manuscript. All coauthors revised the manuscript for important intellectual content, and approved the final version to be published. Rebecca C. Knickmeyer agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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REFERENCES

1. De Vries G, Simerly RB. Anatomy, development, and function of sexually dimorphic neural circuits in the mammalian brain. In: Pfaff D, Arnold A, Etgen A, Fahrbach S, Rubin R, editors. *Hormones, Brain and Behavior* (Vol. IV), New York, NY: Academic Press (2002). p. 137–91.
2. Padmanabhan V, Manikkam M, Recabarren S, Foster D. Prenatal testosterone excess programs reproductive and metabolic dysfunction in the female. *Mol Cell Endocrinol* (2006) **246**(1–2):165–74. doi:10.1016/j.mce.2005.11.016
3. Grigore D, Ojeda NB, Alexander BT. Sex differences in the fetal programming of hypertension. *Gend Med* (2008) **5**(Suppl A):S121–32. doi:10.1016/j.genm.2008.03.012
4. Hines M. Sexual differentiation of human brain and behavior. In: Pfaff D, Arnold A, Etgen A, Fahrbach S, Rubin R, editors. *Hormones, Brain and Behavior* (Vol. IV), New York, NY: Academic Press (2002). p. 425–62.
5. Knickmeyer RC, Davenport M. Turner syndrome and sexual differentiation of the brain: implications for understanding male-biased neurodevelopmental disorders. *J Neurodev Disord* (2011) **3**(4):293–306. doi:10.1007/s11689-011-9089-0
6. Savic I. Advances in research on the neurological and neuropsychiatric phenotype of Klinefelter syndrome. *Curr Opin Neurol* (2012) **25**(2):138–43. doi:10.1097/WCO.0b013e32835181a0
7. Handelsman DJ. Update in andrology. *J Clin Endocrinol Metab* (2007) **92**(12):4505–11. doi:10.1210/jc.2007-1431
8. McCarthy MM. Molecular aspects of sexual-differentiation of the rodent brain. *Psychoneuroendocrinology* (1994) **19**(5–7):415–27.
9. Hines M, Ahmed SF, Hughes IA. Psychological outcomes and gender-related development in complete androgen insensitivity syndrome. *Arch Sex Behav* (2003) **32**(2):93–101. doi:10.1023/A:1022492106974
10. T'Sjoen G, De Cuypere G, Monstrey S, Hoebeke P, Freedman FK, Appari M, et al. Male gender identity in complete androgen insensitivity syndrome. *Arch Sex Behav* (2011) **40**(3):635–8. doi:10.1007/s10508-010-9624-1
11. Hollier LP, Keelan JA, Hickey M, Maybery MT, Whitehouse AJ. Measurement of androgen and estrogen concentrations in cord blood: accuracy, biological interpretation, and applications to understanding human behavioral development. *Front Endocrinol* (2014) **5**:64. doi:10.3389/fendo.2014.00064
12. Voegtline KM, Granger DA. Dispatches from the interface of salivary bioscience and neonatal research. *Front Endocrinol* (2014) **5**:25. doi:10.3389/fendo.2014.00025
13. Manning J, Kilduff L, Cook C, Crewther B, Fink B. Digit ratio (2D:4D): a biomarker for prenatal sex steroids and adult sex steroids in challenge situations. *Front Endocrinol* (2014) **5**:9. doi:10.3389/fendo.2014.00009
14. Honekopp J. No evidence that 2D:4D is related to the number of CAG repeats in the androgen receptor gene. *Front Endocrinol* (2013) **4**:185. doi:10.3389/fendo.2013.00185
15. Korsoff P, Bogl LH, Korhonen P, Kangas AJ, Soininen P, Ala-Korpela M, et al. A comparison of anthropometric, metabolic, and reproductive characteristics of young adult women from opposite-sex and same-sex twin pairs. *Front Endocrinol* (2014) **5**:28. doi:10.3389/fendo.2014.00028
16. Grinspon RP, Loreti N, Braslavsky D, Valeri C, Schteingart H, Ballerini MG, et al. Spreading the clinical window for diagnosing fetal-onset hypogonadism in boys. *Front Endocrinol* (2014) **5**:51. doi:10.3389/fendo.2014.00051
17. O'Connor TG, Barrett ES. Mechanisms of prenatal programing: identifying and distinguishing the impact of steroid hormones. *Front Endocrinol* (2014) **5**:52. doi:10.3389/fendo.2014.00052
18. Alexander GM. Postnatal testosterone concentrations and male social development. *Front Endocrinol* (2014) **5**:15. doi:10.3389/fendo.2014.00015
19. Wallen K, Maestripieri D, Mann DR. Effects of neonatal testicular suppression with a GnRH antagonist on social behavior in group-living juvenile rhesus monkeys. *Horm Behav* (1995) **29**(3):322–37. doi:10.1006/hbeh.1995.1023
20. Brown GR, Dixson AF. Investigation of the role of postnatal testosterone in the expression of sex differences in behavior in infant rhesus macaques (*Macaca mulatta*). *Horm Behav* (1999) **35**:186–94. doi:10.1006/hbeh.1999.1512
21. Xia K, Yu Y, Ahn M, Zhu H, Zou F, Gilmore JH, et al. Environmental and genetic contributors to salivary testosterone levels in males. *Front Endocrinol* (2014) **5**:187. doi:10.3389/fendo.2014.00187
22. Wang PY, Koishi K, McGeachie AB, Kimber M, MacLaughlin DT, Donahoe PK, et al. Mullerian inhibiting substance acts as a motor neuron survival factor in vitro. *Proc Natl Acad Sci U S A* (2005) **102**(45):16421–5. doi:10.1073/pnas.0502917102
23. Wittmann W, McLennan IS. The male bias in the number of Purkinje cells and the size of the murine cerebellum may require Mullerian inhibiting substance/anti-Mullerian hormone. *J Neuroendocrinol* (2011) **23**(9):831–8. doi:10.1111/j.1365-2826.2011.02187.x
24. Wittmann W, McLennan IS. Anti-Mullerian hormone may regulate the number of calbindin-positive neurons in the sexually dimorphic nucleus of the

preoptic area of male mice. *Biol Sex Differ* (2013) **4**(1):18. doi:10.1186/2042-6410-4-18

25. Pankhurst MW, McLennan IS. Inhibin B and anti-Mullerian hormone/Mullerian-inhibiting substance may contribute to the male bias in autism. *Transl Psychiatry* (2012) **2**:e148. doi:10.1038/tp.2012.72

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Measurement of androgen and estrogen concentrations in cord blood: accuracy, biological interpretation, and applications to understanding human behavioral development

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Accurately measuring hormone exposure during prenatal life presents a methodological challenge and there is currently no "gold standard" approach. Ideally, circulating fetal hormone levels would be measured at repeated time points during pregnancy. However, it is not currently possible to obtain fetal blood samples without significant risk to the fetus, and therefore surrogate markers of fetal hormone levels must be utilized. Umbilical cord blood can be readily obtained at birth and largely reflects fetal circulation in late gestation. This review examines the accuracy and biological interpretation of the measurement of androgens and estrogens in cord blood. The use of cord blood hormones to understand and investigate human development is then discussed.

Keywords: estrogens, androgens, cord blood, human development, prenatal

Prenatal exposure to sex steroids has long been posited to influence human development (1, 2). There are a number of biological differences between males and females, including height, weight, internal reproductive anatomy, and external genitalia. Sex differences have also been observed in neuroanatomy and neurochemistry (3). In addition to biological differences, males and females differ in a number of behavioral and cognitive aspects. Sex differences have been found for aggression (4, 5), childhood play (6), visuospatial ability (7, 8), and verbal ability (9–11). However, the examination of the relationship between these sex differences and sex steroid exposure has been limited due to inherent difficulties in providing accurate and reliable measurements of hormone exposure during prenatal life.

Findings from animal models illustrate that exposure to sex steroids during critical periods of gestational development can have a significant long-term impact (12). However, due to the many developmental differences between species (e.g., duration of pregnancy, maturity at birth, and susceptibility to a variety of environmental conditions), it is difficult to extrapolate animal model findings to human development. Human brain and tissue samples cannot realistically be collected for research purposes during normal pregnancy, and it is not ethical to manipulate hormone levels in human fetuses. Therefore, surrogate markers of fetal hormone levels, such as the second-to-fourth digit (2D:4D) ratio or the examination or clinical populations exposed to atypical hormone levels (e.g., congenital adrenal hyperplasia), have been used to investigate the relationship between sex steroids and human development. However, there is doubt as to whether the 2D:4D ratio is a reliable proxy measure of fetal testosterone levels (13, 14) and it is difficult to extrapolate findings from clinical populations to typical human development.

Alternatively, fetal hormones can be measured in amniotic fluid from the second trimester of pregnancy onward. Amniotic samples provide an approximation of circulating fetal hormones by gaging the hormone levels that have entered the amniotic fluid via fetal urination or diffusion through fetal skin (15). While amniotic hormones are thought to relate to hormone levels in fetal blood, the strength of the relationship remains unclear. In addition, the sampling procedure (amniocentesis) is generally only performed in high-risk pregnancies and could not ethically be performed in low risk pregnancies solely for research purposes.

Currently, there is no "gold standard" approach to the measurement of prenatal hormone exposure (16). In response to the methodological difficulties, researchers have sought alternative means through which prenatal hormone levels can be approximated. Umbilical cord plasma collected at birth allows for the collection of large representative samples and analysis of archived samples. The current review will examine the biological interpretation of cord blood hormones, issues with assay, and steroid comparisons in cord blood sex steroids and applications of this measurement approach to understanding human development.

BIOLOGICAL INTERPRETATION OF CORD BLOOD HORMONES

Pregnancy represents a unique phase of human life where circulating hormone concentrations are derived from maternal, placental, and fetal origins. The developing fetal endocrine environment is a function of gonadal, adrenal, and placental biosynthesis, metabolism, and biodistribution, modulated by protein binding, biological activity, and receptor affinity (17). The placenta is a highly steroidogenic organ responsible for the production of large amounts of free androgens and estrogens from fetal adrenal and gonadal precursors (18, 19). The placenta is also a very active metabolic organ with respect to phase II metabolism (conjugation). While steroids are lipophilic and cross the placenta in both directions, most of them are metabolized by the placenta *en route* (20). Fetal blood leaves the placenta (enriched with placental steroid metabolites and some maternal steroids) via the umbilical vein (UV) and returns from the fetus to the placenta via the umbilical artery [UA; (21)].

Umbilical cord blood is typically collected after delivery near term, and so cord plasma or serum hormone concentrations are thought to reflect the levels in the fetal circulation at late gestation (22). Umbilical cord blood samples contain approximately equal amounts of venous (UV) and arterial (UA) components, although the relative proportions are usually not known precisely and are not controlled for. Nevertheless, despite the differences in UV and UA steroid concentrations, they are strongly correlated (19). Ideally, fetal blood would be sampled earlier in pregnancy, during critical periods of development, but this is not feasible for ethical reasons. Hormone concentrations from maternal blood samples collected during normal pregnancy have been suggested as a possible approximation of circulating fetal concentrations (16). However, several studies have shown that maternal sex steroid concentrations do not reflect those in fetal circulation and the relationships are weak (23–25). Amniotic fluid sampled during mid gestation provides a proxy measure of blood hormone concentration in the fetus (15). However, the exact relationship between hormone concentrations in amniotic-fluid and circulating fetal concentrations remains unclear. Furthermore, due to the invasive nature of amniocentesis it is restricted to high-risk pregnancies. Currently, cord blood is the only practical means of assessing fetal hormone levels during a typical pregnancy (26, 27).

It is important to note that the measurement of umbilical cord sex steroids may be affected by a number of obstetric and maternal factors. Fetal adrenal steroid production changes with gestational age and labor, while levels of steroid-metabolizing enzymes in the placenta are regulated by factors known to be associated with labor and delivery such as glucocorticoids, pro-inflammatory cytokines, and exposure to reactive oxygen species (18). Hence, it is highly likely that factors such as prematurity, labor onset, placental weight, intrauterine infection, and pre-eclampsia could influence umbilical cord androgen levels, although the nature and extent of their influence have not yet been fully determined. However, these relationships have recently been investigated in a large unselected birth cohort; it was found that the presence and duration of labor and gestational age at delivery significantly impact upon androgen and estrogen concentrations in cord blood (22, 28). In addition, birth weight and the presence of ante-partum hemorrhage or pre-eclampsia significantly impact cord estrogen levels (28). Some studies also suggest that smoking in pregnancy may increase circulating postnatal testosterone and cortical concentrations and reduce estriol concentrations (29, 30), although these relationships were not observed in the large cohort study of Hickey et al. (28). Other maternal factors such as ethnicity, age, and parity should also be controlled for when examining cord blood hormones (22).

Another important consideration is that the major circulating sex steroids (i.e., testosterone, estradiol, and estrone) are bound to proteins such as sex hormone binding globulin (SHBG) and albumin, which impacts upon their bioactivity (31). Albumin and SHBG values vary according to gestational age at delivery and onset of labor (22, 26). Therefore, accurate evaluation and interpretation of prenatal sex steroid concentrations requires adjustment for pregnancy concentrations of albumin and SHBG in order to determine the biologically active fraction (22, 26).

Normal values for sex steroid concentrations have been derived from birth cohorts and consistently demonstrate that testosterone concentrations are higher in males compared to females (25, 32, 33). Umbilical cord blood androgens are likely to reflect the fetal androgen environment during late gestation. However, a major limitation of the use of cord blood is that it is possible that androgen influences on development may occur earlier in gestation (34).

Unlike androgens, umbilical cord estrogen concentrations do not consistently differ significantly between males and females. Studies have reported inconsistent results including no sex differences (24, 25, 28, 32, 33, 35), higher estrogen concentrations in females (36), and higher estrogen concentrations in males (16, 37). The lack of consistent sex differences in estrogen concentrations is biologically and clinically significant. Sex differences in estrogen exposure and in the ratio of estrogen to testosterone have been postulated to be responsible for a variety of sexually dimorphic neurodevelopmental and behavioral characteristics including sexual orientation (38), reproductive function (12), and cardiovascular disease (39).

It is clear from the literature reviewed that the collection of cord blood plasma is useful as a measure of late gestation circulating fetal hormones. However, it is important when measuring cord hormone concentrations, to adjust for obstetric and maternal confounding factors as well as to calculate the biological active fractions of the sex steroids in order to derive valid conclusions.

ISSUES RELATED TO THE DETERMINATION OF STEROID CONCENTRATIONS IN CORD BLOOD

Cord serum or plasma represents a particularly challenging medium to analyze as it has an unusual steroid profile due to the unique combination of placental and fetal steroid biosynthesis and metabolism [conjugation and deconjugation; (40, 41)]. Therefore, assays require particularly careful validation to ensure that the results are accurate. Due to the presence of a variety of steroid isomers in cord blood with almost identical structures and fragmentation patterns, efficient chromatographic discrimination of analytes during analysis can be particularly important – even when using mass spectrometry as the detection methodology. Most published studies have used radioimmunoassay (RIA) to measure concentrations of estrogens and androgens in cord blood (16, 24,

25, 37, 42–55). Organic solvent extraction has been employed in most studies to remove interfering factors in order to improve specificity, accuracy, and sensitivity. A few studies have used additional column purification techniques (24, 25, 53) to address concerns regarding the accuracy of RIAs for the measurement of low concentrations of steroids, in particular, testosterone (56, 57). However, while the assays are sometimes tested and validated for use in female or pediatric samples, their suitability for umbilical cord blood analysis is usually assumed and invariably not adequately tested.

Increasing awareness of the limitations of RIA for the measurement of low concentrations of sex steroids (56, 58, 59) has led to the adoption of mass spectrometry as the preferred methodology for the measurement of circulating testosterone levels in women and children, and reported concentrations are consistently lower than those derived by RIA (58, 60–63). A comparison of estradiol and testosterone concentrations in cord blood reported over the past few decades in numerous studies reveals the significant impact of assay methodology on the results obtained, and thus the robustness of the data and any conclusions drawn from it (**Figure 1**). Firstly, it is clear that some assays have reported far higher ranges of these hormones than others, even for the same type of assay; this is mostly apparent in the studies that have employed RIA and raises real concerns around the validity of some of these studies. Secondly, it is clear that much lower values for testosterone are consistently reported in studies that have employed mass spectrometry (either LC–MS/MS or GC–MS) compared to immunoassay-derived values. On the other hand, estradiol levels are overall not dramatically different between RIA and mass spectrometry-based studies [with the exception of the study by Lagiou et al. (46)]. Closer inspection of the immunoassay studies, which reported the lowest testosterone values reveals that these assays employed column purification to help remove interfering cross-reacting steroids (24, 25, 53). Nevertheless, these studies still reported values about 40% higher than those reported by the largest study to date which employed an extensively validated and internally controlled LC–MS/MS assay (22), suggesting that chromatographic treatment was not completely effective in removing all cross-reacting compounds prior to RIA analysis.

Even within the mass-spectrometry studies, there are sometimes quite marked variations in reported values (22, 28, 35, 36, 41, 60). This can reflect variations in internal standardization and extraction efficiency, with some studies measuring multiple hormones and conjugates without controlling for extraction efficiency of each specific analyte (35, 41). Inadequate chromatographic separation of similar steroid species prior to mass spectrometry is also an important source of variation. However, there is also a great deal

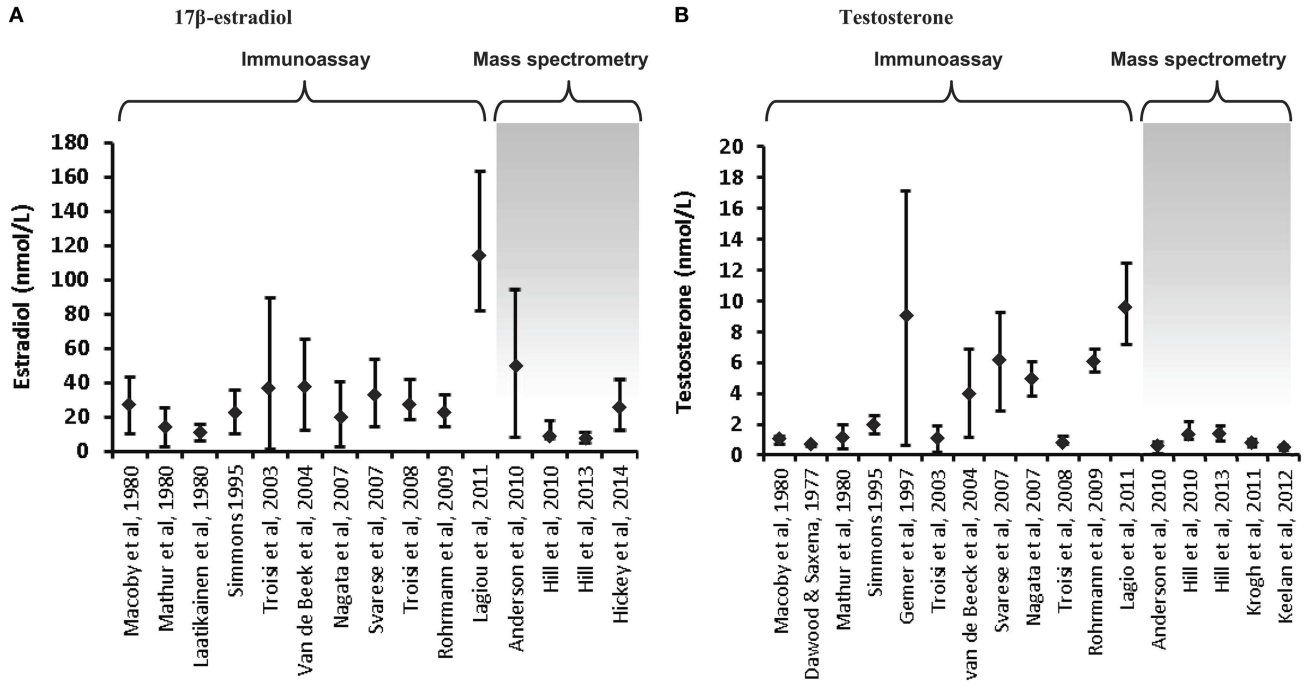

FIGURE 1 | Variations in umbilical cord blood steroid concentrations according to mode of assay. Steroid concentrations in cord blood are displayed as mean ± standard deviation or median and interquartile range, depending on the data available. **(A)** 17β-estradiol concentrations in cord blood (males and females combined) are displayed from 15 studies. The first 11 utilized immunoassay (predominantly RIA); the remaining 4 used LC–MS/MS, apart from the studies by Hill et al. (41), which used GC–MS. **(B)** Testosterone concentrations in cord blood (males or mixed male/female samples). Of the 17 studies included, the final 5 employed mass spectrometry. The last study displayed on each chart has the largest samples size ($n = 860$) and extensive assay validation for cord blood. The steroid values from the studies by Hill et al. (41) are the mean of the published umbilical artery and vein values. The figure demonstrates the influence of assay characteristics on sex steroid values, although the lack of assay specificity is much more significant for measurement of testosterone compared to estradiol.

of variation in the nature of the specimens analyzed in the studies, such that differences in mode of collection, ethnicity, gestational age, labor onset and duration, rates of pregnancy complications, twinning, and sample number/power are all likely to have a major effect on the values obtained (22, 28).

APPLICATIONS OF CORD BLOOD TO UNDERSTANDING HUMAN DEVELOPMENT

The literature reviewed provides general support for the use of cord blood measurements to approximate prenatal sex steroid concentrations, although limitations regarding confounding by mode of delivery and method of analysis need to be considered in the interpretation. The remainder of this review will examine extant applications of cord blood to understanding human behavioral development.

To date, two research groups have used cord blood to examine the relationship between human development and exposure to prenatal hormones (see **Table 1**). Jacklin and colleagues from Stanford University examined a group of participants combined from three longitudinal cohorts. Cohort one comprised infants born at a university hospital during July and August 1973 (40 males, 35 females). The infants in cohorts two and three were born at a nearby general hospital during January–March 1974 (32 males, 42 females) and August–November 1974 (53 males, 54 females), respectively. For offspring to be included in the study, there needed to be no complications of pregnancy or delivery and a 5-min Apgar score of 7 or above. Umbilical cord blood (venous and arterial) was collected at birth and androstenedione, testosterone, estrone, estradiol, and progesterone concentrations were determined by RIA. Cord testosterone concentrations were significantly higher in males than females. No sex differences were observed for the androstenedione, estrone, estradiol, or progesterone levels (33).

Timidity was measured through observation in 162 offspring (84 males, 78 females) at 6, 9, 12, and 18 months (64). No sex difference was found for the timidity measure. For boys,

Table 1 | Summary of the studies examining the relationship between cord hormone concentrations and human development.

Study	Number of participants	Age of participants	Measures			Findings
			Hormones	Assay technique	Outcome measure	
Jacklin et al. (64)	84 males; 78 females	6, 9, 12, and 18 months	Androstenedione; testosterone; estrone; estradiol; progesterone	RIA	Timidity	**Males:** significant negative relationship with testosterone and progesterone, positive relationship with estradiol **Females:** no significant relationship
Jacklin et al. (65)	127 children;	Birth, 3, 6, 9, 12, 18, and 33 months	Androstenedione; testosterone; estrone; estradiol; progesterone	RIA	Muscular strength	**Males:** significant negative relationship with androstenedione, significant positive relationship with progesterone **Females:** significant negative relationship with androstenedione and progesterone
Jacklin et al. (66)	53 males; 43 females	6 years	Androstenedione; testosterone; estrone; estradiol; progesterone	RIA	Reading; Numeracy; Listening; Spatial ability	**Males:** no significant relationship **Females:** significant inverse relationship between testosterone and androstenedione and spatial ability
Hollier et al. (67)	224 males; 199 females	2 years	Testosterone	LC–MS	Vocabulary	**Males:** significant inverse relationship **Females:** no significant relationship
Whitehouse et al. (68)	372 males; 395 females	1–3 years	Testosterone	LC–MS	Language delay	**Males:** significant inverse relationship **Females:** no significant relationship
Farrant et al. (69)	235 males; 232 females	1 and 5 years	Testosterone	LC–MS	Socio-emotional engagement; Vocabulary;	**Males:** no significant relationship after control for covariates **Females:** no significant relationship
Whitehouse et al. (70)	184 males; 190 females	19–20 years	Testosterone	LC–MS	Autism Quotient	**Males:** no significant relationship **Females:** no significant relationship
Robinson et al. (71)	429 males; 430 females	2, 5, 8, and 10 years	Testosterone	LC–MS	Child Behavior Checklist	**Males:** negative relationship between testosterone and attention problems at ages 5, 8, and 10 years **Females:** negative association between testosterone and withdrawal symptoms at age 5

testosterone and progesterone levels were significant negative predictors of timidity, and estradiol levels were a significant positive predictor. No significant relationships were observed between cord hormones concentrations and timidity for girls.

Jacklin et al. (65) investigated the relationship between umbilical cord hormones and muscular strength measured at birth, 3, 6, 9, 12, 18, and 33 months in 127 children. On average, males had greater strength scores than females. A significant negative relationship was found between androstenedione and strength in males and females. However, this relationship was found to be reversed in one of the three cohorts, indicating the relationship is not stable. A significant sex by progesterone interaction was found in all three cohorts, where higher progesterone levels were related to greater strength for males, but to lower strength for females.

The final study by Jacklin and colleagues examined cognitive abilities in a subsample of 96 children (53 males, 43 females) when they were 6 years old. No significant sex differences were found for cognitive ability. A significant inverse relationship was found between cord androgens (testosterone and androstenedione) and spatial ability in girls. No significant associations were found for boys (66).

The data yielded by Jacklin and colleagues indicate that the direction of the association between cord blood hormones and outcome measures may be different between males and females. For boys, cord progesterone concentrations were positively associated with strength in early childhood and negatively related to timidity. For girls, cord progesterone was negatively associated with early childhood strength and cord androgens (testosterone and androstenedione) were inversely related to spatial ability. It should be noted that Jacklin and colleagues measured cord blood hormone concentrations by RIA following extraction and purification. In addition, they used total hormone concentrations, rather than the biologically active proportion. However, the hormone levels reported were similar to a number of studies using mass spectrometry (see **Figure 1**) and their data appear reliable.

The second research group utilized data from the Western Australian Pregnancy Cohort (Raine) study. Between May 1989 and November 1991, 2900 pregnant women were recruited from King Edward Memorial Hospital or nearby private practices. To be included in the study, pregnant women had to have sufficient English language skills, a gestational age between 16 and 18 weeks, an expectation to give birth at King Edward Memorial Hospital Perth and an intention to remain within the state so as to enable follow-up testing. Parents provided written informed consent to participate at each follow-up. Approximately half of the cohort ($n = 1415$) was randomly allocated to an intensive investigation group, and within this group, mixed (venous and arterial) umbilical cord blood was randomly collected from 861 live deliveries. Testosterone, androstenedione, dehydroepiandrosterone (DHEA), estrone, estradiol, estriol, and estetrol concentrations were estimated using LC–MS/MS. The bioavailable proportion of testosterone was calculated, representing the fraction of total testosterone either free (unsequestered by SHBG) or bound to serum albumin. Males had significantly higher levels of cord testosterone than females, while females had significantly higher DHEA concentrations than males (22). There were no significant sex differences for androstenedione, estrone, estradiol, estriol, or estetrol (22, 28).

When offspring were 2 years of age, parents of 426 children (224 male; 199 female) completed the Language Development Survey [LDS; (72)]. Consistent with extensive published literature (73–75), it was found that on average boys had a smaller expressive vocabulary than girls at 2 years of age. In addition, an inverse relationship was found between cord blood testosterone and expressive vocabulary, where higher levels of cord blood testosterone predicted lower vocabulary size in the boys. No relationship was observed in girls (67).

When the offspring were 1, 2, and 3 years of age, parents of 767 children (395 males; 372 females) completed the 12, 24, and 36 month versions of the Infant Monitoring Questionnaire [IMQ; (76)]. The IMQ is a parent report checklist, designed to screen for delayed child development during the early years. Cut-off scores are provided for each scale at each age to indicate a "clinically significant" delay in the development of that particular skill. It was found that males were between two and three times more likely than females to experience language delay at 1, 2, and 3 years of age. In addition, there was a significant association between cord blood testosterone and the rate of language delay in both males and females (70). In line with Hollier et al. (67), it was found that higher levels of cord blood testosterone increased the risk of language delay in males. Interestingly, for females, higher levels of cord blood testosterone were found to be protective for language delay. No significant relationship was observed between the other IMQ scales and cord blood testosterone. The non-significant finding on the Personal–Social scale of the IMQ is in contrast with an inverse association reported by Knickmeyer et al. (77) between amniotic-fluid testosterone levels and parent-reported social skills in their sample of children at 4 years.

Farrant et al. (69) further explored the relationship between cord blood testosterone, socio-emotional engagement, and language development in a subset of 467 children from the Raine sample (235 males, 232 females). Socio-emotional engagement was assessed when the children were 1-year-old using a 14-item Australian revision of the Toddler Temperament Scale (78). Vocabulary was measured using the Peabody Picture Vocabulary Test – Revised [PPVT-R; (79)], when the children were 5 years old. It was found that cord blood testosterone was significantly negatively correlated with socio-emotional engagement and vocabulary in boys. In addition, for boys, socio-emotional engagement was found to completely mediate the relationship between fetal testosterone and vocabulary development. However, when various covariates were included (e.g., maternal age and education, parity, and parent–child book reading) neither fetal testosterone nor socio-emotional engagement were significant predictors of vocabulary development. No significant relationships were observed for girls.

To further extend, the research examining the link between socio-emotional engagement and cord blood testosterone, Whitehouse et al. (68) examined the relationship between cord testosterone and autistic-like traits in adulthood. When the offspring were 19–20 years old 184 males and 190 females completed the Autism-spectrum Quotient [AQ; (80)]. The AQ is a self-report questionnaire, where individuals are provided with 50 statements

and asked to indicate on a four-point scale how well that statement applies to them. No significant relationship was found between cord blood testosterone concentrations and AQ scores for either males or females. This finding is in contrast with results from the Cambridge Fetal Testosterone Project, which include reports of significant associations between amniotic testosterone concentrations and a range of autistic-like traits during early (77, 81, 82) and middle (83) childhood.

The findings from Whitehouse et al. (70), Farrant et al. (69), and Whitehouse et al. (68) indicate there may be no relationship between socio-emotional development and cord blood testosterone at birth. These findings contrast with studies of mid-pregnancy amniotic-fluid steroids, which indicate that socio-emotional development may be related to testosterone exposure during the earlier stages of gestation. The cord blood studies have the advantage that they were conducted on a very large cohort of typically developing infants, although steroid levels were measured at delivery, many weeks after the period expected to be critical for steroid-modulated neurodevelopment. The amniotic-fluid studies, on the other hand, were on relatively small numbers of selected pregnancies with elevated risk of genetic abnormalities, although samples were taken closer to the expected steroid-sensitive developmental window.

Finally, Robinson et al. (71) investigated the relationship between cord blood testosterone and internalizing and externalizing behavior in childhood for the Raine sample. Externalizing and internalizing behaviors were measured when the children (429 males; 430 females) were 2, 5, 8, and 10 years of ages using the Child Behavior Checklist [CBCL; (84, 85)]. The CBCL is a parent-rated questionnaire that includes a Total Behavior Problem scale, an Externalizing Behavior scale (e.g., antisocial or under-controlled behavior), and an Internalizing Behavior scale (e.g., inhibited or over-controlled behavior). In addition, there are eight subscales: withdrawn behavior, somatic complaints, anxiety/depression, delinquency, aggression, social thought, and attention problems. No significant relationships were found between cord blood testosterone and the CBCL total, internalizing or externalizing scales at all ages. When examining the individual subscales, a negative relationship was found between cord testosterone and attention problems for boys at ages 5, 8, and 10 years. For girls, there was a negative association between cord testosterone and withdrawal symptoms at age five. The findings from Robinson et al. (71) did not demonstrate a consistent relationship between fetal testosterone exposure and behavioral difficulties in childhood. However, significant relationships were observed for particular behaviors, which suggest there may be links between fetal testosterone exposure and behavioral development. Further research is warranted to more thoroughly understand the relationship between fetal testosterone exposure and behavioral difficulties in childhood.

In summary, significant negative relationships have been consistently observed between cord blood testosterone and early language development. However, relationships between cord testosterone and other forms of development are still unclear. Some studies indicate that high fetal testosterone exposure is related to more male-typical behavior, while others have found that high fetal testosterone is associated with less male-typical behavior. It is important to note that the analysis of the Raine study samples utilized the more accurate LC–MS/MS method, and calculated the bioavailable proportion of testosterone, both of which add strength to the quality of the data. The studies by Jacklin and colleagues, on the other hand, used a robust RIA but were limited to the use of total hormone concentrations uncorrected for protein binding. To date, only a few studies have been conducted in this area and relationships between cord testosterone and childhood development should be investigated more thoroughly in the future. Furthermore, only Jacklin and colleagues have investigated relationships between cord blood estrogens and early development. Cord estrogen levels have been measured in the Western Australian Pregnancy Cohort; however, the relationship between cord estrogen concentrations and human development has not yet been examined in the cohort.

CONCLUSION

The purpose of the current review was to examine the use of cord blood to measure androgen and estrogen concentrations, and the applicability of this method to understanding human development. From the literature reviewed, it is apparent that cord blood is useful in providing direct measurement of late gestational androgen and estrogen concentrations. However, fetal steroid levels around the time of birth are influenced by obstetric and perinatal factors. It is essential for the accuracy of analyses to take these factors into account when examining associations between cord steroid levels and biological endpoints.

Furthermore, it is important that research using cord blood to measure sex steroids employs properly validated assays. This is to ensure that cross-reacting substances are not measured in error, which is particularly important for the measurement of testosterone concentrations. Most studies to date have used RIA, which has recognized limitations for the measurement of low sex steroid concentrations (56, 58, 59). Mass spectrometry is currently the preferred method for the measurement of circulating hormones in cord blood.

Relatively little research has been performed on cord blood to examine the relationship between fetal hormone exposure and human neurodevelopment. Most of the research in this area has focused on testosterone concentrations. A significant negative relationship between cord testosterone and early language development has been consistently observed (67, 70). However, the relationships between cord testosterone and other aspects of development are still unclear. One of the main criticisms of the use of cord blood to examine the hormonal influences of human development is that hormone effects may occur earlier in gestation. The strength of the relationship between fetal plasma levels at birth and those in early to mid pregnancy is not known. One advantage of cord blood is that it provides a measure of hormone exposure later in gestational development. This is of particular importance given that animal studies have found that the effects of hormones on development are not restricted to the first two trimesters (12, 86). Examining the congruence between hormones obtained during an earlier period of gestation (e.g., via amniotic fluid) and hormones obtained from cord blood is an urgent priority for this field of research.

To date, only Jacklin and colleagues have examined the relationship between cord blood estrogens and human development. However, cord blood hormone concentrations were measured using RIA (64–66). In addition, adjustments for protein binding were not taken into account. Therefore, it is difficult to draw definitive conclusions from their findings. More research needs to be conducted to examine the relationship between human development and cord estrogen concentrations. Furthermore, based on conflicting evidence of sex differences in cord estrogen concentrations (16, 24, 25, 28, 32, 33, 35–37), it has been suggested that the ratio of androgens to estrogens may underlie sex-associated developmental outcomes, rather than absolute concentrations. Future research should investigate possible relationships between the androgen to estrogen ratio and aspects of human development.

In conclusion, the collection of cord blood is currently the most practical means of approximating circulating fetal hormones in normal pregnancy. The use of cord blood to examine the relationship between fetal hormones and human development is promising, but requires validation and further investigation. It is important that future research in this area uses properly validated assays to determine hormone concentrations and takes into account any possible confounding factors. The results of studies that do not take these steps may lack accuracy and valid interpretation.

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REFERENCES

1. Collaer ML, Hines M. Human behavioral sex differences: a role for gonadal hormones during early development? *Psychol Bull* (1995) **118**(1):55–107. doi:10.1037/0033-2909.118.1.55
2. Finegan J-A, Bartleman B, Wong P. A window for the study of prenatal sex hormone influences on postnatal development. *J Genet Psychol* (1989) **150**(1):101–12. doi:10.1080/00221325.1989.9914580
3. Ngun TC, Ghahramani N, Sánchez FJ, Bocklandt S, Vilain E. The genetics of sex differences in brain and behavior. *Front Neuroendocrinol* (2011) **32**(2):224–46. doi:10.1016/j.yfrne.2010.10.001
4. Bettencourt B, Miller N. Gender differences in aggression as a function of provocation: a meta-analysis. *Psychol Bull* (1996) **119**(3):422. doi:10.1037/0033-2909.119.3.422
5. McIntyre MH, Barrett ES, McDermott R, Johnson DDP, Cowden J, Rosen SP. Finger length ratio (2D:4D) and sex differences in aggression during a simulated war game. *Pers Individ Dif* (2007) **42**(4):755–64. doi:10.1016/j.paid.2006.08.009
6. Hines M. *Brain Gender*. New York: Oxford University Press (2004).
7. Collaer ML, Nelson JD. Large visuospatial sex difference in line judgment: possible role of attentional factors. *Brain Cogn* (2002) **49**(1):1–12. doi:10.1006/brcg.2001.1321
8. Voyer D, Voyer S, Bryden MP. Magnitude of sex differences in spatial abilities: a meta-analysis and consideration of critical variables. *Psychol Bull* (1995) **117**(2):250. doi:10.1037/0033-2909.117.2.250
9. Herlitz A, Airaksinen E, Nordström E. Sex differences in episodic memory: the impact of verbal and visuospatial ability. *Neuropsychology* (1999) **13**(4):590. doi:10.1037/0894-4105.13.4.590
10. Lewin C, Wolgers G, Herlitz A. Sex differences favoring women in verbal but not in visuospatial episodic memory. *Neuropsychology* (2001) **15**(2):165. doi:10.1037/0894-4105.15.2.165
11. Sherwin BB. Estrogen and cognitive functioning in women. *Endocr Rev* (2003) **24**(2):133–51. doi:10.1210/er.2001-0016
12. Zambrano E, Guzmán C, Rodríguez-González GL, Durand-Carbajal M, Nathanielsz PW. Fetal programming of sexual development and reproductive function. *Mol Cell Endocrinol* (2014) **382**(1):538–49. doi:10.1016/j.mce.2013.09.008
13. Dean A, Sharpe RM. Anogenital distance or digit length ratio as measures of fetal androgen exposure: relationship to male reproductive development and its disorders. *J Clin Endocrinol Metab* (2013) **98**(6):2230–8. doi:10.1210/jc.2012-4057
14. Knickmeyer RC, Woolson S, Hamer RM, Konneker T, Gilmore JH. 2D:4D ratios in the first 2 years of life: stability and relation to testosterone exposure and sensitivity. *Horm Behav* (2011) **60**(3):256–63. doi:10.1016/j.yhbeh.2011.05.009
15. Nagami M, McDonough P, Ellegood J, Mahesh V. Maternal and amniotic fluid steroids throughout human pregnancy. *Am J Obstet Gynecol* (1979) **134**:674–80.
16. van de Beek C, Thijssen JHH, Cohen-Kettenis PT, van Goozen SHM, Buitelaar JK. Relationships between sex hormones assessed in amniotic fluid, and maternal and umbilical cord serum: what is the best source of information to investigate the effects of fetal hormonal exposure? *Horm Behav* (2004) **46**(5):663–9. doi:10.1016/j.yhbeh.2004.06.010
17. Kuijper EAM, Ket JCF, Caanen MR, Lambalk CB. Reproductive hormone concentrations in pregnancy and neonates: a systematic review. *Reprod Biomed Online* (2013) **27**(1):33–63. doi:10.1016/j.rbmo.2013.03.009
18. Albrecht ED, Pepe GJ. Placental steroid hormone biosynthesis in primate pregnancy. *Endocr Rev* (1990) **11**(1):124–50. doi:10.1210/edrv-11-1-124
19. Pašková A, Parízek A, Hill M, Velíková M, Kubátová J, Dušková M, et al. Steroid metabolome in the umbilical cord: is it necessary to differentiate between arterial and venous blood? *Physiol Res* (2014) **63**(1):115–26.
20. Reis FM, Florio P, Cobellis L, Luisi S, Severi FM, Bocchi C, et al. Human placenta as a source of neuroendocrine factors. *Biol Neonate* (2001) **79**(3–4):150–6. doi:10.1159/000047083
21. Ishimoto H, Jaffe RB. Development and function of the human fetal adrenal cortex: a key component in the feto-placental unit. *Endocr Rev* (2011) **32**(3):317–55. doi:10.1210/er.2010-0001
22. Keelan JA, Mattes E, Tan H, Dinan A, Newnham JP, Whitehouse AJO, et al. Androgen concentrations in umbilical cord blood and their association with maternal, fetal and obstetric factors. *PLoS One* (2012) **7**(8):e42827. doi:10.1371/journal.pone.0042827
23. Cohen-Bendahan CCC, van Goozen SHM, Buitelaar JK, Cohen-Kettenis PT. Maternal serum steroid levels are unrelated to fetal sex: a study in twin pregnancies. *Twin Res Hum Genet* (2005) **8**:173–7. doi:10.1375/twin.8.2.173
24. Troisi R, Potischman N, Roberts J, Siiteri P, Daftary A, Sims C, et al. Associations of maternal and umbilical cord hormone concentrations with maternal, gestational and neonatal factors (United States). *Cancer Causes Control* (2003) **14**(4):347–55. doi:10.1023/A:1023934518975
25. Troisi R, Potischman N, Roberts JM, Harger G, Markovic N, Cole B, et al. Correlation of serum hormone concentrations in maternal and umbilical cord samples. *Cancer Epidemiol Biomarkers Prev* (2003) **12**(5):452–6.
26. Hickey M, Sloboda DM, Atkinson HC, Doherty DA, Franks S, Norman RJ, et al. The relationship between maternal and umbilical cord androgen levels and polycystic ovary syndrome in adolescence: a prospective cohort study. *J Clin Endocrinol Metab* (2009) **94**(10):3714–20. doi:10.1210/jc.2009-0544
27. Sloboda DM, Hickey M, Hart R. Reproduction in females: the role of the early life environment. *Hum Reprod Update* (2011) **17**(2):210–27. doi:10.1093/humupd/dmq048
28. Hickey M, Hart R, Keelan JA. The relationship between umbilical cord estrogens and perinatal characteristics. *Cancer Epidemiol Biomarkers Prev* (2014). doi:10.1158/1055-9965.EPI-13-1321
29. Smith LM, Cloak CC, Poland RE, Torday J, Ross MG. Prenatal nicotine increases testosterone levels in the fetus and female offspring. *Nicotine Tob Res* (2003) **5**(3):369–74. doi:10.1080/14622203100009419 6
30. Varvarigou AA, Liatsis SG, Vassilakos P, Decavalas G, Beratis NG. Effect of maternal smoking on cord blood estriol, placental lactogen, chorionic gonadotropin, FSH, LH, and cortisol. *J Perinat Med* (2009) **37**(4):364–9. doi:10.1515/JPM.2009.028
31. Pardridge WM. Serum bioavailability of sex steroid hormones. *Clin Endocrinol Metab* (1986) **15**(2):259–78. doi:10.1016/S0300-595X(86)80024-X
32. Herruzo AJ, Mozas J, Alarcón JL, López JM, Molina R, Molto L, et al. Sex differences in serum hormone levels in umbilical vein blood. *Int J Gynecol Obstet* (1993) **41**(1):37–41. doi:10.1016/0020-7292(93)90152-M

33. Maccoby EE, Doering CH, Jacklin CN, Kraemer H. Concentrations of sex hormones in umbilical-cord blood: their relation to sex and birth order of infants. *Child Dev* (1979) **50**(3):632–42. doi:10.2307/1128928
34. Beek C, Goozen SM, Buitelaar J, Cohen-Kettenis P. Prenatal sex hormones (maternal and amniotic fluid) and gender-related play behavior in 13-month-old infants. *Arch Sex Behav* (2009) **38**(1):6–15. doi:10.1007/s10508-007-9291-z
35. Hill M, Pašková A, Kanceva R, Velíková M, Kubátová J, Kancheva L, et al. Steroid profiling in pregnancy: a focus on the human fetus. *J Steroid Biochem Mol Biol* (2014) **139**(0):201–22. doi:10.1016/j.jsbmb.2013.03.008
36. Anderson H, Fogel N, Grebe SK, Singh RJ, Taylor RL, Dunaif A. Infants of women with polycystic ovary syndrome have lower cord blood androstenedione and estradiol levels. *J Clin Endocrinol Metab* (2010) **95**(5):2180–6. doi:10.1210/jc.2009-2651
37. Simmons D, France JT, Keelan JA, Song L, Knox BS. Sex differences in umbilical cord serum levels of inhibin, testosterone, oestradiol dehydroepiandrosterone sulphate, and sex hormone-binding globulin in human term neonates. *Neonatology* (1994) **65**(5):287–94. doi:10.1159/000244074
38. Balthazart J. Minireview: hormones and human sexual orientation. *Endocrinology* (2011) **152**(8):2937–47. doi:10.1210/en.2011-0277
39. Sathishkumar K, Elkins R, Yallampalli U, Balakrishnan M, Yallampalli C. Fetal programming of adult hypertension in female rat offspring exposed to androgens in utero. *Early Hum Dev* (2011) **87**(6):407–14. doi:10.1016/j.earlhumdev.2011.03.001
40. Clifton VL, Bisits A, Zarzycki PK. Characterization of human fetal cord blood steroid profiles in relation to fetal sex and mode of delivery using temperature-dependent inclusion chromatography and principal component analysis (PCA). *J Chromatogr B Analyt Technol Biomed Life Sci* (2007) **855**(2):249–54. doi:10.1016/j.jchromb.2007.05.041
41. Hill M, Pařízek A, Kancheva R, Dušková M, Velíková M, Kříž L, et al. Steroid metabolome in plasma from the umbilical artery, umbilical vein, maternal cubital vein and in amniotic fluid in normal and preterm labor. *J Steroid Biochem Mol Biol* (2010) **121**(3–5):594–610. doi:10.1016/j.jsbmb.2009.10.012
42. Dawood MY, Saxena BB. Testosterone and dihydrotestosterone in maternal and cord blood and in amniotic fluid. *Am J Obstet Gynecol* (1977) **129**(1):37–42.
43. Gemer O, Sevillia J, Zalis J, Segal S. Umbilical cord androgens in infants of diabetic mothers. *Arch Gynecol Obstet* (1997) **259**(3):139–41. doi:10.1007/BF02505322
44. Hickey M, Doherty DA, Hart R, Norman RJ, Mattes E, Atkinson HC, et al. Maternal and umbilical cord androgen concentrations do not predict digit ratio (2D:4D) in girls: a prospective cohort study. *Psychoneuroendocrinology* (2010) **35**(8):1235–44. doi:10.1016/j.psyneuen.2010.02.013
45. Laatikainen TJ, Pelkonen JM, Pesonen AK. Steroid concentrations in placenta and fetal membranes at elective caesarean section and after spontaneous labour. *Placenta* (1982) **3**(3):319–24. doi:10.1016/S0143-4004(82)80008-8
46. Lagiou P, Samoli E, Okulicz W, Xu B, Lagiou A, Lipworth L, et al. Maternal and cord blood hormone levels in the United States and china and the intrauterine origin of breast cancer. *Ann Oncol* (2011) **22**(5):1102–8. doi:10.1093/annonc/mdq565
47. Mathur RS, Landgrebe S, Moody LO, Powell S, Williamson HO. Plasma steroid concentrations in maternal and umbilical circulation after spontaneous onset of labor. *J Clin Endocrinol Metab* (1980) **51**(6):1235–8. doi:10.1210/jcem-51-6-1235
48. Nagata C, Iwasa S, Shiraki M, Sahashi Y, Shimizu H. Association of maternal fat and alcohol intake with maternal and umbilical hormone levels and birth weight. *Cancer Sci* (2007) **98**(6):869–73. doi:10.1111/j.1349-7006.2007.00464.x
49. Rohrmann S, Sutcliffe CG, Bienstock JL, Monsegue D, Akereyeni F, Bradwin G, et al. Racial variation in sex steroid hormones and the insulin-like growth factor axis in umbilical cord blood of male neonates. *Cancer Epidemiol Biomarkers Prevent* (2009) **18**(5):1484–91. doi:10.1158/1055-9965.EPI-08-0817
50. Savarese T, Strohsnitter W, Low H, Liu Q, Baik I, Okulicz W, et al. Correlation of umbilical cord blood hormones and growth factors with stem cell potential: implications for the prenatal origin of breast cancer hypothesis. *Breast Cancer Res* (2007) **9**(3):1–10. doi:10.1186/bcr1674
51. Simmons D. Interrelation between umbilical cord serum sex hormones, sex hormone-binding globulin, insulin-like growth factor i, and insulin in neonates from normal pregnancies and pregnancies complicated by diabetes. *J Clin Endocrinol Metab* (1995) **80**(7):2217–21. doi:10.1210/jc.80.7.2217
52. Troisi R, Hoover RN, Thadhani R, Hsieh CC, Sluss P, Ballard-Barbash R, et al. Maternal, prenatal and perinatal characteristics and first trimester maternal serum hormone concentrations. *Br J Cancer* (2008) **99**(7):1161–4. doi:10.1038/sj.bjc.6604639
53. Troisi R, Lagiou P, Trichopoulos D, Xu B, Chie L, Stanczyk FZ, et al. Cord serum estrogens, androgens, insulin-like growth factor-i, and insulin-like growth factor binding protein-3 in Chinese and U.S. Caucasian neonates. *Cancer Epidemiol Biomarkers Prev* (2008) **17**(1):224–31. doi:10.1158/1055-9965.EPI-07-0536
54. Troisi R, Potischman N, Johnson CN, Roberts JM, Lykins D, Harger G, et al. Estrogen and androgen concentrations are not lower in the umbilical cord serum of pre-eclamptic pregnancies. *Cancer Epidemiol Biomarkers Prev* (2003) **12**(11):1268–70.
55. Troisi R, Potischman N, Roberts JM, Ness R, Crombleholme W, Lykins D, et al. Maternal serum oestrogen and androgen concentrations in preeclamptic and uncomplicated pregnancies. *Int J Epidemiol* (2003) **32**(3):455–60. doi:10.1093/ije/dyg094
56. Rosner W, Auchus RJ, Azziz R, Sluss PM, Raff H. Utility, limitations, and pitfalls in measuring testosterone: an endocrine society position statement. *J Clin Endocrinol Metab* (2007) **92**(2):405–13. doi:10.1210/jc.2006-1864
57. Stanczyk FZ. Measurement of androgens in women. *Paper Presented at the Seminars in Reproductive Medicine*. New York (2006).
58. Demers LM. Androgen deficiency in women; role of accurate testosterone measurements. *Maturitas* (2010) **67**(1):39–45. doi:10.1016/j.maturitas.2010.04.019
59. Stanczyk FZ, Cho MM, Endres DB, Morrison JL, Patel S, Paulson RJ. Limitations of direct estradiol and testosterone immunoassay kits. *Steroids* (2003) **68**(14):1173–8. doi:10.1016/j.steroids.2003.08.012
60. Krogh C, Cohen AS, Basit S, Hougaard DM, Biggar RJ, Wohlfahrt J, et al. Testosterone levels in umbilical-cord blood and risk of pyloric stenosis. *Pediatrics* (2011) **127**(1):e197–201. doi:10.1542/peds.2010-2127
61. Soldin OP, Guo T, Weiderpass E, Tractenberg RE, Hilakivi-Clarke L, Soldin SJ. Steroid hormone levels in pregnancy and 1 year postpartum using isotope dilution tandem mass spectrometry. *Fertil Steril* (2005) **84**(3):701–10. doi:10.1016/j.fertnstert.2005.02.045
62. Stanczyk FZ, Clarke NJ. Advantages and challenges of mass spectrometry assays for steroid hormones. *J Steroid Biochem Mol Biol* (2010) **121**(3–5):491–5. doi:10.1016/j.jsbmb.2010.05.001
63. Vicente FB, Smith FA, Sierra R, Wang S. Measurement of serum testosterone using high-performance liquid chromatography/tandem mass spectrometry. *Clin Chem Lab Med* (2006) **44**:70. doi:10.1515/CCLM.2006.014
64. Jacklin CN, Maccoby EE, Doering CH. Neonatal sex-steroid hormones and timidity in 6-18-month-old boys and girls. *Dev Psychobiol* (1983) **16**(3):163–8. doi:10.1002/dev.420160302
65. Jacklin CN, Maccoby EE, Doering CH, King DR. Neonatal sex-steroid hormones and muscular strength of boys and girls in the first three years. *Dev Psychobiol* (1984) **17**(3):301–10. doi:10.1002/dev.420170309
66. Jacklin CN, Wilcox KT, Maccoby EE. Neonatal sex-steroid hormones and cognitive abilities at six years. *Dev Psychobiol* (1988) **21**(6):567–74. doi:10.1002/dev.420210607
67. Hollier LP, Mattes E, Maybery MT, Keelan JA, Hickey M, Whitehouse AJO. The association between perinatal testosterone concentration and early vocabulary development: a prospective cohort study. *Biol Psychol* (2013) **92**(2):212–5. doi:10.1016/j.biopsycho.2012.10.016
68. Whitehouse AJO, Mattes E, Maybery M, Dissanayake C, Sawyer M, Jones R, et al. Perinatal testosterone exposure and autistic-like traits in the general population: a longitudinal pregnancy-cohort study. *J Neurodev Disord* (2012) **4**(1):25. doi:10.1186/1866-1955-4-25
69. Farrant BM, Mattes E, Keelan JA, Hickey M, Whitehouse AJO. Fetal testosterone, socio-emotional engagement and language development. *Infant Child Dev* (2013) **22**(2):119–32. doi:10.1002/icd.1771
70. Whitehouse AJO, Mattes E, Maybery MT, Sawyer MG, Jacoby P, Keelan JA, et al. Sex-specific associations between umbilical cord blood testosterone levels and language delay in early childhood. *J Child Psychol Psychiatry* (2012) **53**(7):726–34. doi:10.1111/j.1469-7610.2011.02523.x
71. Robinson M, Whitehouse AJO, Jacoby P, Mattes E, Sawyer MG, Keelan JA, et al. Umbilical cord blood testosterone and childhood internalizing and externalizing behavior: a prospective study. *PLoS One* (2013) **8**(4):e59991. doi:10.1371/journal.pone.0059991
72. Rescorla L. The language development survey: a screening tool for delayed language in toddlers. *J Speech Hear Disord* (1989) **54**(4):587–99.
73. Berglund EVA, Eriksson M, Westerlund M. Communicative skills in relation to gender, birth order, childcare and socioeconomic status in 18-month-old

children. *Scand J Psychol* (2005) **46**(6):485–91. doi:10.1111/j.1467-9450.2005.00480.x
74. Feldman HM, Dollaghan CA, Campbell TF, Kurs-Lasky M, Janosky JE, Paradise JL. Measurement properties of the Macarthur communicative development inventories at ages one and two years. *Child Dev* (2000) **71**(2):310–22. doi:10.1111/1467-8624.00146
75. Zubrick SR, Taylor CL, Rice ML, Slegers DW. Late language emergence at 24 months: an epidemiological study of prevalence, predictors, and covariates. *J Speech Lang Hear Res* (2007) **50**(6):1562–92. doi:10.1044/1092-4388(2007/106)
76. Squires J, Bricker D, Potter A. *Infant/Child Monitoring Questionnaires Procedures Manual*. Eugene: University of Oregon (1990).
77. Knickmeyer RC, Baron-Cohen S, Raggatt P, Taylor K. Foetal testosterone, social relationships, and restricted interests in children. *J Child Psychol Psychiatry* (2005) **46**(2):198–210. doi:10.1111/j.1469-7610.2004.00349.x
78. Fullard W, McDevitt SC, Carey WB. Assessing temperament in one-to three-year-old children. *J Pediat Psychol* (1984) **9**(2):205–17. doi:10.1093/jpepsy/9.2.205
79. Dunn LM, Dunn LM. *Peabody Picture and Vocabulary Test*. 4th ed. Bloomington: Pearson (2007).
80. Baron-Cohen S, Wheelwright S, Skinner R, Martin J, Clubley E. The autism-spectrum quotient (aq): evidence from Asperger syndrome/high-functioning autism, males and females, scientists and mathematicians. *J Autism Dev Disord* (2001) **31**(1):5–17. doi:10.1023/A:1005653411471
81. Auyeung B, Taylor K, Hackett G, Baron-Cohen S. Foetal testosterone and autistic traits in 18 to 24-month-old children. *Mol Autism* (2010) **1**(1):11. doi:10.1186/2040-2392-1-11
82. Lutchmaya S, Baron-Cohen S, Raggatt P. Foetal testosterone and eye contact in 12-month-old human infants. *Infant Behav Dev* (2002) **25**(3):327–35. doi:10.1016/S0163-6383(02)00094-2
83. Auyeung B, Baron-Cohen S, Ashwin E, Knickmeyer R, Taylor K, Hackett G. Fetal testosterone and autistic traits. *Br J Psychol* (2009) **100**:1–22. doi:10.1348/000712608X311731
84. Achenbach TM. *Manual for the Child Behaviour Checklist/4-18*. Burlington: University of Vermont (1991).
85. Achenbach TM, Edelbrock C, Howell CT. Empirically based assessment of the behavioral/emotional problems of 2- and 3-year-old children. *J Abnorm Child Psychol* (1987) **15**(4):629–50. doi:10.1007/BF00917246
86. Roselli CE, Estill CT, Stadelman HL, Meaker M, Stormshak F. Separate critical periods exist for testosterone-induced differentiation of the brain and genitals in sheep. *Endocrinology* (2011) **152**(6):2409–15. doi:10.1210/en.2010-1445

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Dispatches from the interface of salivary bioscience and neonatal research

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The emergence of the interdisciplinary field of salivary bioscience has created opportunity for neonatal researchers to measure multiple components of biological systems non-invasively in oral fluids. The implications are profound and potentially high impact. From a single oral fluid specimen, information can be obtained about a vast array of biological systems (e.g., endocrine, immune, autonomic nervous system) and the genetic polymorphisms related to individual differences in their function. The purpose of this review is to describe the state of the art for investigators interested in integrating these unique measurement tools into the current and next generation of research on gonadal steroid exposure during the prenatal and neonatal developmental periods.

Keywords: saliva analytes, saliva collection, testosterone, DHEA, estrogen, neonatal, analytical strategy

INTRODUCTION

The direction of research in the field of human development is being influenced by theoretical models that champion the study of individual differences using multi-level analyses of the confluence of prenatal, biological, behavioral, and social contextual forces [e.g., Ref. (1–4)]. Only recently have advances in salivary bioscience enabled investigators to test some of the core theoretical assumptions directly. Technical innovations now reveal that information may be obtained from oral fluid specimens about a broad array of physiologic systems including adrenal and gonadal steroids, inflammatory proteins of various types, infectious disease antibodies, environmental chemical exposures, metabolic markers, and genetic variability relevant to basic biological function, health, and disease. The attention that saliva has received as a biospecimen is largely due to the perceptions of sample collection as quick, uncomplicated, cost-efficient, and safe, and of salivary assays as reliable and accurate (see **Table 1**). The purpose of this report is to provide a roadmap for investigators interested in pushing cutting-edge integration of these measurement tools into the next generation of studies on gonadal steroid exposure and child development.

To date, the range of salivary analytes that have been integrated into developmental science has been restricted relative to the possibilities. The majority of studies with infants and children have primarily targeted cortisol as the salivary marker of choice given known associations with prenatal exposure [e.g., Ref. (5)], child behavior [e.g., Ref. (6, 7)], and in following with developmental origins theory, that early calibration of stress systems impacts health and development across the life course (8). However, the salivary proteome contains more than 1000 detectable analytes (9), including more understudied outputs of the hypothalamic–pituitary–gonadal (HPG) axis. Saliva contains measurable estrogens, androgens, and progesterone, yet few studies have collected oral fluid for this purpose in women during the antepartum and postpartum periods or in neonates (see **Table 2**); most have relied on serum.

The extant literature on gonadal steroids focuses largely on levels observed during puberty and adulthood [see reviews (30, 31)]; we know far less about gonadal steroid exposure in early life and the majority of studies have relied on quantification from serum or plasma. However, not only do gonadal steroids of maternal origin cross the placental barrier and target the developing fetal brain (32, 33), but also birth marks a significant hormonal transition as the influence of the maternal endocrine milieu is withdrawn. In the first month following birth, infants exhibit a postnatal surge in gonadal steroids that parallel levels reached in adolescence, referred to as "mini-puberty" (34, 35). Further, steroid levels found in saliva, albeit lower in concentration, are correlated with serum constituents, particularly the free fraction which exerts physiological effects (12, 36).

To this end, we propose measurement considerations for oral fluid and applications of salivary analytes in studies of maternal and neonatal gonadal steroid activity for future investigation.

SALIVARY MEASUREMENT CONSIDERATIONS

PRIMER ON ORAL FLUID AS A BIOSPECIMEN

"Saliva" is a composite of oral fluids secreted from many different glands. The source glands are located in the upper posterior area of oral cavity, lower area of the mouth between the cheek and jaw, and under the tongue. There are also many minor secretory glands in the lip, cheek, tongue, and palate. A small fraction of oral fluid (crevicular fluid) also comes from serum leakage in the cleft area between each tooth and its surrounding gums, or via leakage from serum due to mucosal injury or inflammation. Under

healthy conditions, the contribution from serum is minimal but under conditions of mucosal or epithelial inflammation, serum constituents can represent a substantial portion of the analytes in the oral fluid pool. Each secretory gland produces a fluid that differs in volume, composition, and constituents [e.g., Ref. (37)]. Each source gland's contribution to the pool of oral fluid varies (38). Oral fluid is water-like in composition and has a pH (acidity) range between 6 and 9. Foods and substances placed in the mouth are capable of changing specimen acidity because oral fluid has minimal buffering capacity. These changes in sample integrity have been shown to compromise measurement validity [e.g., Ref. (39)].

Table 1 | Perceived "advantages" of oral fluids as a research specimen.

"Minimally invasive"	Considered "acceptable and non-invasive" by research participants and patients
	Collection is quick, non-painful, uncomplicated
"Safety"	Reduces transmission of infectious disease by eliminating the potential for accidental needle sticks
	CDC does not consider saliva a class II biohazard unless visibly contaminated with blood
"Self-collection"	Allows for community- and home-based collection
	Enables specimen collection in special populations
"Economics"	Eliminates the need for a health care intermediary (e.g., phlebotomist, nurse)
	Resources for collection and processing samples are low cost and available
"Accuracy"	Salivary levels of many analytes represent the "free unbound fraction" or biological active fraction in the general circulation

Gonadal hormones are exemplars of salivary analytes that reflect levels in general circulation, otherwise known as serum constituents. Serum constituents are transported into saliva either by *filtration* between the tight spaces between cells in the salivary glands or *diffusion* through cell membranes thereby enabling investigators to make interferences about systemic physiological states. Other analytes found in oral fluids are synthesized, stored, and released from the granules within the secretory cells of the saliva glands (i.e., enzymes, mucins, cystatins, histatins). Still others are components of humoral (antibodies, complement) immunity or compounds (cytokines) secreted by cells (neutrophils, macrophages, lymphocytes) of the mucosal immune system. An understanding of whether an analyte is transported into oral fluid by filtration or passive diffusion, secreted from salivary glands, or released or derived from cells locally in the oral mucosa is key to interpreting the meaning of individual differences in that measure.

The secretion of oral fluids is influenced by the day–night cycle; chewing movement of the mandibles; taste and smell; iatrogenic effects of medications that cause xerostomia (dry mouth); as well as medical interventions and conditions that affect saliva gland function. Saliva glands are directly innervated by parasympathetic and sympathetic nerves (40). Not surprisingly, activation of the ANS component of the psychobiology of the stress response affects saliva flow rates. The levels of salivary analytes that migrate into saliva from blood by filtration through the junctions between cells in the salivary gland (e.g., dehydroepiandrosterone-sulfate and other conjugated steroids) are influenced by the rate of saliva secretion [e.g., Ref. (41)].

The U.S. Centers for Disease Control notes that unless visibly contaminated with blood, oral fluid is not a class II biohazard. This statement has contributed to the perception that, among behaviorally scientists, saliva is *safer* to work with than blood. In

Table 2 | Gonadal steroid salivary analytes of interest to developmental science and past human studies utilizing salivary assessment in the prenatal and neonatal periods.

	Study population			Reference
	Antepartum women	Postpartum women	Neonates	
ESTROGENS				
Estradiol	x	x		(10–12)
Esterone	x	x		(10–12)
Estriol	x	x		(10–20)
ANDROGENS				
Testosterone	x	x	x	(11, 12, 21)
Dehydroepiandrosterone and -sulfate	x	x		(12)
Androstenedione[a]				
PROGESTAGENS				
Progesterone; 17-OH progesterone	x	x	x	(10, 12, 15, 16, 20, 22–24)
Aldosterone	x			(25)

Sources to generate list of gonadal steroid analytes: Tabak (26); Malamud and Tabak (27); Cone and Huestis (28); U.S. Department of Health and Human Services (29).
[a]To our knowledge, past studies have exclusively sampled plasma androstenedione; no studies to date have indexed salivary androstenedione in women during the antepartum or postpartum periods or in neonates.

reality, even under normative-healthy conditions, more than 250 species of bacteria are present in oral fluids (42). During upper respiratory infections, oral fluids are highly likely to contain agents of disease (43). An informal survey of biosafety policies at North American and UK academic institutions reveals a consensus that oral fluid specimens should be handled with *universal precautions* when employed for use in research or diagnostic applications.

SAMPLE COLLECTION

In the past, saliva collection devices have involved cotton-based absorbent materials [e.g., Ref. (44, 45)]. Placed in the mouth for 2–3 min, oral fluids rapidly saturate the cotton; the specimen is expressed into collection vials by centrifugation or compression [e.g., Ref. (46)]. Most of the time, this approach is convenient, simple, and time-efficient. However, when the absorbent capacity is large and sample volume is small, as is the case for neonates, the specimen absorbed can be diffusely distributed in the cotton fibers, making sample recovery problematic [e.g., Ref. (47)]. The process of absorbing oral fluid with cotton, and other materials, also has the potential to interfere with the immunoassay of several salivary analytes (48, 49).

Early studies with neonates employed serum assays modified for use with saliva by, among other things, requiring large saliva test volumes (200–400 μl). To collect sufficient test volumes, saliva flow was often stimulated using techniques that involved tasting (sugar crystals, citric acid drops) substances. When not used minimally and/or consistently, some of these methods are capable of changing immunoassay performance [e.g., Ref. (39)]. Indirectly, stimulants also influence measurement of the levels of salivary analytes that are dependent on saliva flow rate (dehydroepiandrosterone-sulfate, DHEA-S). Saliva collected from neonates requires a technique that uses an absorbent material (50). Current collection methods use oral swabs, which are ideal for neonates and infants. This is because the swab can be narrower in diameter, particularly well-suited for the small mouths of neonates, and the material is non-toxic and durable for use with older infants that may gum the device during collection.

SAMPLE HANDLING, TRANSPORT, AND STORAGE

Typically, once specimens are collected, they should be kept cold or frozen. Refrigeration prevents degradation of some salivary analytes and restricts the activity of proteolytic enzymes and growth of bacteria. We explored the impact of bacteria-related issues on the measurement of salivary analytes. Significant declines occur in the levels of some salivary analytes when samples are stored at RT or 4°C in comparison to −60°C after 96 h (51). Whembolua and colleagues (52) showed that changes in salivary analytes related to storage temperatures were associated with growth in bacteria, but not baseline bacteria levels. The way in which samples are handled, stored, and transported after collection has the potential to influence sample integrity and measurement validity. Our recommendation is conservative. After collection, saliva samples should be kept frozen. If freezing is not possible, then at a minimum, samples should be kept cold (on ice or refrigerated) until they can be frozen that day. Repeated freeze–thaw cycles should be avoided with saliva samples. In our experience, DHEA, estradiol, and progesterone are very sensitive to freeze–thaw, whereas testosterone is robust (up to at least three cycles). This position is consistent with aliqouting and archiving frozen samples in anticipation that biotechnology advances will enable different markers to be assayed in the future. It should also be noted that some salivary analytes may require specimens to be directly collected into storage vials that are chilled [Ref. (53); but see Ref. (54)] or treated with neuropeptidase inhibitors (such as EDTA or aprotinin) to minimize rapid degradation (55, 56). For large-scale national surveys, investigators working in remote areas [e.g., Ref. (57, 58)], or patients collecting samples at home (59), freezing and shipping these frozen samples can be logistically complex and cost-prohibitive. Under special circumstances, saliva may not be the biospecimen of choice; alternatively, the degree of the impact of the handling and storage conditions should be documented by pilot work.

CONTAMINANTS IN ORAL FLUID

Most of the time, to meaningfully index *systemic* biological activity, quantitative estimates of an analyte (e.g., hormone) in saliva must be highly correlated with the levels measured in serum. The magnitude of this serum–saliva association depends, in part, on consistency in the processes (27) used to transport circulating molecules into oral fluids. When the integrity of diffusion or filtration is compromised, the level of the serological marker in saliva will be affected because of the differential concentration gradient. It also depends on the presence of contaminants in oral fluid, which investigators should be aware of, control for if possible, and in all cases, document.

Blood and blood products can leak into oral fluids via burns, abrasions, or cuts to the cheek, tongue, or gums. Blood in oral fluid is more prevalent among individuals who suffer from poor oral health (i.e., open sores, periodontal disease, gingivitis), endure certain infectious diseases [e.g., HIV; Ref. (60)], and engage in behavior known to influence oral health negatively [e.g., tobacco use; Ref. (61)]. Saliva contaminated with blood will present varying degrees of yellow-brownish hue (62). Utilizing salivary transferrin as a surrogate marker, our bench (spiking blood into saliva) and experimental studies (inducing microinjury to the mouth by brushing the gums) have shown the degree of blood contamination needed to influence salivary hormone levels. Differences among the lengths of the effect (10–45 min) depend on the salivary analyte of interest (62–64). Gonadal steroids appear to be more susceptible to blood leakage in the oral cavity, with testosterone in particular showing an increase in response to microinjury in the oral cavity (62). At the same time, $<0.1\%$ of statistical outliers ($+2.5$ SDs) in salivary hormone distributions have been shown to be associated with elevated transferrin (65). Thus, blood contamination of oral fluid was a characteristic of individual specimens and not consistently detected within all samples from individuals. In studies of neonates and children, blood contamination is rare (65), however, studies of antepartum women who may present oral health risk should take heed to document and control for blood contamination.

A more universal concern, and one especially relevant in neonates, is contamination of samples from particulate matter and interfering substances placed in the mouth. Breastmilk, formula, and solids introduced later in infancy create residue in the oral cavity after drinking or eating may include particulate matter,

change salivary pH or composition (viscosity), and/or contain substances (e.g., bovine hormones, enzymes) that cross-react in immune- or kinetic-reaction assays. Given the variation of infant feeding schedules and the difficulty in restricting intake, particularly when young infants are fed on demand, we recommend a simple solution: children should not be fed and adults should refrain from eating and drink with exception of water within the 20-min prior to sample donation. For repeated measure designs, saliva collection should be carefully planned and scheduled, and prior feedings noted.

Prescription and over-the-counter medications are also known contaminants to oral fluid, including those that are applied intranasally, inhaled or applied as oral topicals (e.g., teething gels). These substances have the potential to change saliva composition due to residue left by their use in the oral cavity, cross-reaction with antibody–antigen binding in immunoassays, and reduced salivary flow rate (66, 67). Confounding the effort to control for the potential effects of medications on salivary analytes is the possibility that the condition for which the medication is prescribed or taken may also influence individual differences in the analytes levels or activity (68). Few behaviorally oriented studies that involve salivary analytes have comprehensively documented medication usage. In one study of infants, nearly half (44%; $n = 852$) of the 6-month-old infants were given acetaminophen in the 48 h prior to saliva collection. Acetaminophen was shown to attenuate cortisol reactivity (69); implications for gonadal steroids remain unknown.

The lack of normative data and the wide-ranging individual differences in salivary analytes levels make case-by-case exclusion of samples from individuals taking any particular medication questionable (unless the deviation is obviously not physiologically plausible). Of course, the simplest approach would be to exclude anyone who is taking *any* medication from participation in research. This includes sampling from neonates of breastfeeding mothers who are themselves on a medication. Although appropriate from the perspective of rigorous experimental design, this conservative approach yields no information that may help us develop our knowledge of which medications are, or are not, problematic. Also, in studies of some specialized populations (e.g., pregnant patients with physical or mental illnesses), prescription medication use is so highly prevalent that it is considered normative. Withholding treatment raises ethical questions while excluding those taking medications increases the potential that findings may be confounded by selection bias. Future work is encouraged to document participant medication use and evaluate associations with steroid levels measured in saliva to build knowledge and document pharmaceutical contamination effects or lack thereof.

APPLICATIONS OF SALIVARY ANALYTES IN STUDIES OF MATERNAL AND NEONATAL GONADAL STEROID ACTIVITY

Dependent on the nature of the research question, several sampling schemes are common for studies involving oral fluid. At the individual level, a single sample may be collected to index basal level, or multiple samples may be collected across the course of the day to capture diurnal rhythm. Concurrent sampling of a dyad is an additional scheme designed to evaluate associative relations over time. In further discussion of each sampling scheme below, we have embedded existing research drawn from **Table 2** utilizing saliva to index maternal and neonatal gonadal steroid levels to encourage additional reading. Existing studies are relatively scant, yet saliva offers a wide array of application as a minimally invasive, accurate, and cost-effective approach; we offer suggestions for areas of future investigation with salivary analytes.

BASAL OR "TRAIT-LIKE" LEVELS

The basal level or activity of an analyte represents the stable state of the host during a resting period. One approach to assessing basal levels has been to sample early in the morning before the events of the day are able to contribute variation or specify a restricted time window for collection to reduce variation between participants [e.g., ±1200 h, Ref. (12)].

Single sampling is the most common approach for saliva collection, and the majority of studies referenced in **Table 2** have employed this scheme. While overall the literature on salivary steroids during pregnancy and in the postpartum is lacking, studies examining estriol levels among antepartum women are most common (see **Table 2**). Estriol monitoring via saliva has been utilized as a clinical tool to assess the vitality of fetoplacental unit and a marker of preterm birth (17, 18). For this analyte, repeated measures of the "basal" level have been employed to detect atypical change [e.g., Ref. (18); weekly sampling from 22 weeks GA until birth]. As seen, the studies by Marrs and colleagues (11) as well as the more recent Hampson and colleagues (12) offer the most comprehensive report on gonadal steroids concentrations in antepartum and postpartum women and in comparison to serum, though limited to a single measurement during the third trimester of pregnancy. Future work is needed to elucidate reference values for gonadal steroids earlier in gestation, to evaluate normative endocrine changes of pregnancy and serve as a clinical tool for diagnosis of adrenal disorders during pregnancy.

We are only aware of a single study measuring neonatal salivary testosterone basal levels; findings revealed a positive association between salivary testosterone and neonatal health problems as well as growth delays among a high-risk preterm sample (21). A multitude of studies have explored neonatal cortisol reactivity, but more research is needed to understand the significance of the gonadal hormonal transition surrounding birth. Beyond the neonatal period, a handful of studies have sampled gonadal steroids to characterize "mini-puberty" observed during early infancy (70, 71). Testosterone levels of male infants peak between 1 and 3 months of age, and female infants show a spike between 2 and 4 months in follicle stimulating hormone (71, 72). The physiological relevance of this phenomenon, which exhibits universality across mammalian species, is theorized to activate sexual dimorphisms in the brain (72), remains underexplored to date. These analyses relied on single measures at varying ages to identify peak levels.

While a start, a single-time point measure of salivary analytes (other than invariant genetic polymorphisms), except under very unique circumstances, is *unlikely* to yield meaningful results for "basal levels" given that salivary analytes may vary depending on the inherent moment-to-moment, diurnal and/or monthly variation in their production/release, rate of their metabolism/degradation, and sensitivity to environmental influence. Given these issues, in combination with the moving target gonadal steroids present during the antepartum and neonatal/infant

periods during which physiological shifts in hormone concentration are expected, cluster sampling for a series of three consecutive days is recommended. The association of salivary analyte measurements across days is likely to be stronger for some salivary analytes than others yet employing cluster sampling enhances overall reliability of the estimates by aggregating (e.g., average assay results or physically pool specimens). The minimally invasive nature of oral fluid collection is ideal for cluster sampling. Future studies are needed to describe the observed surge in gonadal steroids during infancy, investigate sex differences, and test predictive associations linking gonadal steroid activation to behavior.

DIURNAL RHYTHM

An important component of variability within and between individuals in salivary analyte levels is the diurnal rhythm of production [e.g., Ref. (73, 74)]. Most salivary hormone levels (e.g., cortisol, testosterone) are high in the morning, decline before noon, and then decline more slowly in the afternoon and evening hours (75). The non-linear nature of these patterns requires multiple sampling time points to create adequate statistical models (76).

The diurnal rhythm of gonadal steroid output in antepartum or postpartum women remains largely unexplored to date. There is evidence that the diurnal pattern is retained during pregnancy for other hormone outputs such as cortisol (77–79). For neonates, recent evidence suggests that in the first postnatal week the adrenal circadian rhythm becomes unsynchronized with clock time, peaking in late afternoon or at a time parallel to the birth time (80). The typical 24-h rhythm is generally not observed until after a few months for cortisol production, potentially coinciding with stabilization of the sleep–wake cycle. One study of salivary 17-OH progesterone indexed in neonates on the first or second day of life confirmed this, finding no evidence of a diurnal pattern (22). In a second cross sectional study designed to establish diurnal reference values for salivary progesterone and 17-OH progesterone, a diurnal pattern was observed among neonates <4 weeks age ($n = 13$, age range = 4–27 days) for 17-OH progesterone but not progesterone (23). Less is known about at what age gonadal steroids begin to show a characteristic diurnal rhythm; future studies documenting this would represent a considerable contribution to the literature, particularly during this rapid phase of development.

Analytical techniques that have been used to model individual differences in diurnal rhythm range from average levels, to focusing specifically on the awakening component of the overall pattern, to estimating AUC. Growth curve modeling (81) may be an optimal method of parameterizing individual differences because: (1) it allows the level and slope of the diurnal rhythm to be examined in the same model; (2) unsystematic error variance is partialed out of the "true" score; (3) the presence of individual differences in the diurnal rhythm is statistically tested; (4) predictors of the diurnal rhythm are related to level and slope in the same model (partialing out the effects of one another); and (5) changes in the variance estimates of the level and slope act as indicators of the overall contribution of the predictors [e.g., Ref. (81, 82)].

ASSOCIATIVE RELATIONS OF SALIVARY ANALYTES BETWEEN DYADS

Physiological attunement or concordance of maternal and fetal hormones is documented before birth, *in utero*. For example, maternal prenatal testosterone serum levels are correlated with fetal levels ascertained from samples of amniotic fluid for both sexes (83). Further, women carrying male fetuses show higher testosterone concentrations relative to those carrying females (84) suggesting bidirectionality of observed associative relations.

Although recognized that in the postpartum period gonadal steroids may be decreasing in concentration among women while concurrently increasing among neonates, postnatal dyadic attunement of gonadal steroids remains an underexplored, yet promising, avenue for future research. The study noted in **Table 2** that indexed neonatal salivary testosterone did find a positive correlation between testosterone levels in neonates and mothers in the first week after birth (21). Examining predictive associations from maternal levels during pregnancy to neonatal levels in the postpartum would also be of interest; for example, is there a greater or faster increase in gonadal steroid activity among neonates that experience more significant withdrawal from the maternal prenatal endocrine context (i.e., exposure to higher concentrations in the prenatal period).

The examination of associative relations need not be constrained to the dyad; at the individual level, this scheme carries over to studies exploring the coaction of two or more hormones. A single saliva sample of sufficient volume may be assayed for multiple analytes. For example, there is known functional cross-talk occurring between the HPG and HPA axes where gonadal steroids are postulated to induce a negative feedback loop in HPA reactivity (85). Gonadal steroids have also been theorized to stimulate oxytocin and in turn promote maternal behaviors in the postpartum period among mammals (86), as well as modulate immune and autoimmune response in general (87, 88). A dual systems approach that examines associative relations among two or more hormonal outputs (e.g., cortisol, oxytocin, SIgA) holds great promise for a deeper understanding of hormonal coaction during the antepartum and postpartum periods as well as calibration of early gonadal steroid activity in neonates and infants.

Typically, examination of relationships between two continuous variables involves the use of a summary statistic, such as a correlation; however, this is not always the most advantageous method. Nagin and Tremblay (89) used a model that jointly estimates growth mixture models for two distinct but related measurement series. The joint probabilities generated allow investigators to characterize the relationship between two dependent variables as they unfold over a specified period of time. Physiological attunement between members of the mother–child dyad appears to be a worthwhile application for this dual trajectory model.

CONCLUSION

Parturition marks a host of physiological adaptations associated with changing environments; systems of respiration, circulation, homeostasis, and state regulation are just a few examples. While widely acknowledged that endocrine factors play an important role in this transition, the interrelationship of mother and infant gonadal steroids during this transition is not well understood. Beyond organizational effects, we know even less about how the transient activation of gonadal steroids in early postnatal life serves to differentiate child behavior and development.

As prenatal and neonatal gonadal steroid exposure can be reliably ascertained from saliva, and collected in a non-invasive manner particularly well-suited for research designs involving neonates and infants, oral fluid may become the biospecimen of choice for students of early human development. The purpose of this review was to provide a conceptual and tactical roadmap for investigators interested in integrating these measurement tools surrounding saliva into research on gonadal steroid exposure and health and human development. We continue to believe that the implications of the information divulged via this research will have a profound impact on developmental science in general, and the study of neonatal health and development in particular.

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REFERENCES

1. Belsky J. The determinants of parenting: a process model. *Child Dev* (1984) **55**:83–96. doi:10.2307/1129836
2. Belsky J. Differential susceptibility to rearing influence. In: Ellis BJ, Bjorklund DF, editors. *Origins of the Social Mind: Evolutionary Psychology and Child Development*. New York: Guildford Press (2005). p. 139–63.
3. Boyce WT, Ellis BJ. Biological sensitivity to context: I. An evolutionary-developmental theory of the origins and functions of stress reactivity. *Dev Psychopathol* (2005) **17**:271–301. doi:10.1017/S0954579405050145
4. Johnson SB, Riley AW, Granger DA, Riis J. The science of early life toxic stress for pediatric practice and advocacy. *Pediatrics* (2013) **131**:319–27. doi:10.1542/peds.2012-0469
5. O'Connor TG, Bergman K, Sarkar P, Glover V. Prenatal cortisol exposure predicts infant cortisol response to acute stress. *Dev Psychobiol* (2012) **55**(2):145–55. doi:10.1002/dev.21007
6. Ramsay D, Lewis M. Reactivity and regulation in cortisol and behavioral responses to stress. *Child Dev* (2003) **74**(2):456–64. doi:10.1111/1467-8624.7402009
7. Stansbury K, Gunnar MR. Adrenocortical activity and emotion regulation. *Monogr Soc Res Child Dev* (1994) **59**(2–3):108–34. doi:10.1111/j.1540-5834.1994.tb01280.x
8. Gluckman P, Hanson M. The conceptual basis for the developmental origins of health and disease. In: Gluckman P, Hanson M, editors. *Developmental Origins of Health and Disease*. New York: Cambridge University Press (2006). p. 33–50.
9. Hu S, Loo JA, Wong DT. Human saliva proteome analysis. *Ann N Y Acad Sci* (2007) **1098**:323–9. doi:10.1196/annals.1384.015
10. Darne J, McGarrigle HHG, Lachelin GC. Saliva oestriol, oestradiol, oestrone and progesterone levels in pregnancy: spontaneous labour at term is preceded by a rise in the saliva oestriol: progesterone ratio. *BJOG* (1987) **94**:227–35. doi:10.1111/j.1471-0528.1987.tb02359.x
11. Marrs CR, Ferraro DP, Cross CL. Salivary hormones and parturition in healthy, primigravid women. *Int J Gynecol Obstet* (2007) **99**:59–60. doi:10.1016/j.ijgo.2007.03.032
12. Hampson E, Phillips SD, Soares CN, Steiner M. Steroid concentrations in antepartum and postpartum saliva: normative values in women and correlations with serum. *Biol Sex Differ* (2013) **4**:7. doi:10.1186/2042-6410-4-7
13. Kundu N, Novak N, Petersen LP. Salivary unconjugated estriol levels in normal third trimester pregnancy-direct correlation with serum levels. *Steroids* (1983) **41**:145–53. doi:10.1016/0039-128X(83)90003-X
14. Lechner W, Heim K, Zech J, Daxenbichler G, Marth C. The relation between saliva estriol levels in pregnancy and infant birth weight. *Arch Gynecol Obstet* (1987) **241**:9–12. doi:10.1007/BF00931435
15. Lewis PR, Galvin PM, Short RV. Salivary oestriol and progesterone concentrations in women during late pregnancy, parturition and the puerperium. *J Endocrinol* (1987) **115**:177–81. doi:10.1677/joe.0.1150177
16. Scott EM, Thomas A, McGarrigle HHG, Lachelin GC. A comparison of serial ultrasound measurements of fetal adrenal glands with maternal plasma and saliva oestriol and progesterone levels in normal pregnancy. *J Obstet Gynecol* (1991) **11**:381–5. doi:10.3109/01443619109013573
17. McGregor JA, Jackson GM, Lachelin GC, Goodwin TM, Artal R, Hastings C, et al. Salivary estriol as risk assessment for preterm labor: a prospective trial. *Am J Obstet Gynecol* (1995) **173**:1337–42.
18. Heine RP, McGregor JA, Goodwin TM, Artal R, Hayashi RH, Robertson PA, et al. Serial salivary estriol to detect an increased risk of preterm birth. *Obstet Gynecol* (2000) **96**:490–7. doi:10.1016/S0029-7844(00)01004-8
19. Hedriana HL, Munro CJ, Eby-Wilkens EM, Lasley BL. Changes in rates of salivary estriol increases before parturition at term. *Am J Obstet Gynecol* (2001) **184**:123–30. doi:10.1067/mob.2001.108338
20. Klebanoff MA, Meis PJ, Dombrowski MP, Zhao Y, Moawad AH, Northen A, et al. Salivary progesterone and estriol among pregnant women treated with 17-α-hydroxyprogesterone caproate or placebo. *Am J Obstet Gynecol* (2008) **199**:506–e1. doi:10.1016/j.ajog.2008.03.003
21. Cho JI, Carlo WA, Su X, McCormick KL. Associations between salivary testosterone and cortisol levels and neonatal health and growth outcomes. *Early Hum Dev* (2012) **88**:789–95. doi:10.1016/j.earlhumdev.2012.05.002
22. Klug I, Dressendörfer R, Strasburger C, Kühl GP, Reiter HL, Reich A, et al. Cortisol and 17-hydroxyprogesterone levels in saliva of healthy neonates. *Neonatology* (2000) **78**:22–6. doi:10.1159/000014242
23. Gröschl M, Rauh M, Dörr HG. Circadian rhythm of salivary cortisol, 17α-hydroxyprogesterone, and progesterone in healthy children. *Clin Chem* (2003) **49**:1688–91. doi:10.1373/49.10.1688
24. Priya B, Mustafa MD, Guleria K, Vaid NB, Banerjee BD, Ahmed RS. Salivary progesterone as a biochemical marker to predict early preterm birth in asymptomatic high-risk women. *BJOG* (2013) **120**:1003–11. doi:10.1111/1471-0528.12217
25. Few JD, Paintin DB, James VHT. The relation between aldosterone concentrations in plasma and saliva during pregnancy. *BJOG* (1986) **93**:928–32. doi:10.1111/j.1471-0528.1986.tb08010.x
26. Tabak LA. Point-of-care diagnostics enter the mouth. *Ann N Y Acad Sci* (2007) **1098**:7–14. doi:10.1196/annals.1384.043
27. Malamud D, Tabak L. Saliva as a diagnostic fluid. *Ann N Y Acad Sci* (1993) **694**:216–33.
28. Cone EJ, Huestis MA. Interpretation of oral fluid tests for drugs of abuse. *Ann N Y Acad Sci* (2007) **1098**:51–103. doi:10.1196/annals.1384.037
29. U.S. Department of Health and Human Services. *Oral Health in America: A Report of the Surgeon General*. Rockville, MD: U.S. Department of Health and Human Services, National Institute of Dental and Craniofacial Research, National Institutes of Health (2000). 74–94 p.
30. Sisk CL, Zehr JL. Pubertal hormones organize the adolescent brain and behavior. *Front Neuroendocrinol* (2005) **26**(3):163–74. doi:10.1016/j.yfrne.2005.10.003
31. Pike CJ, Carroll JC, Rosario ER, Barron AM. Protective actions of sex steroid hormones in Alzheimer's disease. *Front Neuroendocrinol* (2009) **30**(2):239–58. doi:10.1016/j.yfrne.2009.04.015
32. McEwen B, Davis P, Parsons B, Pfaff D. The brain as a target for steroid hormone action. *Annu Rev Neurosci* (1979) **2**:65–112. doi:10.1146/annurev.ne.02.030179.000433
33. Peper J, Koolschijn C. Sex steroids and the organization of the human brain. *J Neurosci* (2012) **32**:6745–6. doi:10.1523/JNEUROSCI.1012-12.2012
34. Forest M, Cathiard A, Bertrand J. Evidence of testicular activity in early infancy. *J Clin Endocrinol* (1973) **37**:148–51. doi:10.1210/jcem-37-1-148
35. Forest M, Sizonenko P, Cathiard A, Bertrand J. Hypophyso-gonadal function in humans during the first year of life. *J Clin Invest* (1974) **53**:819–28. doi:10.1172/JCI107621
36. Gröschl M. Current status of salivary hormone analysis. *Clin Chem* (2008) **54**:1759–69. doi:10.1373/clinchem.2008.108910

37. Veerman ECI, van den Keijbus PAM, Vissink A, Nieuw Amerongen AV. Human glandular salivas: their separate collection and analysis. *Eur J Oral Sci* (1996) **104**:346–52. doi:10.1111/j.1600-0722.1996.tb00090.x
38. Nieuw Amerongen AVM, Bolscher JGM, Veerman ECI. Salivary mucins: protective functions in relation to their diversity. *Glycobiology* (1995) **5**:733–40. doi:10.1093/glycob/5.8.733
39. Schwartz EB, Granger DA, Susman EJ, Gunnar MR, Laird B. Assessing salivary cortisol in studies of child development. *Child Dev* (1998) **69**:1503–13. doi:10.2307/1132128
40. Garrett JR. The proper role of nerves in salivary secretion: a review. *J Dent Res* (1987) **66**:387–97. doi:10.1177/00220345870660020201
41. Kugler J, Hess M, Haake D. Secretion of salivary immunoglobulin A in relation to age, saliva flow, mood states, secretion of albumin, cortisol, and catecholamines in saliva. *J Clin Immunol* (1992) **12**:45–9. doi:10.1007/BF00918272
42. Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, et al. Bacterial diversity in human subgingival plaque. *J Bacteriol* (2001) **183**:3770–83. doi:10.1128/JB.183.12.3770-3783.2001
43. Scannapieco FA, Papandonatos GD, Dunford RG. Associations between oral conditions and respiratory disease in a national sample survey population. *Ann Periodontol* (1998) **3**:251–6. doi:10.1902/annals.1998.3.1.251
44. Dabbs JM Jr. Salivary testosterone measurements: collecting, storing, and mailing saliva samples. *Physiol Behav* (1991) **49**:815–7. doi:10.1016/0031-9384(91)90323-G
45. Haeckel R, Bucklitsch I. Procedures for saliva sampling. *J Clin Chem Biochem* (1987) **25**:199–204.
46. Gunnar M, Mangelsdorf S, Larson M, Hertsgaard L. Attachment, temperament, and adrenocortical activity in infancy: a study of psychoendocrine regulation. *Dev Psychol* (1989) **3**:355–63. doi:10.1037/0012-1649.25.3.355
47. Harmon AG, Hibel LC, Rumyansteva O, Granger DA. Measuring salivary cortisol in studies of child development: watch out – what goes in my not come out of saliva collection devices. *Dev Psychobiol* (2007) **49**:495–500. doi:10.1002/dev.20231
48. Gröschl M, Rauh M. Influence of commercial collection devices for saliva on the reliability of salivary steroids analysis. *Steroids* (2006) **71**:1097–100. doi:10.1016/j.steroids.2006.09.007
49. Shirtcliff EA, Granger DA, Schwartz EB, Curran MJ. Use of salivary biomarkers in biobehavioral research: cotton based sample collection methods can interfere with salivary immunoassay results. *Psychoneuroendocrinology* (2001) **26**:165–73. doi:10.1016/S0306-4530(00)00042-1
50. Granger DA, Kivlighan KT, Fortunato C, Harmon AG, Hibel LC, Schwartz EB, et al. Integration of salivary biomarkers into developmental and behaviorally-oriented research: problems and solutions for collecting specimens. *Physiol Behav* (2007) **92**:583–90. doi:10.1016/j.physbeh.2007.05.004
51. Schwartz EB, Eppihimer L, Nelson VJ, Granger DA. Effects of sample storage temperature on the measurement of salivary analytes: degradation of testosterone, estradiol, and progesterone. *Poster presented at the annual meeting of the American Association for Clinical Chemistry*, Orlando, FL. *Clin Chem* (2005) **51**(6 Suppl):A2–261.
52. Whembolua GL, Granger DA, Singer S, Kivlighan KT, Marguin JA. Bacteria in the oral mucosa and its effects on the measurement of cortisol, dehydroepiandrosterone, and testosterone in saliva. *Horm Behav* (2006) **49**:478–83. doi:10.1016/j.yhbeh.2005.10.005
53. Carter CS, Pournajafi-Nazarloo H, Kramer KM, Ziegler TE, White-Traut R, Bello D, et al. Oxytocin: behavioral associations and potential as a salivary biomarker. *Ann N Y Acad Sci* (2007) **1098**:312–22. doi:10.1196/annals.1384.006
54. Horvat-Gordon M, Granger DA, Schwartz EB, Nelson V, Kivlighan KT. Oxytocin is not a valid biomarker when measured in saliva by immunoassay. *Physiol Behav* (2005) **16**:445–8. doi:10.1016/j.physbeh.2005.01.007
55. Dawidson I, Blom M, Lundeberg T, Theodorsson E, Angmar-Mansson B. Neuropeptides in the saliva of healthy subjects. *Life Sci* (1997) **60**:269–78. doi:10.1016/S0024-3205(96)00627-3
56. Santavirta N, Konttinen YT, Tornwall J, Segerberg M, Santavirta S, Matucci-Cerinic M, et al. Neuropeptides of the autonomic nervous system in Sjogren's syndrome. *Ann Rheum Dis* (1997) **56**:737–40. doi:10.1136/ard.56.12.737
57. Beall CM, Worthman CM, Stallings J, Strohl KP, Brittenham GM, Barragan M. Salivary testosterone concentration of Aymara men native to 3600 m. *Ann Hum Biol* (1992) **19**:67–78. doi:10.1080/03014469200001932
58. Flinn MV, England BG. Childhood stress and family environment. *Curr Anthropol* (1995) **36**:854–66. doi:10.1086/204444
59. Raff H, Homar PJ, Skoner DP. New enzyme immunoassay for salivary cortisol. *Clin Chem* (2003) **49**:203–4. doi:10.1373/49.1.203
60. Arendorf TM, Bredekamp B, Cloete CA, Sauer G. Oral manifestations of HIV infection in 600 South African patients. *J Oral Pathol Med* (1998) **27**:176–9. doi:10.1111/j.1600-0714.1998.tb01936.x
61. Reibel J. Tobacco and oral diseases. Update on the evidence, with recommendations. *Med Princ Pract* (2003) **1**(Suppl):22–32. doi:10.1159/000069845
62. Kivlighan KT, Granger DA, Schwartz EB, Nelson V, Curran M, Shirtcliff EA. Quantifying blood leakage into the oral mucosa and its effects on the measurement of cortisol, dehydroepiandrosterone, and testosterone in saliva. *Horm Behav* (2004) **46**:39–46. doi:10.1016/j.yhbeh.2004.01.006
63. Kivlighan KT, Granger DA, Schwartz EB. Blood contamination and the measurement of salivary progesterone and estradiol. *Horm Behav* (2005) **47**:367–70. doi:10.1016/j.yhbeh.2004.12.001
64. Schwartz EB, Granger DA. Transferrin enzyme immunoassay for quantitative monitoring of blood contamination in saliva. *Clin Chem* (2004) **50**:654–6. doi:10.1373/clinchem.2003.028266
65. Granger DA, Cicchetti D, Rogosch F, Hibel LC, Teisl M, Flores E. Blood contamination in children's saliva: prevalence, stability, and impact on the measurement of salivary cortisol, testosterone, and dehydroepiandrosterone. *Psychoneuroendocrinology* (2007) **32**:724–33. doi:10.1016/j.psyneuen.2007.05.003
66. Rees TD. Oral effects of drug abuse. *Crit Rev Oral Biol Med* (1992) **3**:163–84.
67. Sreebny LM, Schwartz SS. A reference guide to drugs and dry mouth – 2nd edition. *Gerodontology* (1997) **14**:33–47. doi:10.1111/j.1741-2358.1997.00033.x
68. Granger DA, Hibel LC, Fortunato CK, Kapelewski CH. Medication effects on salivary cortisol: tactics and strategy to minimize impact in behavioral and developmental science. *Psychoneuroendocrinology* (2009) **34**:1437–48. doi:10.1016/j.psyneuen.2009.06.017
69. Hibel LC, Granger DA, Kivlighan KT, Blair C, FLP Investigators. Individual differences in salivary cortisol: associations with common over-the-counter and prescription medication status in infants and their mothers. *Horm Behav* (2006) **50**:293–300. doi:10.1016/j.yhbeh.2006.03.014
70. Hines M. Gender development and the human brain. *Annu Rev Neurosci* (2011) **34**:69–88. doi:10.1146/annurev-neuro-061010-113654
71. Lamminmaki A, Hines M, Kuiri-Hanninen T, Kilpelainen L, Dunkel L, Sankilampi U. Testosterone measured in infancy predicts subsequent sex-typed behavior in boys and in girls. *Horm Behav* (2012) **61**:611–6. doi:10.1016/j.yhbeh.2012.02.013
72. Quigley C. Editorial: the postnatal gonadotropin and sex steroid surge-insights from the androgen insensitivity syndrome. *J Clin Endocrinol Metab* (2002) **87**:24–8. doi:10.1210/jcem.87.1.8265
73. Gunnar MR, Vasquez D. Low cortisol and a flattening of expected daytime rhythm: potential indices of risk in human development. *Dev Psychopathol* (2001) **13**:515–38. doi:10.1017/S0954579401003066
74. Smyth JM, Ockenfels MC, Gorin AA, Cately D, Porter LS, Kirschbaum C, et al. Individual differences in the diurnal cycle of cortisol. *Psychoneuroendocrinology* (1997) **22**:89–105. doi:10.1016/S0306-4530(96)00039-X
75. Nelson RJ. *An Introduction to Behavioral Endocrinology*. Sunderland, MA: Sinauer Associates (1995).
76. Hellhammer J, Fries E, Schweusthal OW, Schlotz W, Stone AA, Hagemann D. Several daily measurements are necessary to reliably assess the cortisol rise after awakening: state- and trait components. *Psychoneuroendocrinology* (2007) **32**:80–6. doi:10.1016/j.psyneuen.2006.10.005
77. Allolio B, Hoffmann J, Linton EA, Winkelmann W, Kusche M, Schulte HM. Diurnal salivary cortisol patterns during pregnancy and after delivery: relationship to plasma corticotrophin-releasing-hormone. *Clin Endocrinol* (1990) **33**:279–89. doi:10.1111/j.1365-2265.1990.tb00492.x
78. de Weerth C, Buitelaar JK. Cortisol awakening response in pregnant women. *Psychoneuroendocrinology* (2005) **30**:902–7. doi:10.1016/j.psyneuen.2005.05.003

79. Kivlighan KT, DiPietro JA, Costigan KA, Laudenslager ML. Diurnal rhythm of cortisol during late pregnancy: associations with maternal psychological well-being and fetal growth. *Psychoneuroendocrinology* (2008) **33**:1225–35. doi:10.1016/j.psyneuen.2008.06.008
80. Iwata O, Okamura H, Saitsu H, Saikusa M, Kanda H, Eshima N, et al. Diurnal cortisol changes in newborn infants suggesting entrainment of peripheral circadian clock in utero and at birth. *J Clin Endocrinol Metab* (2013) **98**:E25–32. doi:10.1210/jc.2012-2750
81. McArdle JJ, Bell RQ. An introduction to latent growth models for developmental data analysis. In: Little TD, Schnabel KU, Baumert J, editors. *Modeling Longitudinal and Multiple-Group Data: Practical Issues, Applied Approaches, and Specific Examples*. Hillsdale, NJ: Lawrence Erlbaum Associates (2000). p. 69–107.
82. McArdle JJ, Nesselroade J. Using multivariate data to structure developmental change. In: Cohen SH, Reese HW, editors. *Life-Span Developmental Psychology*. Hillsdale, NJ: Lawrence Erlbaum Associates (1994). p. 223–67.
83. Gitau R, Adams D, Risk N, Glover V. Fetal plasma testosterone correlates positively with cortisol. *Arch Dis Child Fetal Neonatal Ed* (2005) **90**:F166–9. doi:10.1136/adc.2004.049320
84. Meulenberg PMM, Hofman JA. Maternal testosterone and fetal sex. *J Steroid Biochem Mol Biol* (1991) **39**(1):51–4. doi:10.1016/0960-0760(91)90012-T
85. Viau V. Functional cross-talk between the hypothalamic-pituitary-gonadal and -adrenal axes. *J Neuroendocrinol* (2002) **14**:506–13. doi:10.1046/j.1365-2826.2002.00798.x
86. Pedersen CA. Oxytocin control of maternal behavior. Regulation by sex steroids and offspring stimuli. *Ann N Y Acad Sci* (1997) **807**:126–45. doi:10.1111/j.1749-6632.1997.tb51916.x
87. Grossman CJ. Interactions between the gonadal steroids and the immune system. *Science* (1985) **227**:257–61. doi:10.1126/science.3871252
88. Tanriverdi F, Silveira LF, MacColl GS, Bouloux PM. The hypothalamic-pituitary-gonadal axis: immune function and autoimmunity. *J Endocrinol* (2003) **176**:293–304. doi:10.1677/joe.0.1760293
89. Nagin DS, Tremblay RE. Analyzing developmental trajectories of distinct by related behaviors: a group-based method. *Psychol Methods* (2001) **6**:18–34. doi:10.1037/1082-989X.6.1.18

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Digit ratio (2D:4D): a biomarker for prenatal sex steroids and adult sex steroids in challenge situations

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Digit ratio (2D:4D) denotes the relative length of the second and fourth digits. This ratio is considered to be a biomarker of the balance between fetal testosterone (T) and estrogen (E) in a narrow window of early ontogeny. Evidence for this assertion is derived from direct and indirect measures of prenatal hormonal exposure (in experimental animals, via amniotic fluid samples and in the study of sex-typical traits) in relation to 2D:4D. In contrast, the relationships between 2D:4D and levels of sex steroids in adults are less clear, as many correlational studies of 2D:4D and adult sex steroids have concluded that this association is statistically non-significant. Here, we suggest that in order to understand the link between 2D:4D and sex hormones, one must consider both fetal organizing and adult activating effects of T and E. In particular, we hypothesize that 2D:4D correlates with organizing effects on the endocrine system that moderate activating effects in adulthood. We argue that this is particularly evident in "challenging" conditions such as aggressive and sexual encounters, in which individuals show increased levels of T. We discuss this refinement of the 2D:4D paradigm in relation to the links between 2D:4D and sports performance, and aggression.

Keywords: digit ratio, testosterone, estrogen, performance, aggression, organizing effects, activating effects

INTRODUCTION

Digit ratio (or 2D:4D) is the relative lengths of the second digit (the "index" finger) and the fourth digit (the "ring" finger). It has been known for many years that 2D:4D varies according to sex, such that males tend to have longer fourth digits relative to second digits (low 2D:4D) than females [high 2D:4D; see Ref. (1)]. The effect size of this sex difference is small to moderate (Cohen's *d* is about 0.50), and 2D:4D attracted little attention until 1998, when it was suggested that a balance of fetal testosterone (FT) and fetal estrogen (FE) influences the formation of 2D:4D, such that low 2D:4D indicates high FT and low FE and high 2D:4D indicates low FT and high FE [(2); see Ref. (3) for a detailed development of the hypothesis]. Following this suggestion, there was a marked increase in the number of 2D:4D studies, from 1 in 1998 to 51 in 2007, and from 2008 to 2010 the numbers of studies have averaged about 60 papers per year (4).

The initial statement of the hypothesis that 2D:4D is a morphological correlate of FT and FE was derived from two data sets (2, 3) reporting the following relationships: (i) age effects; a longitudinal sample of 800 children and adults aged from 2 to 25 years showed a sex difference in 2D:4D, such that males tended to have lower 2D:4D than females, and mean 2D:4D did not change significantly with age. Therefore, it is likely that the sexual dimorphism is determined early in ontogeny, probably *in utero*, (ii) hormone and fertility effects; data from 131 participants (69 males) attending an infertility clinic showed that high 2D:4D was linked to germ cell failure, low sperm numbers, and high levels of estrogen (E), while low 2D:4D was linked to high testosterone (T) and high sperm numbers. It was concluded that the sex difference in 2D:4D was likely to be determined *in utero* by a balance of T and E and that adult levels of these hormones echo prenatal concentrations of sex steroids. Subsequent studies have provided support for the link between 2D:4D and FT and FE, but have not confirmed the associations between 2D:4D and adult concentrations of T and E.

2D:4D AND PRENATAL TESTOSTERONE AND ESTROGEN

The sex difference in 2D:4D is now well understood [see Ref. (5) for a meta-analysis]. It is found in fetuses as early as the end of the first trimester (6, 7) and although it may change postnatally as the fingers grow, this change is in the form of a gentle increase in 2D:4D (8, 9). Fetal levels of T are sexually dimorphic with male fetuses having higher T than female fetuses. Therefore, this pattern of prenatal determination of the sexual dimorphism in 2D:4D is consistent with – but does not prove that – the sex difference in 2D:4D reflects fetal levels of sex steroids.

It is difficult to measure the effect of FT and FE on 2D:4D in humans directly because of ethical constraints. This is why a readily measured indirect correlate of the balance between FT and FE (such as 2D:4D) is valuable in investigating organizing effects on sex-typical traits. However, the difficulties associated with measuring fetal hormones and fetal 2D:4D means that the link between FT/FE and 2D:4D is not easily demonstrated. Attempts

to investigate this relationship may be considered as of two kinds, i.e., correlational studies and experimental studies.

Correlational studies have considered relationships between 2D:4D and sexually dimorphic physical and behavioral traits that are thought to be linked to FT and FE. There are many of these; here we focus on some of the more important ones, i.e., those that are very likely to be affected by FT and/or FE. Congenital adrenal hyperplasia (CAH) is a trait associated with an increase in the size of the fetal adrenal glands and an elevated level of fetal androgens. To date, there have been four studies of CAH and 2D:4D. All have shown a tendency for low 2D:4D (i.e., "masculinized" 2D:4D) to be linked to CAH and in three such studies, the effects were significant [see Ref. (5) for a meta-analysis of these studies]. In contrast to CAH patients, individuals with Klinefelter's syndrome (males with 47 chromosomes, including XXY) have low fetal androgen levels. In Klinefelter patients, 2D:4D is significantly higher (i.e., "feminized" 2D:4D) than that of the population norm (10). This pattern of "feminized" 2D:4D has also been found in individuals who suffer from androgen insensitivity (11), i.e., a clinical condition that results in a partial or complete inability of cells in their response to androgens. All these studies have focused on FT. However, Lutchmaya et al. (12) obtained both FT and FE concentrations from amniotic fluid samples in order to investigate relationships with 2D:4D. It was found that 2D:4D of neonates was related to a balance of FT and FE, such that high FT and low FE were linked to "masculinized" 2D:4D.

The link of digit ratio to CAH, Klinefelter's syndrome, and androgen insensitivity is strong evidence for a link between 2D:4D and prenatal sex steroids. However, one trait, the anogenital distance (AGD), may well be influenced by prenatal T, but shows little or no correlation with 2D:4D in rodents [for mice, see Ref. (13, 14); for rats, see Ref. (15)]. Why is this so? In humans the sex difference in 2D:4D is of medium effect size (5). It is determined toward the end of the first trimester of pregnancy (6, 7) in a narrow time window [for mice see Ref. (14)] and its magnitude changes little with subsequent growth (8, 9). In contrast, AGD shows a large sex difference, which varies from 1.4-fold-longer in males at 11–13 weeks to 2.0-fold-longer at 17–20 week gestation and a smaller difference is found in adults (16). This variability suggests that – unlike 2D:4D – the sex difference in AGD is not fixed early *in utero* but is influenced by fluctuations in second-trimester and post-natal androgens. Indeed, there has been one study in mice that experimentally confirmed these suggested effects [see Ref. (4, 14) for discussion]. There have been reports that both AGD and 2D:4D change when fetuses are exposed to endocrine disruptors [AGD, see Ref. (16); 2D:4D, see Ref. (15)]. The study by Auger et al. is of particular relevance here, as these authors compared the effect of estrogenic and anti-androgenic compounds on 2D:4D and AGD in rats. The authors reported a feminization effect for 2D:4D, but not so for AGD, which again suggests different times of developmental fixation of the sexual dimorphism in 2D:4D and AGD.

Correlational studies have also focused on the relationship between 2D:4D and the structure of the androgen receptor gene (AR), with emphasis on the number of CAG repeats in the AR.

Sensitivity to T is negatively associated to CAGn, such that in general population samples the highest sensitivity is found for CAGn of about 10 and lowest sensitivity for CAGn of about 30. Therefore, we might expect that 2D:4D is positively correlated with CAGn. There is mixed evidence from studies that have investigated this relationship and a recent meta-analysis including 14 samples and 1904 participants found no association between 2D:4D and CAGn (17). However, a closer inspection of the link between CAGn and T-dependent phenotypic traits suggests that normal variability of CAGn has mostly no, very small, or inconsistent effects [for example see Ref. AGD; (18)]. Thus, Hönekopp (17) concluded that "the lack of a clear correlation between CAGn and 2D:4D has no negative implications for the latter's validity as a marker of prenatal testosterone effects."

Experimental studies of the effects of FT and FE on 2D:4D are based on the assumption that the effects of prenatal hormones on human 2D:4D are essentially similar to those observed in other mammals. Consistent with this assumption, the 2D:4D of mammals, such as chimpanzees and bonobos (19), mice (20, 21), and rats (15) have been reported to show a sexual dimorphism, which is similar to that observed in humans (i.e., lower 2D:4D in males compared to females). In addition, comparative studies of primates showed that selection for high FT, resulting from a polygynous mating system, leads to the evolution of low 2D:4D (22). Moreover, the manipulation of FT and FE in animal models provides persuasive evidence for the developmental origins of 2D:4D. In rodents, there have been three such studies. Talarovicova et al. (23) reported that maternal enhancement of PT during pregnancy increased 4D length and reduced 2D:4D in both male and female rats. Zheng and Cohn (14) found that in mice a balance of FT to FE controlled 2D:4D, such that high FT increased 4D (leading to a reduction in 2D:4D) and high FE reduced 4D (leading to an increase in 2D:4D). The ratio of FT/FE had a marked effect on 4D because this fetal digit was richly supplied with receptors for FT and FE. Hence, Zheng and Cohn concluded that "digit ratio is a lifelong signal of prenatal hormonal exposure." This model was developed further by Auger et al. (15) who exposed rat fetuses to environmental levels of estrogenic and anti-androgenic disruptors. They found that, in comparison to controls, such disruptors feminized digit ratios in male rats and concluded 2D:4D was a biomarker of prenatal exposure to low-dose environmental levels of endocrine disruptors.

RIGHT–LEFT DIFFERENCES IN 2D:4D

Zheng and Cohn's (14) study has been influential in clarifying the relationship between 2D:4D and T/E ratios in the fetus. It has also shed some light on right–left differences (Dr–l) and associated effects on sex differences in 2D:4D. In their initial study, Manning et al. (2) reported that in humans right 2D:4D showed stronger relationships with target traits (such as T, E, and sperm numbers) than did left 2D:4D, suggesting that right 2D:4D is more sensitive to prenatal sex steroids than left 2D:4D [(2); see also Ref. (3), p.21]. More recently, Hönekopp and Watson (5) reported that the sex difference in right 2D:4D was greater than that of left 2D:4D. In order to determine whether 2D and 4D length is specified differently in males and females, Zheng and Cohn (14) used expression of Sox9, the earliest molecular marker of cartilage differentiation, to label the primordium of each digit in a sample of mice. They then measured the length of the Sox9 domain in the second and fourth

rays. At embryonic day (ED) 12.5, when cartilage condensations first appeared, males and females did not differ significantly in 2D:4D, indicating that the sexual dimorphism arises after condensation of the digit primordia. By ED17, however, a significant sex difference in 2D:4D had emerged in the right hind paw. Of interest here, the left hind paw showed no significant difference between males and females, which is similar to the right–left asymmetry in adult humans; however, in mice both right and left hind paws exhibited a significant dimorphism by P2. These results indicate that the sexual dimorphism of 2D:4D in mice develops during a narrow window of embryonic development and that 2D:4D of the right paw is, at least initially, more sensitive to prenatal sex steroids than 2D:4D of the left paw [see also Ref. (4)]. Studies of 2D:4D usually consider both right and left 2D:4D, but also right–left 2D:4D or Dr–l as an additional negative marker for prenatal testosterone and a positive marker for prenatal estrogen (24, 25).

2D:4D AND ADULT TESTOSTERONE AND ESTROGEN

Digit ratio has been reported to show associations with a number of behavioral and morphological traits expressed in adults (3, 26). This is likely to be because 2D:4D is supposed to be a biomarker for the organizing effects of FT and FE, although this conclusion does not explain how such organizing effects influence adult traits. It may be that 2D:4D is in some way associated with adult levels of T and E through its links with FT and FE. However, most studies suggest that 2D:4D correlations with adult T and E provide at best a very faint echo of associations between 2D:4D and FT/FE. A few studies have reported a negative correlation between 2D:4D and T [e.g., Ref. (2, 27, 28)] and a positive correlation with E [e.g., Ref. (2, 29)]. Yet a review of the evidence (30) and a meta-analysis (31) concluded that in the normal non-clinical population 2D:4D is not associated with adult sex hormone levels. This view was supported by a recent study of 1036 men and 620 women aged between 39 and 70 years (32). As predicted, T and T/E ratio was found to be significantly negatively related to right 2D:4D in men, but the effects were very weak. There were no significant associations in women. In conclusion, this large study confirmed that 2D:4D is negatively associated with high T in men but the association is weak and no more than an echo of the prenatal position.

2D:4D AND "CHALLENGE" ASSOCIATED SPIKES IN TESTOSTERONE

Testosterone has energetic and immunosuppressive costs and maintaining high resting levels in the absence of challenges may be maladaptive (33, 34). Therefore, T levels tend to show spikes in response to "challenges," such as aggressive (35) and sexual (36) encounters and to competitive sports such as soccer (37). It is now becoming clear that improved muscular performance results from such spikes (38, 39). Based on the literature on relationships of 2D:4D with aggression and sports performance, we suggest that 2D:4D is not related to resting T but is associated with the magnitude, and perhaps the response to, T spikes.

There have been two studies that have considered 2D:4D and T spikes induced by exercise or by an aggressive video. (i) The participants in the former were 79 professional rugby union players. Of these, 54 players served as controls and 25 were challenged using a repeated sprint agility test. In the experimental group T was measured immediately before the test and 5 and 20 min after completion. It was found that players with low right 2D:4D relative to left 2D:4D (low Dr–l) produced the highest amount of T at all three time points. The controls showed no association between 2D:4D and testosterone (24). (ii) With regard to the latter study, 45 participants were exposed to an aggressive video (rugby tackles and a "*haka*") and a control video (a blank screen). Testosterone was assayed before and after each video and an aggression questionnaire completed after each video. The aggressive video was associated with a marginally significant increase in T, but the control video was not. Low 2D:4D (this time left 2D:4D) predicted high aggression scores after the aggressive video, and the association was particularly strong in participants showing the highest increase in T. However, there were no associations between 2D:4D and aggression after the control video (25).

If low 2D:4D predicts high T spikes in response to competition in aggressive sports and to aggressive stimuli then we may expect that low 2D:4D is associated with performance in many competitive sports.

2D:4D AND THE "CHALLENGE" LINK WITH SPORTS

Digit ratio shows relationships with many traits, but the effect sizes of reported associations are far from uniform. For example, in sports there are considerable relationships with negative correlations of about 0.4–0.6 reported in some sports such as distance running, rowing, rugby, and surfing, but also weak associations in sprinting and strength events [see Ref. (40) for a meta-analysis]. We suggest that many of these associations are driven by the link between low 2D:4D and pronounced spikes of T after challenge. For example, it is known that low Dr–l is predictive of high performance in elite rugby union players, with low Dr–l associated with high representation at international level (number of "caps") and high number of tries scored (41). Low Dr–l is also a predictor of high T spikes, and this correlation may underlie the link between 2D:4D and sports. There are also behavioral traits, which are associated with 2D:4D, but in general the effect sizes of such relationships are much weaker than the link with sports. An appropriate and related example is aggression. Aggression is important in many sports, and low 2D:4D has been reported to be correlated with high physical aggression. However, in a non-sporting context the association is generally considered to be weak and requires large sample sizes to be demonstrated convincingly (5, 42). Given this dichotomy of results, we suggest that low 2D:4D is robustly linked to high aggression, but it is the context in which aggression is measured that is important here. The work of Millet (43) illustrates this inter-actionist perspective, suggesting that low 2D:4D does predict high aggression if the participants are subject to provocation or are placed in a threatening context (43, 44). In such situations, we expect marked spikes in T and robust correlations between 2D:4D and aggression. However, if participants are tested in neutral conditions then links between 2D:4D and aggression should be tenuous. Another example of such context-dependent findings is the intensely competitive environment of short-term financial trading. We should not be surprised that in this setting a strong negative correlation between 2D:4D and financial success was reported (45). On the contrary, in neutral

laboratory conditions the links between 2D:4D and aggression are typically much weaker and should be seen with caution (46).

Studies on the associations between 2D:4D and sport are often focused on male participants. However, there is some evidence that low 2D:4D is linked to high levels of performance in females also (40), yet the overall picture with regard to 2D:4D in female athletes remains obscure [e.g., for handgrip strength, see Ref. (47)]. Little is known with regard to "challenge-related" spikes of T in females, although there is some support for a link between 2D:4D and sensitivity to administered T in women. In three studies, Van Honk and colleagues have shown that administered T modulates empathy (48), cooperation (49), and moral judgments (50) in women, and that 2D:4D strongly moderates the effects. The sample sizes in these studies are small, but the effect sizes are very large with 2D:4D explaining 25–44% of the variance of the effects of T. It is yet unclear why 2D:4D predicts response to administered spikes in T in such studies, but similar relationships between 2D:4D and response to T may also be found in men.

CONCLUSION

We conclude that 2D:4D is a biomarker for the balance between FT and FE, such that high FT and low FE is linked to low 2D:4D. There is evidence that 2D:4D is fixed in a relatively narrow developmental window at the end of the first trimester of pregnancy and that it does not change substantially with age. Considering 15 years of work on this topic, we feel that there is quite strong evidence for this link. However, more contentiously, we hypothesize that the relative levels of FT and FE have organizing effects on the adult endocrine system, which are particularly evident in "challenging" situations, such as aggressive or sexual encounters. This means that 2D:4D should correlate with T spikes produced under challenge and it may also be linked to response to such spikes. In consequence, low 2D:4D may be a predictor of high performance in sports and high aggression when provoked. We suggest that future studies regarding the links between 2D:4D and such traits as adult T levels, sports performance, and aggression should include aggressive and/or sexual stimuli in their protocols. In this way our hypothesized link between 2D:4D and T spikes may be tested.

REFERENCES

1. Phelps VR. Relative index finger length as a sex-influenced trait in man. *Am J Hum Genet* (1952) **4**:72–89.
2. Manning JT, Scutt D, Wilson J, Lewis-Jones DI. The ratio of 2nd to 4th digit length: a predictor of sperm numbers and concentrations of testosterone, luteinizing hormone and oestrogen. *Human Reprod* (1998) **13**:3000–4. doi:10.1093/humrep/13.11.3000
3. Manning JT. *Digit Ratio: A Pointer to Fertility, Behavior, and Health*. New Brunswick: Rutgers University Press (2002).
4. Manning JT. Resolving the role of prenatal sex steroids in the development of digit ratio. *Proc Natl Acad Sci U S A* (2011) **108**:16143–4. doi:10.1073/pnas.1113312108
5. Hönekopp J, Watson S. Meta-analysis of digit ratio 2D:4D shows greater sex difference in the right hand. *Am J Hum Biol* (2010) **22**:619–30. doi:10.1002/ajhb.21054
6. Malas MA, Dogan S, Evcil EH, Desdicioglu K. Fetal development of the hand, digits and digit ratio (2D:4D). *Early Hum Dev* (2006) **82**(7):469–75. doi:10.1016/j.earlhumdev.2005.12.002
7. Galis F, Ten Broek CMA, Van Dongen S, Wijnaendts LCD. Sexual dimorphism in the prenatal digit ratio (2D:4D). *Arch Sex Behav* (2010) **39**:57–62. doi:10.1007/s10508-009-9485-7
8. McIntyre MH, Ellison PT, Liebermann DE, Demerath E, Towne B. The development of sex differences in digital formula from infancy in the Fels Longitudinal Study. *Proc Roy Sob B* (2005) **272**:1473–79. doi:10.1098/rspb.2005-3100
9. Trivers R, Manning JT, Jacobson A. A longitudinal study of digit ratio (2D:4D) and other finger ratios in Jamaican children. *Horm Behav* (2006) **49**(2):150–6. doi:10.1016/j.yhbeh.2005.05.023
10. Manning JT, Kilduff LP, Trivers R. Digit ratio (2D:4D) in Klinefelter's syndrome. *Andrology* (2013) **1**:94–9. doi:10.1111/j.2047-2927.2012.00013.x
11. Berenbaum SA, Bryk KK, Nowak N, Quigley CA, Moffat S. Fingers as a marker of prenatal androgen exposure. *Endocrinology* (2009) **150**:4819–22. doi:10.1210/en.2009-0774
12. Lutchmaya S, Baron-Cohen S, Raggatt P, Knickmeyer R, Manning JT. 2nd to 4th digit ratios, fetal testosterone and estradiol. *Early Hum Dev* (2004) **77**(1-2):23–8. doi:10.1016/j.earlhumdev.2003.12.002
13. Manno FA III. Measurement of the digit lengths and the anogenital distance in mice. *Physiol Behav* (2008) **93**:364–8. doi:10.1016/j.physbeh.2007.09.011
14. Zheng Z, Cohn MJ. Developmental basis of sexually dimorphic digit ratios. *Proc Natl Acad Sci U S A* (2011) **108**(39):16289–94. doi:10.1073/pnas.1108312108
15. Auger J, Le Denmat D, Berges R, Doridot L, Salmon B, Canivenc-Lavier MC, et al. Environmental levels of oestrogenic and antioestrogenic compounds feminize digit ratios in male rats and their unexposed male progeny. *Proc Biol Sci* (2013) **280**:20131532. doi:10.1098/rspb.2013.1532
16. Dean A, Sharpe RM. Anogenital distance or digit length ratio as measures of fetal androgen exposure: relationship to male reproductive development and its disorders. *J Clin Endocrin Metab* (2013) **98**(6):2230–8. doi:10.1210/jc.2012-4057
17. Hönekopp J. No evidence that 2D:4D is related to the number of CAG repeats in the androgen receptor gene. *Front Endocrin* (2013) **4**:185. doi:10.3389/fendo.2013.00185
18. Eisenberg ML, Hsieh TC, Pastuszak AW, McIntyre MG, Walters RC, Lamb DJ, et al. The relationship between anogenital distance and the androgen receptor CAG repeat length. *Asian J Androl* (2013) **15**:286–9. doi:10.1038/aja.2012.126
19. McIntyre MH, Herrmann E, Wobber V, Halbwax M, Mohamba C, de Sousa N, et al. Bonobos have a more human-like second to fourth finger length ratio (2D:4D) than chimpanzees: a hypothesized indication of lower prenatal androgens. *J Hum Evol* (2009) **56**:361–5. doi:10.1016/j.jhevol.2008.12.004
20. Brown WM, Finn CJ, Breedlove SM. Sexual dimorphism in digit-length ratios of laboratory mice. *Anat Rec* (2002) **267**(3):231–4. doi:10.1002/ar.10108
21. Manning JT, Callow M, Bundred PE. Finger and toe ratios in humans and mice: implications for the aetiology of diseases influenced by HOX genes. *Med Hypotheses* (2003) **60**(3):340–3. doi:10.1016/S0306-9877(02)00400-0
22. Nelson E, Shultz S. Finger length ratios (2D:4D) in anthropoids implicate reduced prenatal androgens in social bonding. *Am J Phys Anthropol* (2010) **141**:395–405. doi:10.1002/ajpa.21157
23. Talarovicova A, Krskova L, Blazekova J. Testosterone enhancement during pregnancy influences the 2D:4D ratio and open field motor activity of rat siblings in adulthood. *Horm Behav* (2009) **55**(1):235–9. doi:10.1016/j.yhbeh.2008.10.010
24. Kilduff L, Cook CJ, Bennett M, Crewther B, Bracken RM, Manning JT. Right-left digit ratio (2D:4D) predicts free testosterone levels associated with a physical challenge. *J Sports Sci* (2013) **31**:677–83. doi:10.1080/02640414.2012.747690
25. Kilduff LP, Hopp RN, Cook CJ, Crewther BT, Manning JT. Digit ratio (2D:4D), aggression and testosterone in men exposed to an aggressive video stimulus. *Evol Psych* (2013) **11**:953–64.
26. Manning JT. *The Finger Ratio*. London: Faber and Faber (2009).
27. Coco M, Perciavalle V, Maci T, Nicoletti F, Di Corrado D, Perciavalle V. The second-to-fourth digit ratio correlates with the rate of academic performance in medical school students. *Mol Med Rep* (2011) **4**(3):471–6. doi:10.3892/mmr.2011.456
28. Perciavalle V, Di Corrado D, Petralia MC, Gurrisi L, Massimino S, Coco M. The second-to-fourth digit ratio correlates with aggressive behaviour in professional soccer players. *Mol Med Rep* (2013) **7**(3):1733–8. doi:10.3892/mmr.2013.1426
29. McIntyre MH, Chapman JF, Lipson SF, Ellison PT. Index-to-ring finger length ratio (2D:4D) predicts levels of salivary estradiol, but not progesterone, over the menstrual cycle. *Am J Hum Biol* (2007) **19**(3):434–6. doi:10.1002/ajhb.20623
30. Manning JT, Wood S, Vang E, Walton J, Bundred PE, van Heyningen C, et al. Second to fourth digit ratio (2D:4D) and testosterone in men. *Asian J Androl* (2004) **6**(3):211–5.

31. Hönekopp J, Bartholdt L, Beier L, Liebert A. Second to fourth digit length ratio (2D:4D) and adult sex hormone levels: new data and a meta-analytic review. *Psychoneuroendocrinology* (2007) **32**:313–21. doi:10.1016/j.psyneuen.2007.01.007
32. Muller DC, Giles GG, Bassett J, Morris HA, Manning JT, Hopper JL, et al. Second to fourth digit ratio (2D:4D) and concentrations of circulating sex hormones in adulthood. *Reprod Biol Endocrin* (2011) **9**:57. doi:10.1186/1477-7827-9-57
33. Folstad I, Karter AJ. Parasites, bright males, and the immunocompetence handicap. *Am Nat* (1992) **139**:603–22. doi:10.1086/285346
34. Muehlenbein MP, Bribiescas R. Testosterone-mediated immune functions and male life histories. *Am J Hum Biol* (2005) **17**:527–58. doi:10.1002/ajhb.20419
35. Elias M. Serum cortisol, testosterone and testosterone binding globulin responses to competitive fighting in human males. *Aggress Behav* (1981) **7**(3):215–24. doi:10.1002/1098-2337(1981)7:33.0.CO;2-M
36. Escasa MJ, Casey JF, Gray PB. Salivary testosterone levels in men at a U.S. sex club. *Arch Sex Behav* (2011) **40**:921–6. doi:10.1007/s10508-010-9711-3
37. Neave N, Wolfson S. Testosterone, territoriality and the 'home advantage'. *Physiol Behav* (2003) **78**(2):269–75. doi:10.1016/s0031-9384(02)00969-1
38. Crewther BT, Cook C, Cardinale M, Weatherby RP, Lowe T. Two emerging concepts for elite athletes: the short-term effects of testosterone and cortisol on the neuromuscular system and the dose-response training role of these endogenous hormones. *Sports Med* (2011) **41**:103–23. doi:10.2165/11539170-000000000-00000
39. Cook CJ, Kilduff LP, Crewther BT, Beaven M, West DJ. Morning based strength training improves afternoon physical performance in rugby union players. *J Sci Med Sport* (2013). doi:10.1016/j.jsams.2013.04.016
40. Hönekopp J, Schuster M. A meta-analysis on 2D:4D and athletic prowess: substantial relationships but neither hand outpredicts the other. *Pers Individ Dif* (2010) **48**:4–10. doi:10.1016/j.paid.2009.08.009
41. Bennett M, Manning JT, Cook CJ, Kilduff LP. Digit ratio (2D:4D) and performance in elite rugby players. *J Sports Sci* (2010) **28**:1415–21. doi:10.1080/02640414.2010.510143
42. Butovskaya M, Fedenok J, Burkova V, Manning J. Sex differences in 2D:4D and aggression in children and adolescents from five regions in Russia. *Am J Phys Anthropol* (2013) **152**:130–9. doi:10.1002/ajpa.22337
43. Millet K. An interactionist perspective on the relation between 2D:4D and behaviour: an overview of (moderated) relationships between 2D:4D and economic decision making. *Pers Individ Dif* (2010) **51**:397–401. doi:10.1016/j.paid.2010.04.005
44. Millet K, Dewitte S. Digit ratio (2D:4D) moderates the impact of an aggressive music video on aggression. *Pers Individ Dif* (2007) **43**:289–94. doi:10.1016/j.paid.2006.11.024
45. Coates JM, Gurnell M, Rustichini A. Second-to-fourth digit ratio predicts success among high-frequency financial traders. *Proc Natl Acad Sci U S A* (2010) **106**(2):623–8. doi:10.1073/pnas.0810907106
46. Austin EJ, Manning JT, McInroy K, Mathews E. A preliminary investigation of associations between personality, cognitive ability and digit ratio. *Pers Individ Dif* (2002) **33**:1115–24. doi:10.1016/S0191-8869(02)00002-8
47. Zhao D, Yu K, Zhang X, Zheng L. Digit ratio (2D:4D) and handgrip strength in Hani ethnicity. *PLoS One* (2013) **8**(10):e77958. doi:10.1371/journal.pone.0077958
48. Van Honk J, Schutter DJ, Bos PA, Kruijt AW, Lentjes EG, Baron-Cohen S. Testosterone administration impairs cognitive empathy in women depending on second-to-fourth digit ratio. *Proc Natl Acad Sci U S A* (2011) **108**:3448–52. doi:10.1073/pnas.1011891108
49. Van Honk J, Montoya ER, Bos PA, van Vogt M, Terburg D. New evidence on testosterone and cooperation. *Nature* (2013) **485**:E4–5. doi:10.1038/nature11136
50. Montoya ER, Terburg D, Bos PA, Will GJ, Buskens V, Raub W, et al. Testosterone administration modulates moral judgements depending on second-to-fourth digit ratio. *Psychoneuroendocrinology* (2013) **38**:1362–9. doi:10.1016/j.psyneuen.2012.12.001

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No evidence that 2D:4D is related to the number of CAG repeats in the androgen receptor gene

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The length ratio of the second to the fourth digit (2D:4D) is a putative marker of prenatal testosterone (T) effects. The number of CAG repeats (CAGn) in the AR gene is negatively correlated with T sensitivity *in vitro*. Results regarding the relationship between 2D:4D and CAGn are mixed but have featured prominently in arguments for and against the validity of 2D:4D. Here, I present random-effects meta-analyses on 14 relevant samples with altogether 1904 subjects. Results were homogeneous across studies. Even liberal estimates (upper limit of the 95% CI) were close to zero and therefore suggested no substantial relationship of CAGn with either right-hand 2D:4D, left-hand 2D:4D, or the difference between the two. However, closer analysis of the effects of CAGn on T dependent gene activation *in vitro* and of relationships between CAGn and T dependent phenotypic characteristics suggest that normal variability of CAGn has mostly no, very small, or inconsistent effects. Therefore, the lack of a clear association between CAGn and 2D:4D has no negative implications for the latter's validity as a marker of prenatal T effects.

Keywords: testosterone, 2D:4D, AR gene, CAG repeats, meta-analysis

INTRODUCTION

In contrast to circulating testosterone (T), perinatal T has long-lasting, "organizational" effects in many species, including humans (1). In the latter, T levels are particularly relevant during the second trimester, but their effects are notoriously difficult to study (2). However, 2D:4D (i.e., the length of the second digit divided by the length of the fourth digit) seems to track prenatal steroid effects (3), thereby providing an easily accessible, though probably noisy (4–6) index of individuals' hormonal past. In short, this is evidenced by experiments in mice [e.g., Ref. (7)]; correlations between steroid levels in amniotic fluid and 2D:4D at age 2 years (8); masculinized (i.e., lowered) 2D:4D in females exposed to high prenatal T levels caused by congenital adrenal hyperplasia [$d \approx 0.8$; Ref. (9)]; and feminized 2D:4D in (i) genetic males with complete androgen insensitivity syndrome [$d \approx 0.5$; Ref. (4)] and (ii) in males with Klinefelter's syndrome [$d \approx 0.8$; Ref. (10)], a condition associated with low T levels throughout development. The sex difference in 2D:4D seen in adults [$d \approx 0.4$; Ref. (9)] is established *in utero* [$d \approx 0.6$; Ref. (11, 12)]; individual 2D:4D scores show stability during development, including puberty [e.g., Ref. (13)] and are unrelated to baseline circulating T levels in adults (14, 15).

Testosterone effects depend on a structure called androgen receptor (AR), which comes in different variants, some of them leading to stronger T effects than others. The relationship between these AR variants and 2D:4D has received considerable attention, based on the notion that if 2D:4D reflects prenatal T effects and if AR variants moderate T effects, AR variants should show systematic relationships with 2D:4D [e.g., Ref. (16, 17)]. The current paper seeks to describe this relationship. However, before this is addressed further, it is necessary to look at the link between the AR and T effects in greater detail.

Testosterone regulates the transcription of genes, and this depends on the AR. In the cytoplasm, the AR is bound to heat-shock proteins and therefore inactive. When binding with T or dihydrotestosterone, the AR sheds its heat-shock proteins, changes into an active shape and migrates to the cell nucleus. There, it connects with coactivators and another AR and then binds in this dimerized form to specific sites in the DNA where it regulates the transcription of target genes (18, 19).

The AR is produced by the AR gene, which is located on the X-chromosome. On exon 1, this gene repeats the nucleotide sequence CAG; the number of these repeats (CAGn) varies interindividually in length and codes for the length of a polyglutamine stretch on the N-terminal domain of the AR. Most humans have CAGn between 15 and 30, the average is about 22 with a standard deviation of about 3.5 (20). Experiments *in vitro* demonstrated that longer polyglutamine stretches make the AR less effective, resulting in less AR-regulated genetic activity [e.g., Ref. (21–23)]. In such studies, cell lines from either monkey kidneys or human prostate cancer are transfected with AR gene variants that differ in CAGn. Subsequent activity of a target gene is then measured in the presence and absence of androgen. How strong is the effect of CAGn on target gene activity? I used figures in relevant reports (20–26) to calculate regression slopes that reflect by what proportion target gene activity drops for each additional CAG repeat (cf. **Figure 1**); activity at "normal" CAGn (around 20) served as the 100% baseline in each case. Where non-linear effects occurred at CAGn outside the normal human range (20, 25), I restricted the computation of the regression slope to the CAGn range that produced a linear effect. **Figure 1** illustrates this process based on a fictitious *in vitro* study. As can be seen from **Table 1**, which provides an overview of the results, regression slopes averaged −2.3%

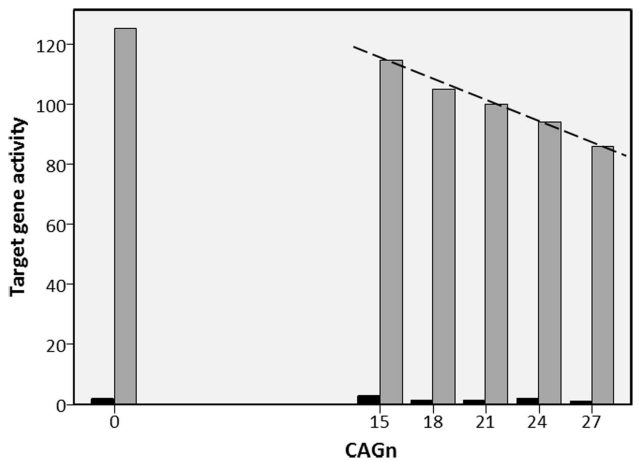

FIGURE 1 | Fictitious example of *in vitro* study into the effect of CAGn on target gene activity. Example for regression of the activity of a testosterone regulated target gene on CAGn in *in vitro* studies (cf. **Table 1**). ARs with CAGn of 0, 15, 18, 21, 24, or 27 are used either with testosterone (gray bars) or without testosterone (black bars). Target gene activity observed at CAGn most typical in humans (21) is set to 100%. Then the regression slope (dashed line, −2.3%) is calculated to describe target gene activity as a function of CAGn. In cases like the present, where CAGn outside the human range produce a deviation from linearity (here 0 CAGn), the regression slope was calculated only for those CAGn that showed a linear function.

Table 1 | Change in androgen driven target gene activity per additional CAG repeat in the AR gene in *in vitro* studies.

Study	Cell type	CAGn range tested	Change (%)
Beilin et al. (21)	Monkey kidney	15–31	−2.8
Callewaert et al. (24)	Monkey kidney	0–9	−3.9
Chamberlain et al. (22)	Monkey kidney	25–77	−0.7
Kazemi-Esfarjani et al. (26)	Monkey kidney	0–50	−1.9
Beilin et al. (21)	Prostate cancer	15–31	−1.4
Buchanan et al. (20)	Prostate cancer	16–35	−1.8
Ding et al. (23)	Prostate cancer	14–25	−1.0
Irvine et al. (25)	Prostate cancer	9–42	−0.7

for cell lines from monkey kidneys and −1.2% for prostate cancer cell lines.

In short then, high CAGn is associated with low androgen sensitivity *in vitro*; hence, a positive relationship between CAGn and 2D:4D might be expected. The first report of such a correlation (17) became one of the most frequently cited papers in the 2D:4D literature; however, later studies showed an inconsistent picture with a mixture of positive and negative findings [e.g., Ref. (27, 16)]. The relationship between CAGn and 2D:4D has often played a prominent role in discussions of the validity of 2D:4D as a marker of prenatal T effects. For example Breedlove (5) argued, "the strongest evidence that androgens affect digit ratios is the report (17) that normal polymorphism in the AR gene correlates with digit ratios in men" (p. 4117); conversely, Hampson and Sankar (16) concluded that their failure to find a positive relationship between CAGn and 2D:4D "call[s] into question the widespread assumption that small differences in the size of [. . .] [2D:4D] are an accurate gage of relative differences across individuals in fetal testosterone exposure" (p. 560).

This paper has two purposes. First, to clarify the relationship between 2D:4D and CAGn; to this end, I present a meta-analysis of the relevant literature. And second, to discuss in greater detail the implications of this relationship for the validity of 2D:4D as a marker of prenatal T effects.

MATERIALS AND METHODS

Studies were retrieved with the search terms *2D:4D* OR *digit ratio* in conjunction with *CAG* OR *AR* in the *topics* field in *ISI Web of Science* and in the *MeSH Major Topic* field in *PubMed*; this resulted in nine relevant studies from which 14 samples with 792 females and 1331 males entered the analyses. For all samples, CAGn was treated as a continuous measure and I report Pearson correlations with 2D:4D in all cases. As females (but not males) have two AR gene copies, either the shorter allele, the longer allele, or the bi-allelic mean can be used. One report (28) reported all three analyses (which led to very similar results), and I used the result for the bi-allelic mean in the present analysis. For two other reports that involved females (29, 30) it remained unclear on which of the three measures their analysis was based.

In line with the approach in the primary studies, separate meta-analyses were run for 2D:4Dr (right-hand 2D:4D), 2D:4Dl (left-hand 2D:4D), and $D_{r\text{-}l}$ (2D:4Dr × 2D:4Dl). One longitudinal study (29) reported multiple results for each 2D:4D measure and CAG repeats in the same sample. These were averaged so that each sample contributed only one effect size in each meta-analysis. The Knickmeyer samples and the Loehlin et al. (30) study contained sib-pairs. Although this creates statistical dependencies, the weighting of these samples in the analyses was not corrected downwards, mostly because it did not matter, as I will discuss later. Typically, samples showed little or no ethnic heterogeneity; for one atypical study (31), results with ethnic group as a covariate were used. Where relevant information was missing in the publications, authors were contacted (cf. note in **Table 1**). Random-effects meta-analyses were performed (32), which model the population correlation as a random variable with mean ρ and variance τ^2. Due to chance effects in sampling, multiple studies into the same phenomenon are expected to produce different results, resulting in variance of the correlations in primary studies. If the observed variance exceeds the variance to be expected due to random sampling, this suggests that primary studies differ in a systematic fashion, i.e., that not all tap into the same population correlation. E.g., the correlation between CAGn and 2D:4D might differ for females and males, young and old, etc. τ^2 reflects to what extent the observed variance in correlations exceeds the variance expected due to random sampling. The *Q*-statistic is used to test if this excess variance deviates significantly from zero. In the results, I report the standard deviation τ instead of the variance τ^2 because the former is easier to interpret. Analyses were carried out with *Comprehensive Meta-Analysis* (2.2.064).

Table 2 | Primary studies investigating the relationship between 2D:4D and CAGn.

Study	Country	Age	Sex	CAGn	*N*	*r*
2D:4D RIGHT HAND						
Manning et al. (17)	England	32.6 ± 14.2	M	21.4 ± 2.3	50	0.29*
Butovskaya et al. (27)[a]	Tanzania	≈34 ± 13	M	22.5 ± 2.2	107	0.135
Folland et al. (33)	England	20.1 ± 2.2	M	26 ± 4	77	0.10
Loehlin et al. (30)	Australia	≈14	F		218	0.08
Hurd et al. (31)	Canada	≈19 ± 2	M		155	0.05
Knickmeyer et al. (29)[a]	USA/Asian	≈1	M/F		6	0.04
Zhang et al. (28)	China	19.9 ± 1.4	F		391	0.030
Mas et al. (34)[a]			M		70	0.005
Zhang et al. (28)	China	19.9 ± 1.4	M		294	0.003
Knickmeyer et al. (29)[a]	USA/Black	≈1	M/F		31	−0.01
Knickmeyer et al. (29)[a]	USA/White	≈1	M/F	≈19.7 ± 2.5	108	−0.04
Mas et al. (34)[a]			M[b]		63	−0.04
Loehlin et al. (30)	Australia	≈14	M	22.1 ± 3.1	182	−0.06
Hampson and Sankar (16)	Canada	18.7 ± 1.6	M		152	−0.085
2D:4D LEFT HAND						
Folland et al. (33)	England	20.1 ± 2.2	M	26 ± 4	77	0.2
Butovskaya et al. (27)[a]	Tanzania	≈34 ± 13	M	22.5 ± 2.2	107	0.191*
Loehlin et al. (30)	Australia	≈14	F		218	0.14*
Zhang et al. (28)	China	19.9 ± 1.4	M		294	0.016
Manning et al. (17)	England	32.6 ± 14.2	M	21.4 ± 2.3	50	0.005
Mas et al. (34)[a]			M		70	−0.014
Zhang et al. (28)	China	19.9 ± 1.4	F		391	−0.018
Knickmeyer et al. (29)[a]	USA/White	≈1	M/F	≈19.7 ± 2.5	111	−0.03
Hampson and Sankar (16)	Canada	18.7 ± 1.6	M	22.1 ± 3.1	152	−0.063
Hurd et al. (31)	Canada	≈19 ± 2	M		153	−0.08
Knickmeyer et al. (29)[a]	USA/Black	≈1	M/F		31	−0.08
Mas et al. (34)[a]			M[b]		63	−0.081
Loehlin et al. (30)	Australia	≈14	M		181	−0.13
Knickmeyer et al. (29)[a]	USA/Asian	≈1	M/F		6	−0.41
D_{r-l}						
Knickmeyer et al. (29)[a]	USA/Asian	≈1	M/F		6	0.41
Manning et al. (17)	England	32.6 ± 14.2	M	21.4 ± 2.3	50	0.36***
Hurd et al. (31)	Canada	≈19 ± 2	M		153	0.14
Loehlin et al. (30)	Australia	≈14	M		181	0.10
Knickmeyer et al. (29)[a]	USA/Black	≈1	M/F		30	0.10
Zhang et al. (28)	China	19.9 ± 1.4	F		391	0.055
Knickmeyer et al. (29)[a]	USA/White	≈1	M/F	≈19.7 ± 2.5	105	0.04
Mas et al. (34)[a]			M		70	−0.021
Zhang et al. (28)	China	19.9 ± 1.4	M		294	−0.022
Hampson and Sankar (16)	Canada	18.7 ± 1.6	M	22.1 ± 3.1	152	−0.047
Mas et al. (34)[a]			M[b]		63	−0.057
Loehlin et al. (30)	Australia	≈14	F		218	−0.06
Butovskaya et al. (27)[a]	Tanzania	≈34 ± 13	M	22.5 ± 2.2	107	−0.080

D_{r-l} indicates right-hand 2D:4D minus left-hand 2D:4D.

[a] *Plus personal communication.*

[b] *Male-to-female transsexuals.*

*$p < 0.05$. ***$p < 0.001$.*

RESULTS

The results for individual studies are listed in **Table 2**. The results of the three meta-analyses are summarized in **Table 3**. As can be seen from column ρ, estimates for all population correlations were close to (and not significantly different from) zero, and all upper limits of the 95% CI were $r < 0.09$. Estimates for random variance around ρ were zero or small, and not statistically significant. Therefore, no attempt was made to explain differences

Table 3 | Results of meta-analyses for the relationship between CAGn and 2D:4D.

	Mean effect size				Random variance		
	ρ	*Z*	*p*	95% CI upper bound	τ	*Q* (df)	*p*
ALL SAMPLES							
2D:4D right hand	0.023	1	0.318	0.068	0	10.0(13)	0.696
2D:4D left hand	0.004	0.14	0.888	0.059	0.05	17.2(13)	0.188
D_{r-l}	0.027	1	0.320	0.081	0.04	14.5(12)	0.269
MALE SAMPLES ONLY							
2D:4D right hand	0.018	0.58	0.564	0.080	0.03	8.8(8)	0.361
2D:4D left hand	−0.005	0.13	0.896	0.070	0.06	11.9(8)	0.155
D_{r-l}	0.035	0.818	0.414	0.117	0.08	11.9(7)	0.103

Estimates for the population correlation ρ and the upper bound of the 95% confidence interval are Pearson correlations.

across study results via meta-regression. Results remained basically unchanged when: the female and mixed-sex samples ($k = 4$) were removed (cf. **Table 3**); when an unusual sample of male-to-female transsexuals was removed (detailed results not shown here); or when all of the previous were excluded from analysis (detailed results not shown here).

DISCUSSION

Estimates for the population correlations between CAGn and 2D:4D were close to zero and not statistically significant, and even a liberal viewpoint suggests that any relationship is at best very small (largest upper limit for 95% CI in the full data set $r = 0.08$). None or little (and statistically non-significant) random variance was observed. Therefore, sampling error suffices to explain the mixture of significant and non-significant findings and there is no reason to assume that the former meaningfully differ from the latter (35). As mentioned in the method section, the Loehlin et al. (30) and the Knickmeyer et al. (29) samples contained numerous sib-pairs, and this was not reflected in the weighting of these samples in the current analyses. However, **Table 1** shows that the results for these samples were either close to the estimates for ρ or else had very small sample sizes and therefore had little impact on ρ estimates in the first place; consequently, somewhat reduced weights for these samples would not have meaningfully altered the outcome of any of the analyses or any conclusions drawn. This is also illustrated by the result of the analysis that excluded mixed-sex samples (i.e., the three Knickmeyer et al. (29) samples).

Overall, the evidence is quite clear then that 2D:4D and CAGn show no substantial relationship. What does this mean for the validity of 2D:4D as a marker of prenatal T effects? Several authors argued that a relationship between CAGn and 2D:4D is to be expected if the latter indeed reflects prenatal T effects (5, 16, 17), the logic being that if variables *A* and *B* correlate, and variables *B* and *C* do as well, then a correlation between *A* and *C* should emerge. However, if $r_{AB} = 0.40$ and $r_{BC} = 0.20$, a reasonable expectation for r_{AC} is 0.08, and to differentiate this empirically from the null hypothesis ($r = 0.00$) is difficult.

There is considerable indirect evidence that the link between CAGn and T effects is weak, which is relevant in this context. First, as discussed in the introduction, *in vitro* studies suggest that each additional CAGn repeat lowers T effectiveness by about 2% (cf. **Table 1**). Thus, a one standard deviation in CAGn [which is about 3.5, Ref. (20)] would result in a T effect change of only about 7% *in vitro*. Changes of this magnitude might only have a moderate effect on 2D:4D: when Berenbaum et al. (4) looked at the effect of a 100% change in T effects by comparing typically developing men with genetic males affected from complete androgen insensitivity syndrome, the group difference in 2D:4D was about $d = 0.5$, which is equivalent to a correlation of $r = 0.241$[1]. Moreover, *in vitro* studies might overestimate the effects of CAGn *in vivo*, where lower androgen sensitivity due to higher CAGn appears to be counterbalanced by higher circulating T levels, at least in adult men (36, 37).

The second line of indirect evidence stems from relationships between CAGn and other T dependent phenotypes. Androgenetic alopecia (patterned hair loss from the scalp), male infertility, polycystic ovary syndrome, and prostate cancer are conditions in the genesis of which T is clearly implicated (38–41). Following the same line of thought that led to the investigation of a potential link between CAGn and 2D:4D (17), numerous studies looked into the link between CAGn and these conditions. Recent meta-analyses of these studies show that evidence for such a link is at best tentative for prostate cancer and absent for the other three (41–43).

Androgens promote muscle growth and therefore affect FFM (44). A similar picture emerges for the relationship between CAGn and FFM. Pertinent studies (45–49) report results for 11 samples (median $N = 115$). Statistically significant results were only obtained for the two male samples in Walsh et al. (49); in either case a *positive* relationship between CAGn and FFM was observed, which runs against expectations.

In a well-controlled intervention study by Woodhouse et al. (44), 61 eugonadal young men received either 25, 50, 125, 300, or 600 mg/week T enanthate treatment for 20 weeks. FFM gains were statistically modeled by T treatment, CAGn, age, initial strength, and other variables. T treatment explained 64% of the variance in FFM gain. The two next best predictors explained another 2 and 1% of variance, respectively, but CAGn was not among them. When T treatment was excluded as a predictor, the best three-variable model explained only 17% of the variance in FFM change, and again CAGn was not among these predictors. In sum then the results of this study do not suggest a sizable negative effect of CAGn on FFM, which is in line with the correlational studies.

Inferences from androgenetic alopecia, male infertility, polycystic ovary syndrome, prostate cancer, and FFM to 2D:4D are tentative because the former concern adult phenotypes whereas the latter is largely determined *in utero* (11, 12). Nonetheless, these domains demonstrate that a T effect on a phenotype does not necessarily mean that CAGn correlates with this phenotype. Therefore, the lack of a substantial link between CAGn and 2D:4D observed here does not necessarily implicate that 2D:4D is not affected by prenatal T.

[1]This is because $r = \sqrt{\frac{d^2}{d^2+4}}$.

On the contrary, the absence of a strong relationship between CAGn and 2D:4D makes the interpretation of 2D:4D findings less ambiguous. If 2D:4D was substantially linked to CAGn, the former might reflect AR effectiveness to a considerable degree. Consequently, a given relationship between 2D:4D and the study variable could reflect effects of circulating T, effects of prenatal T, or both. In light of the nil or near-nil relationship between CAGn and 2D:4D it seems less likely that observed correlations between 2D:4D and study variables reflect effects of circulating T instead of prenatal T [see also Ref. (14)].

Hampson and Sankar (16) conceded that 2D:4D tracks large prenatal T differences between groups (e.g., CAIS vs. typically developing individuals) but argued that the lack of CAGn and 2D:4D demonstrates the latter's inability to reflect finer prenatal T differences within each sex. But I showed here that a sizable relationship between CAGn and 2D:4D may not be expected even when 2D:4D reflects prenatal T effects well. Further, strong relationships between 2D:4D and performance in sports have been consistently shown (50–54), which also speaks against the idea that 2D:4D cannot explain within-sex differences. However, 2D:4D differences tend to be moderate (*d* about 0.4–0.8) between groups that differ strongly in prenatal T effects (4, 9, 10). This suggests that other factors than prenatal steroids strongly affect 2D:4D (55). Indeed, genetic factors unrelated to T have been implied (56, 57). The use of 2D:4D as a marker for prenatal T effects requires that the non-T variance in 2D:4D is not systematically related to the study variable, and at present we know next to nothing about this point. It would therefore be desirable to better understand the non-T variance in 2D:4D, which might open avenues for its statistical control. Further, a systematic review to what extent 2D:4D and other methods that are less accessible but also less controversial [e.g., Ref. (2)] lead to similar conclusions about prenatal T effects on human behavior would appear helpful.

CONCLUSION

A meta-analysis of the literature showed no evidence for a relationship between 2D:4D and CAGn. However, closer inspection of the effects of CAGn on T dependent gene activation *in vitro* and of relationships between CAGn and T dependent phenotypic characteristics suggests that normal variability of CAGn has no, very small, or inconsistent effects. Therefore, the observed lack of an association between CAGn and 2D:4D does not undermine the latter's validity as an indicator of prenatal T effects.

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REFERENCES

1. Hines M. Gender development and the human brain. *Annu Rev Neurosci* (2011) **34**:69–88. doi:10.1146/annurev-neuro-061010-113654
2. Cohen-Bendahan CCC, van de Beek C, Berenbaum SA. Prenatal sex hormone effects on child and adult sex-typed behavior: methods and findings. *Neurosci Biobehav Rev* (2005) **29**:353–84. doi:10.1016/j.neubiorev.2004.11.004
3. Manning JT. Resolving the role of prenatal sex steroids in the development of digit ratio. *Proc Natl Acad Sci U S A* (2012) **108**:16143–4. doi:10.1073/pnas.1113312108
4. Berenbaum SA, Bryk KK, Nowak N, Quigley CA, Moffat S. Fingers as a marker of prenatal androgen exposure. *Endocrinology* (2009) **150**:4819–22. doi:10.1210/en.2009-0774
5. Breedlove SM. Organizational hypothesis: instances of the fingerpost. *Endocrinology* (2010) **151**:4116–22. doi:10.1210/en.2010-0041
6. Dean A, Sharpe RM. Anogenital distance or digit length ratio as measures of fetal androgen exposure: relationship to male reproductive development and its disorders. *J Clin Endocrinol Metab* (2013) **98**:2230–8. doi:10.1210/jc.2012-4057
7. Zheng Z, Cohn MJ. Developmental basis of sexually dimorphic digit ratios. *Proc Natl Acad Sci U S A* (2011) **108**:16289–94. doi:10.1073/pnas.1108312108
8. Lutchmaya S, Baron-Cohen S, Raggatt P, Knickmeyer R, Manning JT. 2nd to 4th digit ratios, fetal testosterone and estradiol. *Early Hum Dev* (2004) **77**:23–8. doi:10.1016/j.earlhumdev.2003.12.002
9. Hönekopp J, Watson S. Meta-Analysis of digit ratio 2D:4D shows greater sex difference in the right hand. *Am J Hum Biol* (2010) **22**:619–30. doi:10.1002/ajhb.21054
10. Manning JT, Kilduff LP, Trivers R. Digit ratio (2D:4D) in Klinefelter's syndrome. *Andrology* (2013) **1**:94–9. doi:10.1111/j.2047-2927.2012.00013.x
11. Galis F, Ten Broek CMA, Van Dongen S, Wijnaendts LCD. Sexual dimorphism in the prenatal digit ratio (2D:4D). *Arch Sex Behav* (2010) **39**:57–62. doi:10.1007/s10508-009-9485-7
12. Malas MA, Dogan S, Evcil EH, Desdicioglu K. Fetal development of the hand, digits and digit ratio (2D:4D). *Early Hum Dev* (2006) **82**:469–75. doi:10.1016/j.earlhumdev.2005.12.002
13. Trivers R, Manning J, Jacobson A. A longitudinal study of digit ratio (2D:4D) and other finger ratios in Jamaican children. *Horm Behav* (2006) **49**:150–6. doi:10.1016/j.yhbeh.2005.05.023
14. Hönekopp J, Bartholdt L, Beier L, Liebert A. Second to fourth digit length ratio (2D:4D) and adult sex hormone levels: new data and a meta-analytic review. *Psychoneuroendocrinology* (2007) **32**:313–21. doi:10.1016/j.psyneuen.2007.01.007
15. Muller DC, Giles GG, Manning JT, Hopper JL, English DR, Severi G. Second to fourth digit ratio (2D:4D) and concentrations of circulating sex hormones in adulthood. *Reprod Biol Endocrinol* (2011) **9**:57. doi:10.1186/1477-7827-9-57
16. *Hampson E, Sankar JS. Re-examining the Manning hypothesis: androgen receptor polymorphism and the 2D:4D digit ratio. *Evol Hum Behav* (2012) **33**:557–61. doi:10.1016/j.evolhumbehav.2012.02.003
17. *Manning JT, Bundred PE, Newton DJ, Flanagan BF. The second to fourth digit ratio and variation in the androgen receptor gene. *Evol Hum Behav* (2003) **24**:399–405. doi:10.1016/S1090-5138(03)00052-7
18. Baculescu N. The role of androgen receptor activity mediated by the CAG repeat polymorphism in the pathogenesis of PCOS. *J Med Life* (2013) **6**:18–25.
19. Zitzmann M, Nieschlag E. The CAG repeat polymorphism within the androgen receptor gene and maleness. *Int J Androl* (2003) **26**:76–83. doi:10.1046/j.1365-2605.2003.00393.x
20. Buchanan G, Yang M, Cheong A, Harris JM, Irvine RA, Lambert PF, et al. Structural and functional consequences of glutamine tract variation in the androgen receptor. *Hum Mol Genet* (2004) **13**:1677–92. doi:10.1093/hmg/ddh181
21. Beilin J, Ball EMA, Favaloro JM, Zajac JD. Effect of the androgen receptor CAG repeat polymorphism on transcriptional activity: specificity in prostate and non-prostate cell lines. *J Mol Endocrinol* (2000) **25**:85–96. doi:10.1677/jme.0.0250085
22. Chamberlain NL, Driver ED, Miesfeld RL. The length and location of CAG trinucleotide repeats in the androgen receptor N-terminal domain affect transactivation function. *Nucleic Acids Res* (1994) **22**:3181–6. doi:10.1093/nar/22.15.3181
23. Ding D, Xu L, Menon M, Reddy JPV, Barrack ER. Effect of a short CAG (glutamine) repeat on human androgen receptor function. *Prostate* (2004) **58**:23–32. doi:10.1002/pros.10316
24. Callewaert L, Christiaens V, Haelens A, Verrijdt G, Verhoeven G, Claessens F. Implications of a polyglutamine tract in the function of the human androgen receptor. *Biochem Biophys Res Commun* (2003) **306**:46–52. doi:10.1016/S0006-291X(03)00902-1
25. Irvine RA, Ma H, Yu MC, Ross RK, Stallcup MR, Coetzee GA. Inhibition of p160-mediated coactivation with increasing androgen receptor polyglutamine length. *Hum Mol Genet* (2000) **9**:267–74. doi:10.1093/hmg/9.2.267
26. Kazemi-Esfarjani P, Trifiro MA, Pinsky L. Evidence for a repressive function of the long polyglutamine tract in the human androgen receptor: possible

*Entries marked by an asterisk appear in the meta-analysis.

pathogenetic relevance for the (CAG)n-expanded neuronopathies. *Hum Mol Genet* (1995) **4**:523–7. doi:10.1093/hmg/4.4.523
27. *Butovskaya ML, Vasilyev VA, Lazebny OE, Burkova VN, Kulikov AM, Mabulla A, et al. Aggression, digit ratio, and variation in the androgen receptor, serotonin transporter and dopamine D4 receptor genes in African foragers: the Hadza. *Behav Genet* (2012) **42**:647–62. doi:10.1007/s10519-012-9533-2
28. *Zhang C, Dang J, Pei L, Guo M, Zhu H, Qu L, et al. Relationship of 2D:4D finger ratio with androgen receptor CAG and GGN repeat polymorphism. *Am J Hum Biol* (2013) **25**:101–6. doi:10.1002/ajhb.22347
29. *Knickmeyer RC, Woolson S, Hamer RM, Konneker T, Gilmore JH. 2D:4D ratios in the first 2 years of life: stability and relation to testosterone exposure and sensitivity. *Horm Behav* (2011) **60**:256–63. doi:10.1016/j.yhbeh.2011.05.009
30. *Loehlin JC, Medland SE, Martin NG. Is CAG sequence length in the androgen receptor gene correlated with finger-length ratio? *Pers Indiv Differ* (2012) **52**:224–7. doi:10.1016/j.paid.2011.09.009
31. *Hurd PL, Vaillancourt KL, Dinsdale NL. Aggression, digit ratio and variation in androgen receptor and monoamine oxidase a genes in men. *Behav Genet* (2010) **41**:543–56. doi:10.1007/s10519-010-9404-7
32. Schmidt FL, Oh IS, Hayes TL. Fixed- versus random-effects models in meta-analysis: model properties and an empirical comparison of differences in results. *Br J Math Stat Psychol* (2009) **62**:97–128. doi:10.1348/000711007X255327
33. *Folland JP, McCauley TM, Phypers C, Hanson B, Mastana SS. Relationship of 2D:4D finger ratio with muscle strength, testosterone, and androgen receptor CAG repeat genotype. *Am J Phys Anthropol* (2012) **148**:81–7. doi:10.1002/ajpa.22044
34. *Mas M, Alonso C, Hernandez P, Fernandez M, Gutierrez P, Salido E, et al. Androgen receptor CAG and GGN polymorphisms and 2D:4D finger ratio in male to female transsexuals. *J Sex Med* (2009) **6**(Suppl 5):419–20.
35. Cumming G. *Understanding the New Statistics. Effect Sizes, Confidence Intervals, and Meta-Analysis*. New York: Routledge (2012).
36. Crabbe P, Bogaert V, de Bacquer D, Goemaere S, Zmierczak H, Kaufman JM. Part of the interindividual variation in serum testosterone levels in healthy men reflects differences in androgen sensitivity and feedback set point: contribution of the androgen receptor polyglutamine tract polymorphism. *J Clin Endocrinol Metab* (2007) **92**:3604–10. doi:10.1210/jc.2007-0117
37. Huhtaniemi IT, Pye SR, Limer KL, Thomson W, O'Neill TW, Platt H, et al. Increased estrogen rather than decreased androgen action is associated with longer androgen receptor CAG repeats. *J Clin Endocrinol Metab* (2009) **94**:277–84. doi:10.1210/jc.2008-0848
38. Davis-Dao CA, Tuazon ED, Sokol RZ, Cortessis VK. Male Infertility and variation in CAG repeat length in the androgen receptor gene: a meta-analysis. *J Clin Endocrinol Metab* (2007) **92**:4319–26. doi:10.1210/jc.2007-1110
39. Morales A. Androgen replacement therapy and prostate safety. *Eur Urol* (2002) **41**:113–20. doi:10.1016/S0302-2838(01)00039-2
40. Randall VA. Physiology and pathophysiology of androgenetic Alopecia. *Dermatol Ther* (2008) **21**:314–28. doi:10.1111/j.1529-8019.2008.00214.x
41. Wang R, Goodarzi MO, Xiong T, Wang D, Azziz R, Zhang H. Negative association between androgen receptor gene CAG repeat polymorphism and polycystic ovary syndrome? A systematic review and meta-analysis. *Mol Hum Reprod* (2012) **18**:498–509. doi:10.1093/molehr/gas024
42. Gu M, Dong X, Zhang X, Niu W. The CAG repeat polymorphism of androgen receptor gene and prostate cancer: a meta-analysis. *Mol Biol Rep* (2012) **39**:2615–24. doi:10.1007/s11033-011-1014-9
43. Zhuo FL, Xu W, Wang L, Wu Y, Xu ZL, Zhao JY. Androgen receptor gene polymorphisms and risk for androgenetic alopecia: a meta-analysis. *Clin Exp Dermatol* (2012) **37**:104–11. doi:10.1111/j.1365-2230.2011.04186.x
44. Woodhouse LJ, Reisz-Porszasz S, Javanbakht M, Storer TW, Lee M, Zerounian H, et al. Development of models to predict anabolic response to testosterone administration in healthy young men. *Am J Physiol Endocrinol Metab* (2003) **284**:E1009–17. doi:10.1152/ajpendo.00536.2002
45. Campbell BC, Gray PB, Eisenberg DTA, Ellison P, Sorenson MD. Androgen receptor CAG repeats and body composition among Ariaal men. *Int J Androl* (2007) **32**:140–8. doi:10.1111/j.1365-2605.2007.00825.x
46. Guadalupe-Grau A, Rodríguez-González FG, Dorado C, Olmedillas H, Fuentes T, Pérez-Gómez J. Androgen receptor gene polymorphisms lean mass and performance in young men. *Br J Sports Med* (2011) **45**:95–100. doi:10.1136/bjsm.2009.060285
47. Lapauw B, Goemaere S, Crabbe P, Kaufman JM, Ruige JB. Is the effect of testosterone on body composition modulated by the androgen receptor gene CAG repeat polymorphism in elderly men? *Eur J Endocrinol* (2007) **156**:395–401. doi:10.1530/EJE-06-0607
48. Voorhoeve PG, van Mechelen W, Uitterlinden AG, Delemarre-van de Waal HA, Lamberts SWJ. Androgen receptor gene CAG repeat polymorphism in longitudinal height and body composition in children and adolescents. *Clin Endocrinol* (2011) **74**:732–5. doi:10.1111/j.1365-2265.2011.03986.x
49. Walsh S, Zmuda JM, Cauley JA, Shea PR, Metter EJ, Hurley BF, et al. Androgen receptor CAG repeat polymorphism is associated with fat-free mass in men. *J Appl Physiol* (2005) **98**:132–7. doi:10.1152/japplphysiol.00537.2004
50. Bennett M, Manning JT, Cook CJ, Kilduff LP. Digit ratio (2D:4D) and performance in elite rugby players. *J Sports Sci* (2010) **28**:1415–21. doi:10.1080/02640414.2010.510143
51. Hönekopp J, Schuster M. A meta-analysis on 2D:4D and athletic prowess: substantial relationships but neither hand out-predicts the other. *Pers Individ Dif* (2010) **48**:4–10. doi:10.1016/j.paid.2009.08.009
52. Kilduff LP, Cook CJ, Manning JT. Digit ratio (2D:4D) and performance in male surfers. *J Strength Cond Res* (2011) **25**:3175–80. doi:10.1519/JSC.0b013e318212de8e
53. Longman D, Stock JT, Wells JCK. Digit Ratio (2D:4D) and rowing ergometer performance in males and females. *Am J Phys Anthropol* (2011) **144**:337–41. doi:10.1002/ajpa.21407
54. Voracek M, Reimer B, Dressler SG. Digit ratio (2D:4D) predicts sporting success among female fencers independent from physical, experience, and personality factors. *Scand J Med Sci Sports* (2010) **20**:853–60. doi:10.1111/j.1600-0838.2009.01031.x
55. Forstmeier W, Mueller JC, Kampenaers B. A polymorphism in the oestrogen receptor gene explains covariance between digit ratio and mating behaviour. *Proc R Soc Lond B Biol Sci* (2010) **277**:3353–61. doi:10.1098/rspb.2010.1007
56. Medland SE, Zayats T, Glaser B, Nyholt DR, Gordon SD, Wright MJ, et al. A variant in LIN28B is associated with 2D:4D finger-length ratio, a putative retrospective biomarker of prenatal testosterone exposure. *Am J Hum Genet* (2010) **86**:519–25. doi:10.1016/j.ajhg.2010.02.017
57. Lawrance-Owen AJ, Bargary G, Bosten JM, Goodbourn PT, Hogg RE, Mollon JD. Genetic association suggests that SMOC1 mediates between prenatal sex hormones and digit ratio. *Hum Genet* (2013) **132**:415–21. doi:10.1007/s00439-012-1259-y

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A comparison of anthropometric, metabolic, and reproductive characteristics of young adult women from opposite-sex and same-sex twin pairs

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Background: Prenatal exposure to androgens has been linked to masculinization of several traits. We aimed to determine whether putative female intra-uterine exposure to androgens influences anthropometric, metabolic, and reproductive parameters using a twin design.

Methods: Two cohorts of Finnish twins born in 1975–1979 and 1983–1987 formed the basis for the longitudinal FinnTwin16 (FT16) and FinnTwin12 (FT12) studies. Self-reported anthropometric characteristics, disease status, and reproductive history were compared between 679 same-sex (SS) and 789 opposite-sex (OS) female twins (mean age ± SD: 34 ± 1.1) from the wave 5 of data collection in FT16. Serum lipid and lipoprotein subclass concentrations measured by nuclear magnetic resonance spectroscopy were compared in 226 SS and 169 OS female twins (mean age ± SD: 24 ± 2.1) from the wave 4 of data collection in FT12 and FT16.

Results: Anthropometric measures, the prevalence of hypertension and diabetes mellitus type 2 did not differ significantly between females from SS and OS twin pairs at age 34. Similarly, the prevalence of infertility, age at first pregnancy and number of induced and spontaneous abortions did not differ significantly between these two groups of women. The serum lipid and lipoprotein profile did not differ between females from SS and OS twins at age 24.

Conclusion: We found no evidence that androgen overexposure of the female fetus affects obesity, metabolic profile, or reproductive health in young adult females. However, these results do not exclude the possibility that prenatal androgen exposure in females could be adversely associated with these phenotypes later in life.

Keywords: prenatal androgen exposure, twin testosterone transfer hypothesis, opposite-sex twin pairs, anthropometrics, reproductive history, lipoprotein profile

INTRODUCTION

Hyperandrogenism and insulin resistance are key features of polycystic ovary syndrome (PCOS), and women with PCOS are consequently at an increased risk of developing type 2 diabetes mellitus and the metabolic syndrome (1). Increased ovarian androgen production leads to premature adrenarche, menstrual irregularity, acne, hirsutism, and infertility by means of elevated luteinizing hormone to follicle stimulating hormone production and hyperinsulinemia (2, 3). In addition to these important reproductive outcomes, hyperandrogenism is associated with an adverse metabolic profile, including obesity, particularly abdominal obesity (4), hypertension (5), insulin resistance (6), type 2 diabetes (7), dyslipidemia (8, 9), and subclinical atherosclerosis leading to increased cardiovascular morbidity (10, 11). The onset and duration of exposure to excess androgens required to induce metabolic and reproductive anomalies is unknown. However, it has been hypothesized that intra-uterine exposure to androgens can accelerate hyperandrogenism-related phenotypes through epigenetic mechanisms (12, 13). A natural experiment to test the intra-uterine exposure hypothesis is the existence of same-sex (SS) and opposite-sex (OS) twin pairs, with the premise that females in OS twin pregnancies may be exposed to androgens from the male

fetus (14), while females from female–female pairs are not. In a Swedish study of over 17,000 female twins, females from OS twin pairs showed a more adverse anthropometric and metabolic profile than females from SS twin pairs. However, the differences between the zygosity groups were only observed in those females who were over 60 years of age (15).

We examined anthropometric and metabolic signs of hyperandrogenism and reproductive health in two large longitudinal studies of twins, which include females from SS and OS DZ twin pairs. The analysis is restricted to DZ pairs to avoid confounding from the greater variability in placentation patterns among monozygotic twin pairs. Variables related to anthropometric characteristics, disease status, and reproductive history were examined in the wave 5 study of the Finntwin16 (FT16) study, when the subjects were aged 34 years on average, while information on lipids was available from two clinical subsamples studied in their 20s. We found no evidence for any phenotype for differences between women from SS and OS twin pairs.

MATERIALS AND METHODS

THE TWIN COHORTS

The sample was derived from two population-based longitudinal studies, FinnTwin16 and FinnTwin12 (FT12) (16, 17). Both are longitudinal studies of behavioral development and health habits of Finnish twins initially enrolled during adolescence, and repeatedly assessed by self-report questionnaires. FT12 included five consecutive birth cohorts of Finnish twins born in 1983–1987. Questionnaires were mailed to twin individuals in the autumn of the year in which their birth cohort reached age 11 (90% of the responses were received by the end of that year), and subsequent follow-up assessments were made at 14, 17.5, and ~22 years. A subsample of the wave four participants was assessed in person in Helsinki, at which time a fasting blood sample was taken. In FT16, the baseline survey questionnaire was sent to all Finnish twins born in 1975–1979 within 2 months after their 16th birthday (response rate of 88%) and individuals were mailed four follow-up questionnaires at 17, 18.5, ~25, and 34 years. After the fourth wave, some of the twins were invited to more detailed clinical assessments in Helsinki, and a blood sample was taken in the morning of the assessment. Serum lipid and lipoprotein measurements were derived from these two sets of blood samples collected when the twins were in their mid-20s. The fifth wave of data collection of the FT16 cohort was done between October 2010 and November 2011 for Finnish speaking subjects and in the spring of 2012 for Swedish speaking subjects. The invitation to take part in an internet survey was sent to all twins in the cohort (born 1975–1979) living in Finland irrespective of earlier participation. Of the 6132 twins that we contacted, 4246 provided adequate data, a response rate of 69%. There were 679 female twins of SS pairs and 789 female twins from OS pairs. Mean age was 34.0 years, SD 1.13, range 31.9–37.3, with no difference by zygosity status.

Subjects completed questionnaires on anthropometric characteristics, medical status, and reproductive history. BMI (kilograms per square meter) was calculated from self-reported height (meters) and weight (kilograms). Waist circumference was self-measured and self-reported at the level midway between the lowest rib and the iliac crest according to the instructions supplied with a picture. Medical history was assessed with the question: "Has a doctor ever told you that you suffer or have suffered from hypertension, diabetes mellitus type 1 or type 2?" Response options were "yes" or "no" for each item. Reproductive history was assessed as follows: are you currently pregnant (yes/no), do you have children of which you are the biological parent (yes/no), have you ever had a spontaneous abortion (yes/no) and the number of children (open answer). The age at first pregnancy was computed from the mother birth year and year of first birth. Infertility was measured by one item: "have you ever tried to become pregnant for more than 1 year without achieving a pregnancy?"

SAMPLE CHARACTERISTICS OF THE TWINS WITH LIPID MEASUREMENTS

Serum lipid and lipoprotein subclass concentrations were measured by proton nuclear magnetic resonance (NMR) spectroscopy (18, 19). The 14 lipoprotein subclass sizes determined by this methodology are as follows: chylomicrons and extremely large VLDL particles (with particle diameters from ~75 nm upwards), five different VLDL subclasses, namely, very large VLDL (average particle diameter of 64.0 nm), large VLDL (53.6 nm), medium VLDL (44.5 nm), small VLDL (36.8 nm), very small VLDL (31.3 nm); IDL (28.6 nm), three LDL subclasses as large LDL (25.5 nm), medium LDL (23.0 nm), and small LDL (18.7); and four HDL subclasses as very large HDL (14.3 nm), large HDL (12.1 nm), medium HDL (10.9 nm), and small HDL (8.7 nm). We grouped extremely large, very large, and large VLDL to "large VLDL," small and very small VLDL to "small VLDL," IDL and large LDL to "large LDL" and very large and large HDL to "large HDL." Thus, three subclasses (large, medium, and small) of VLDL, LDL, and HDL were analyzed. The mean particle size for VLDL, LDL, and HDL particles was calculated by weighting the corresponding subclass diameters with their particle concentrations. Apolipoprotein B (apoB) and apolipoprotein A-1 (apoA-1) were estimated from an extended version of the Friedewald formula (20). NMR spectroscopy measurements were available for 436 DZ female twins (249 SS and 187 OS twins). The exclusion criteria were lipid-lowering medication ($n = 1$) and pregnancy ($n = 40$). Thus, the final sample comprised 395 DZ female twins (226 SS and 169 OS twins). Mean age was 23.9 years, SD 2.1, range 21–29, with no difference by zygosity status. Data collection and analysis were approved by the ethics committee of the Department of Public Health of the University of Helsinki, ethics committee of the Helsinki University Hospital District, and the Institutional Review Board (IRB) of Indiana University. Written informed consent was obtained from all participants.

STATISTICAL METHODS

For the wave 5 questionnaire data of FT16, we tested differences between women from SS and OS pairs by an adjusted Wald test for continuous variables and Chi^2/design-based F for categorized variables to take into account the clustering of twins in twin pairs. Sample sizes varied somewhat due to non-response to selected items.

For the wave 4 lipoprotein data of FT12 and FT16 subjects, differences between SS and OS DZ female twins were tested by the Wald tests for independent samples (*t*-tests adapted for clustered

twin data). Because the distribution of most lipid measures was highly skewed, variables were standardized by cohort and transformed using rank transformation methods in R version 2.14.0. The standard errors were corrected for clustering of twin pairs by survey methods (21). Principal component analysis was used to determine the number of principal components that explain most of the variance of the studied lipids and lipoproteins. As the strong correlations among these metabolites makes the traditional Bonferroni correction for multiple testing too conservative, the number of principal components provides a more permissive *P*-value threshold. In this study, the first five principal components explained more than 95% of the variance, allowing associations to be significant at $P < 0.01$ after the Bonferroni correction. Sample sizes vary slightly in statistical analyses because of missing data ($n = 353$–395). Statistical analyses were conducted using the Stata statistical software package (release 12.0; Stata Corporation, College Station, TX, USA).

RESULTS

Characteristics for females from SS and OS twin pairs are shown in **Table 1**. The females were normal weight based on BMI. There were no significant differences in height or adiposity measures (BMI and waist circumference) between females from SS and OS twin pairs. The prevalence of hypertension, type 1 diabetes mellitus and type 2 diabetes mellitus was low in both zygosity types and did not differ significantly between SS and OS female twins.

The reproductive history was similar between the zygosity groups of females. There were no differences in the age at the first pregnancy, the number of biological children, and the number of spontaneous abortions between females from SS and OS twin pairs. Females from SS pairs were more likely to be currently pregnant (10.2 vs. 7.4%, $P = 0.058$). The prevalence of infertility of more than 1 year duration did not differ significantly between the two groups of women (**Table 1**).

Serum lipids and lipoproteins concentrations for females from SS and OS twin pairs are shown in **Table 2**. Females from SS twin pairs had higher concentrations of serum triglycerides (mean ± SD: 1.13 ± 0.51 vs. 1.01 ± 0.43, $P = 0.038$). However, only a *P*-value below 0.01 was considered statistically significant after Bonferroni correction. None of the other serum lipid and lipoprotein subclass concentrations differed significantly between females from SS and OS twin pairs (**Table 2**).

Table 1 | Obesity measures, disease status, and reproductive characteristics for females from same-sex (SS) and opposite-sex (OS) dizygotic twin pairs.

Variable	SS females ($n = 679$)	OS females ($n = 789$)	*P*-value
Height (m), mean and SD	1.66 ± 6.1	1.66 ± 5.6	0.19
BMI (kg/m^2), mean and SD	24.2 ± 4.9	23.9 ± 4.4	0.34
Waist circumference (cm) mean and SD	81.9 ± 12.3	81.4 ± 11.7	0.48
Hypertension (%)	1.78	1.81	0.97
Type 1 diabetes (%)	0.89	0.65	0.60
Type 2 diabetes (%)	0.75	1.17	0.42
Current pregnancy (%)	10.2	7.4	0.06
Age at the first pregnancy, years	29.6	29.2	0.53
Prevalence of biological children (%)	62.2	66.1	0.12
Number of biological children (%)			
1 Child	32.1	31.3	
2 Children	44.5	46.4	
3 Children	16.4	16.2	
4 Children	4.5	3.8	
5 Children	2.6	2.3	0.97
Spontaneous abortions (%)			
Once	16.2	13.6	
2 Or more	4.1	4.2	0.37
Infertility of >1 year duration (%)	15.8	15.5	0.93

P-value from the Wald test for continuous variables and Chi^2/design-based F for categorized variables. Mean values (±SD) or percentages.
Missing values for height (n = 10), BMI (12), and waist (n = 61).
Wave 5 of FinnTwin16.

DISCUSSION

The hypothesis that prenatal hormone transfer from the male co-twin may result in masculinization of females, and therefore predispose them to endocrine disorders can be tested by comparing females from SS and OS twin pairs, i.e., females *in utero* with a male as compared to a female co-twin. Therefore, if prenatal androgen exposure influences phenotypes related to hyperandrogenism, females from OS twin pairs are expected to have a higher BMI, higher prevalence of type 2 diabetes and hypertension and a more adverse lipid profile than females from SS pairs. However, in the present study we did not find significant differences in anthropometric measures, disease status, and reproductive history between females from SS and OS twin pairs. We cannot exclude the possibility of minor differences despite a fairly substantial sample size.

The concept that individual variability in sex-related traits may be influenced by variations in hormonal exposure during fetal development is interesting and comes from animal studies with placentation patterns, which are quite different from human twin pregnancies (14). Female fetuses developing between two males tend to show masculinized anatomical, physiological, and behavioral traits as adults. Female fetuses developing without adjacent males, on the other hand, tend to show more feminized traits as adults. These traits include permanently altered hormone levels, reproductive organs, aggressive behaviors, secondary sex ratios, and susceptibility to endocrine disruption. This intra-uterine effect is due to the transfer of testosterone from male fetuses to adjacent fetuses (14).

It is questionable whether prenatal testosterone transfer occurs in humans. Thus far direct evidence for the existence of prenatal testosterone transfer in females from OS twin pregnancies is missing. Testosterone is a steroid hormone; therefore it has the ability to diffuse through the amniotic fluid between fetuses (22). In addition, hormones can transfer among fetuses through the mother's bloodstream (23). Evidence is mounting, however, for *in utero* testosterone excess, together with gestational hyperglycemia, contributing to either early differentiation of PCOS or phenotypic amplification of its genotypes. Abnormal endocrine, ovarian, and hyperinsulinemic traits are detectable as early as

Table 2 | Serum lipid and lipoprotein profile for females from same-sex (SS) and opposite-sex (OS) twin pairs.

	SS females ($n = 226$)	OS females ($n = 169$)	*P*-value
LIPOPROTEIN PARTICLE CONCENTRATIONS			
Large VLDL (and chylomicrons) (nmol/l)	4.18 ± 4.01	3.51 ± 3.12	0.14
Medium VLDL (nmol/l)	13.79 ± 8.63	12.28 ± 6.70	0.17
Small VLDL (nmol/l)	54.08 ± 17.59	50.07 ± 16.79	0.05
Large LDL (nmol/l)	241.35 ± 56.90	230.61 ± 58.50	0.07
Medium LDL (nmol/l)	119.18 ± 30.70	114.03 ± 31.03	0.12
Small LDL (nmol/l)	136.64 ± 33.97	130.33 ± 33.99	0.10
Large HDL (μmol/l)	2.05 ± 0.78	2.06 ± 0.68	0.92
Medium HDL (μmol/l)	2.46 ± 0.45	2.37 ± 0.46	0.07
Small HDL (μmol/l)	4.87 ± 0.54	4.76 ± 0.56	0.06
LIPOPROTEIN PARTICLE SIZE			
VLDL diameter (nm)	36.53 ± 1.48	36.39 ± 1.47	0.50
LDL diameter (nm)	23.60 ± 0.18	23.60 ± 0.16	0.86
HDL diameter (nm)	10.10 ± 0.25	10.12 ± 0.23	0.45
APOLIPOPROTEINS			
ApoA-1 (g/l)	1.89 ± 0.22	1.88 ± 0.22	0.63
ApoB (g/l)	0.84 ± 0.19	0.81 ± 0.17	0.31
ApoB/ApoA-1 ratio	0.45 ± 0.10	0.44 ± 0.09	0.57
TRIGLYCERIDES			
Total triglycerides (mmol/l)	1.13 ± 0.51	1.01 ± 0.43	0.04
Extremely large VLDL-TG (mmol/l)	0.01 ± 0.01	0.01 ± 0.01	0.05
Total VLDL-TG (mmol/l)	0.70 ± 0.44	0.62 ± 0.35	0.13
CHOLESTEROL			
Total cholesterol (mmol/l)	4.99 ± 0.90	4.86 ± 0.89	0.23
IDL-C (mmol/l)	0.70 ± 0.16	0.68 ± 0.16	0.38
LDL-C (mmol/l)	1.72 ± 0.48	1.66 ± 0.49	0.20
HDL-C (mmol/l)	2.01 ± 0.42	1.99 ± 0.37	0.67
HDL2-C (mmol/l)	1.49 ± 0.45	1.47 ± 0.39	0.84
HDL3-C (mmol/l)	0.53 ± 0.06	0.52 ± 0.05	0.63
HDL-C/LDL-C ratio	1.26 ± 0.47	1.30 ± 0.44	0.43

P-value from the Wald tests for independent samples. Mean values (±SD).
Statistical significance at $P < 0.01$ after Bonferroni correction.
Wave 4 clinical subsample data from FinnTwin12 and FinnTwin16.

2 months of age in daughters of women with PCOS, with adiposity enhancement of hyperinsulinemia during childhood potentially contributing to hyperandrogenism and luteinizing hormone excess by adolescence (12).

There is indirect evidence that human fetuses gestated with a male co-twin may be masculinized in development, perhaps due to the influence of prenatal androgens: the so-called twin testosterone transfer hypothesis. Results from studies in humans using a number of traits that show distinct sexual dimorphism are conflicting. Generally, the evidence for traits such as perception and cognition is more consistent than for behaviors (24). For example, we have shown a decreased prevalence of left-handedness and better mental rotation performance among females with male co-twins as compared to females with female co-twins (25, 26), a finding consistent with the intra-uterine exposure hypothesis. On the other hand, for a variety of personality and fertility traits, we have shown no differences between females from like and OS DZ pairs (27). Some studies have reported that females from OS twin pairs show an increased tooth size (28), adverse anthropometric, and metabolic parameters (15), increased alcohol use disorder symptoms (29), increased risk for alcohol dependence (30), greater sensation seeking (31), and enhanced aggressive behaviors (32) as compared to female twins from SS twin pairs. However, the present literature is far from consistent, and negative reports exist for several traits, including, anthropometric measures (33), birth weight (34), disordered eating (35, 36), and fertility (27).

The only twin study examining the prenatal exposure effects of androgens on the prevalence of PCOS found no differences between women from OS (480 women) and SS twin pairs (711 women) (37). PCOS was defined as less than nine natural menstrual cycles a year combined with either hirsutism or acne, which is not a fully satisfactory definition of PCOS. Our results were in good agreement with the study of Kuijper et al. (37), although we did not study PCOS *per se*, but compared parameters related to the androgenic phenotype including BMI, waist circumference, hypertension, type 2 diabetes mellitus, infertility, and the serum lipid profile.

According to the prenatal testosterone exposure hypothesis, the women with a twin brother in the present study would be expected to be more prone to the hyperandrogenism-related phenotypes. However, they tended to have a more favorable serum lipoprotein profile than women from SS pairs, albeit these differences did not reach statistical significance after correction for multiple comparisons. Testosterone administration has been shown to decrease measured HDL, which is associated with atherosclerosis (38). It is possible that the higher HDL concentrations associated with female gender contradicts the effect of intra-uterine hyperandrogenism.

An early onset of menarche has been associated with body fatness (39) and early menarche has been suggested to trigger the development of the metabolic syndrome and incidence of PCOS (40). We previously reported that the women from OS DZ twin pairs had a significantly higher mean age at menarche (13.3 years) compared to the women from DS pairs (13.1 years) (41). This is a small difference and one that is unlikely to be of clinical relevance.

Non-classical congenital adrenal hyperplasia (NCAH) due to 21-hydroxylase deficiency is the most common inherited disorder of adrenal steroid biosynthesis. Patients with the classic form of NCAH show androgen excess, with or without salt wasting. The factors that associate with increased risk for adverse metabolic consequences cluster in women with NCAH and include obesity, hypertension, and insulin resistance. The androgen excess may independently contribute to this increased risk due to atherogenic lipid profiles (42). Comparison of metabolic parameters in women with PCOS, women with NCAH, and healthy control women showed that metabolic parameters were comparable among women with NCAH, lean women with PCOS, and healthy control women whereas metabolic dysfunction was evident in the obese women with PCOS (43).

There are some weaknesses in our study. We measured only phenotypes related to hyperandrogenism and not the plasma levels

of androgens. As for PCOS, we did not have any questionnaire items concerning oligomenorrea nor clinical signs for hyperandrogenism (e.g., hirsutism). The subjects of the present study were young adults and mostly healthy, which may explain why we did not observe differences in disease status between SS and OS female twins. Moreover, we cannot extrapolate our findings to middle-aged or older females. The strength of our study includes the twin study design, population-based sampling, and high response rates, as well as the comprehensive analysis of serum lipoprotein subclasses.

In conclusion, anthropometric characteristics, disease status, and reproductive history did not differ between females from SS and OS twin pairs. Thus, we found no evidence for the hypothesis that prenatal hormone transfer from the male co-twin may result in masculinization of females in regard to these PCOS-related phenotypes. However, these results do not exclude the possibility that prenatal androgen exposure in females could be associated with these phenotypes later in life.

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REFERENCES

1. Pinola P, Lashen H, Bloigu A, Puukka K, Ulmanen M, Ruokonen A, et al. Menstrual disorders in adolescence: a marker for hyperandrogenaemia and increased metabolic risks in later life? Finnish general population-based birth cohort study. *Hum Reprod* (2012) **27**:3279–86. doi:10.1093/humrep/des309
2. Kousta E, White DM, Cela E, McCarthy MI, Franks S. The prevalence of polycystic ovaries in women with infertility. *Hum Reprod* (1999) **14**:2720–3. doi:10.1093/humrep/14.11.2720
3. Sam S, Dunaif A. Polycystic ovary syndrome: syndrome XX? *Trends Endocrinol Metab* (2003) **14**:365–70. doi:10.1016/j.tem.2003.08.002
4. Gambineri A, Pelusi C, Vicennati V, Pagotto U, Pasquali R. Obesity and the polycystic ovary syndrome. *Int J Obes Relat Metab Disord* (2002) **26**:883–96.
5. Lima R, Wofford M, Reckelhoff JF. Hypertension in postmenopausal women. *Curr Hypertens Rep* (2012) **14**:254–60. doi:10.1007/s11906-012-0260-0
6. Diamanti-Kandarakis E, Dunaif A. Insulin resistance and the polycystic ovary syndrome revisited: an update on mechanisms and implications. *Endocr Rev* (2012) **33**:981–1030. doi:10.1210/er.2011-1034
7. Legro RS, Kunselman AR, Dodson WC, Dunaif A. Prevalence and predictors of risk for type 2 diabetes mellitus and impaired glucose tolerance in polycystic ovary syndrome: a prospective, controlled study in 254 affected women. *J Clin Endocrinol Metab* (1999) **84**:165–9. doi:10.1210/jc.84.1.165
8. Legro RS, Kunselman AR, Dunaif A. Prevalence and predictors of dyslipidemia in women with polycystic ovary syndrome. *Am J Med* (2001) **111**:607–13. doi:10.1016/S0002-9343(01)00948-2
9. Sidhwani S, Scoccia B, Sunghay S, Stephens-Archer CN, Mazzone T, Sam S. PCOS is associated with atherogenic changes in lipoprotein particle number and size independent of body weight. *Clin Endocrinol (Oxf)* (2011) **75**:76–82. doi:10.1111/j.1365-2265.2011.04015.x
10. Bajuk Studen K, Jensterle Sever M, Pfeifer M. Cardiovascular risk and subclinical cardiovascular disease in polycystic ovary syndrome. *Front Horm Res* (2013) **40**:64–82. doi:10.1159/000341838
11. Wild S, Pierpoint T, Jacobs H, McKeigue P. Long-term consequences of polycystic ovary syndrome: results of a 31 year follow-up study. *Hum Fertil (Camb)* (2000) **3**:101–5. doi:10.1080/1464727002000198781
12. Abbott DH, Bacha F. Ontogeny of polycystic ovary syndrome and insulin resistance in utero and early childhood. *Fertil Steril* (2013) **100**:2–11. doi:10.1016/j.fertnstert.2013.05.023
13. Abbott DH, Dumesic DA, Franks S. Developmental origin of polycystic ovary syndrome – a hypothesis. *J Endocrinol* (2002) **174**:1–5. doi:10.1677/joe.0.1740001
14. Ryan BC, Vandenbergh JG. Intrauterine position effects. *Neurosci Biobehav Rev* (2002) **26**:665–78. doi:10.1016/S0149-7634(02)00038-6
15. Alexanderson C, Henningsson S, Lichtenstein P, Holmäng A, Eriksson E. Influence of having a male twin on body mass index and risk for dyslipidemia in middle-aged and old women. *Int J Obes (Lond)* (2011) **35**:1466–9. doi:10.1038/ijo.2011.18
16. Kaprio J. Twin studies in Finland 2006. *Twin Res Hum Genet* (2006) **9**:772–7. doi:10.1375/twin.9.6.772
17. Kaprio J. The Finnish Twin Cohort Study: an update. *Twin Res Hum Genet* (2013) **16**:157–62. doi:10.1017/thg.2012.142
18. Soininen P, Kangas AJ, Würtz P, Tukiainen T, Tynkkynen T, Laatikainen R, et al. High-throughput serum NMR metabonomics for cost-effective holistic studies on systemic metabolism. *Analyst* (2009) **134**:1781–5. doi:10.1039/b910205a
19. Tukiainen T, Kettunen J, Kangas AJ, Lyytikäinen L-P, Soininen P, Sarin A-P, et al. Detailed metabolic and genetic characterization reveals new associations for 30 known lipid loci. *Hum Mol Genet* (2012) **21**:1444–55. doi:10.1093/hmg/ddr581
20. Niemi J, Mäkinen V-P, Heikkonen J, Tenkanen L, Hiltunen Y, Hannuksela ML, et al. Estimation of VLDL, IDL, LDL, HDL2, apoA-I, and apoB from the Friedewald inputs – apoB and IDL, but not LDL, are associated with mortality in type 1 diabetes. *Ann Med* (2009) **41**:451–61. doi:10.1080/07853890902893392
21. Williams RL. A note on robust variance estimation for cluster-correlated data. *Biometrics* (2000) **56**:645–6. doi:10.1111/j.0006-341X.2000.00645.x
22. Vom Saal FS, Quadagno DM, Even MD, Keisler LW, Keisler DH, Khan S. Paradoxical effects of maternal stress on fetal steroids and postnatal reproductive traits in female mice from different intrauterine positions. *Biol Reprod* (1990) **43**:751761. doi:10.1095/biolreprod43.5.751
23. Gorodeski GI, Sheean LA, Utian WH. Sex hormone modulation of flow velocity in the parametrial artery of the pregnant rat. *Am J Physiol* (1995) **268**:R614R624.
24. Tapp AL, Maybery MT, Whitehouse AJO. Evaluating the twin testosterone transfer hypothesis: a review of the empirical evidence. *Horm Behav* (2011) **60**:713–22. doi:10.1016/j.yhbeh.2011.08.011
25. Vuoksimaa E, Eriksson CJP, Pulkkinen L, Rose RJ, Kaprio J. Decreased prevalence of left-handedness among females with male co-twins: evidence suggesting prenatal testosterone transfer in humans? *Psychoneuroendocrinology* (2010) **35**:1462–72. doi:10.1016/j.psyneuen.2010.04.013
26. Vuoksimaa E, Kaprio J, Kremen WS, Hokkanen L, Viken RJ, Tuulio-Henriksson A, et al. Having a male co-twin masculinizes mental rotation performance in females. *Psychol Sci* (2010) **21**:1069–71. doi:10.1177/0956797610376075
27. Rose RJ, Kaprio J, Winter T, Dick DM, Viken RJ, Pulkkinen L, et al. Femininity and fertility in sisters with twin brothers: prenatal androgenization? Cross-sex socialization? *Psychol Sci* (2002) **13**:263–7. doi:10.1111/1467-9280.00448
28. Dempsey PJ, Townsend GC, Richards LC. Increased tooth crown size in females with twin brothers: evidence for hormonal diffusion between human twins in utero. *Am J Hum Biol* (1999) **11**:577–86. doi:10.1002/(SICI)1520-6300(199909/10)11:5<577::AID-AJHB1>3.0.CO;2-Y
29. Ellingson JM, Slutske WS, Richmond-Rakerd LS, Martin NG. Investigating the influence of prenatal androgen exposure and sibling effects on alcohol use and alcohol use disorder in females from opposite-sex twin pairs. *Alcohol Clin Exp Res* (2013) **37**:868–76. doi:10.1111/acer.12035
30. Lenz B, Müller CP, Kornhuber J. Alcohol dependence in same-sex and opposite-sex twins. *J Neural Transm* (2012) **119**:1561–4. doi:10.1007/s00702-012-0907-7
31. Resnick SM, Gottesman II, McGue M. Sensation seeking in opposite-sex twins: an effect of prenatal hormones? *Behav Genet* (1993) **23**:323–9. doi:10.1007/BF01067432

32. Cohen-Bendahan CCC, Buitelaar JK, van Goozen SH, Orlebeke JF, Cohen-Kettenis PT. Is there an effect of prenatal testosterone on aggression and other behavioral traits? A study comparing same-sex and opposite-sex twin girls. *Horm Behav* (2005) **47**:230–7. doi:10.1016/j.yhbeh.2004.10.006
33. Gaist D, Bathum L, Skytthe A, Jensen TK, McGue M, Vaupel JW, et al. Strength and anthropometric measures in identical and fraternal twins: no evidence of masculinization of females with male co-twins. *Epidemiology* (2000) **11**:340–3. doi:10.1097/00001648-200005000-00020
34. Tul N, Lucovnik M, Novak Ž, Verdenik I, Blickstein I. No "masculinization" effect of a male on birth weight of its female co-twin. *J Perinat Med* (2012) **40**:255–7. doi:10.1515/jpm-2011-0233
35. Raevuori A, Kaprio J, Hoek HW, Sihvola E, Rissanen A, Keski-Rahkonen A. Anorexia and bulimia nervosa in same-sex and opposite-sex twins: lack of association with twin type in a nationwide study of Finnish twins. *Am J Psychiatry* (2008) **165**:1604–10. doi:10.1176/appi.ajp.2008.08030362
36. Lydecker JA, Pisetsky EM, Mitchell KS, Thornton LM, Kendler KS, Reichborn-Kjennerud T, et al. Association between co-twin sex and eating disorders in opposite sex twin pairs: evaluations in North American, Norwegian, and Swedish samples. *J Psychosom Res* (2012) **72**:73–7. doi:10.1016/j.jpsychores.2011.05.014
37. Kuijper EA, Vink JM, Lambalk CB, Boomsma DI. Prevalence of polycystic ovary syndrome in women from opposite-sex twin pairs. *J Clin Endocrinol Metab* (2009) **94**:1987–90. doi:10.1210/jc.2009-0191
38. Schleich F, Legros JJ. Effects of androgen substitution on lipid profile in the adult and aging hypogonadal male. *Eur J Endocrinol* (2004) **151**:415–24. doi:10.1530/eje.0.1510415
39. Feng Y, Hong X, Wilker E, Li Z, Zhang W, Jin D, et al. Effects of age at menarche, reproductive years, and menopause on metabolic risk factors for cardiovascular diseases. *Atherosclerosis* (2008) **196**:590–7. doi:10.1016/j.atherosclerosis.2007.06.016
40. Mastorakos G, Lambrinoudaki I, Creatsas G. Polycystic ovary syndrome in adolescents: current and future treatment options. *Paediatr Drugs* (2006) **8**:311–8. doi:10.2165/00148581-200608050-00004
41. Kaprio J, Rimpelä A, Winter T, Viken RJ, Rimpelä M, Rose RJ. Common genetic influences on BMI and age at menarche. *Hum Biol* (1995) **67**:739–53.
42. Wild RA, Applebaum-Bowden D, Demers LM, Bartholomew M, Landis JR, Hazzard WR, et al. Lipoprotein lipids in women with androgen excess: independent associations with increased insulin and androgen. *Clin Chem* (1990) **36**:283–9.
43. Pall M, Azziz R, Beires J, Pignatelli D. The phenotype of hirsute women: a comparison of poly-cystic ovary syndrome and 21-hydroxylase-deficient nonclassic adrenal hyperplasia. *Fertil Steril* (2010) **94**:684–9. doi:10.1016/j.fertnstert.2009.06.025

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Spreading the clinical window for diagnosing fetal-onset hypogonadism in boys

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In early fetal development, the testis secretes – independent of pituitary gonadotropins – androgens and anti-Müllerian hormone (AMH) that are essential for male sex differentiation. In the second half of fetal life, the hypothalamic–pituitary axis gains control of testicular hormone secretion. Follicle-stimulating hormone (FSH) controls Sertoli cell proliferation, responsible for testis volume increase and AMH and inhibin B secretion, whereas luteinizing hormone (LH) regulates Leydig cell androgen and INSL3 secretion, involved in the growth and trophism of male external genitalia and in testis descent. This differential regulation of testicular function between early and late fetal periods underlies the distinct clinical presentations of fetal-onset hypogonadism in the newborn male: primary hypogonadism results in ambiguous or female genitalia when early fetal-onset, whereas it becomes clinically undistinguishable from central hypogonadism when established later in fetal life. The assessment of the hypothalamic–pituitary–gonadal axis in male has classically relied on the measurement of gonadotropin and testosterone levels in serum. These hormone levels normally decline 3–6 months after birth, thus constraining the clinical evaluation window for diagnosing male hypogonadism. The advent of new markers of gonadal function has spread this clinical window beyond the first 6 months of life. In this review, we discuss the advantages and limitations of old and new markers used for the functional assessment of the hypothalamic–pituitary–testicular axis in boys suspected of fetal-onset hypogonadism.

Keywords: hypopituitarism, cryptorchidism, micropenis, disorder of sex development, testosterone

The concept of male hypogonadism is usually associated with the adult patient, and rarely thought of as a condition in the prepubertal boy. Furthermore, male hypogonadism is most frequently equated to hypoandrogenism. Androgens are the dean of testicular hormones, and the normal testis produces very little or no testosterone during most of infancy and childhood. It is therefore easy to understand why the term hypogonadism is almost absent from the pediatrician's terminology. However, many hypogonadal states in the male bear their origin in fetal life. With the advent of direct markers of Sertoli cell function, hypogonadism can be identified in boys beyond the early postnatal critical window of pituitary–gonadal activation (1) – called "mini-puberty" by some authors – and before pubertal age. In this review, we address the diagnostic approaches of fetal-onset male hypogonadism based on the physiology and pathophysiology of the hypothalamic–pituitary–testicular axis ontogeny.

ONTOGENY OF THE HYPOTHALAMIC–PITUITARY–TESTICULAR AXIS

FETAL LIFE: THE FIRST VERSUS THE SECOND AND THIRD TRIMESTERS

The gonadotropin-releasing hormone (GnRH) neurons derive from cells present in the nasal placode in the sixth fetal week (2), which migrate together with olfactory axons and blood vessels through the cribriform plate and arrive in the developing forebrain in the 9th–10th weeks. Several genes are involved in the development and migration of GnRH neurons, including *KAL1*, *FGF8*, *FGFR1*, *PROK2*, *PROKR2*, *CHD7*, *WDR11*, and *NELF*, and in their homeostasis and function, including *DAX1* (or *NR0B1*), *LEP*, *LEPR*, *KISS1*, *KISS1R*, *TAC3*, *TACR3*, and *GNRH1* [reviewed in Ref. (3, 4)].

The pituitary gonadotropes develop in the Rathke's pouch following a sequential differentiating pathway, which also includes the other pituitary cell lineages, from the oral ectoderm ancestor. Early genes, like *SHH*, *GLI1*, *GLI2*, *LHX3*, *LHX4*, *PITX1*, *PITX2*, *OTX2*, and *HESX1*, are involved in the differentiation of all pituitary cell lineages, whereas *TBX19* (or *TPIT*), *GATA2*, and *SF1* (or *NR5A1*) are more specifically related to the gonadotrope lineage [reviewed in Ref. (5)]. Fully functional gonadotropes are present in the fetal male pituitary and secrete luteinizing hormone (LH) from week 12 and Follicle-stimulating hormone (FSH) from week 14 (6). Circulating levels of both gonadotropins increase to attain peak levels by weeks 20–25 and then decrease toward term (7–9).

The testes differentiate from the adreno-gonadal primordium by the seventh week of gestation. Interestingly, Sertoli cells actively secrete anti-Müllerian hormone (AMH), involved in the regression of the uterine anlage during the eighth and ninth weeks, i.e., before exposure to FSH. In fact, basal AMH expression is triggered by SOX9 and enhanced by SF1, GATA4, and WT1 independent of

FSH [reviewed in Ref. (10)]. Afterward, FSH increases testicular AMH output by inducing Sertoli cell proliferation and AMH transcription following the classic FSH receptor transduction pathway involving protein kinase A and cyclic AMP (10, 11). Sertoli cells also secrete inhibin B, which is present at high levels in the serum of mid-term fetuses and only slightly lower by term (8, 9). Sertoli cells are not directly regulated by androgens during fetal life since they do not express the androgen receptor [reviewed in Ref. (12)].

Approximately 1 week later than Sertoli cells do, Leydig cells differentiate in the interstitial tissue and secrete testosterone, responsible for the differentiation of the male gonaduct, the prostate, and the external genitalia, independently from fetal pituitary LH. In fact, the major regulator of testosterone production during the first trimester is chorionic gonadotropin (hCG), which circulates at high levels in fetuses with a peak at 12–17 weeks subsequently decreasing through term (7, 13). The relevance of fetal LH in Leydig cell function becomes more evident during the second and third trimesters. Both LH and hCG act on the same transmembrane receptor, the LHCG-R, present on the Leydig cell membrane and inducing cell proliferation and differentiation as well as androgen and insulin-like 3 (INSL3) synthesis and secretion. Male differentiation of internal and external genitalia is completed in the first trimester (**Figure 1**). Afterward, androgens induce the growth of the phallus and the trophism of the scrotum, whereas both androgens and INSL3 are important for testicular descent (14).

POST-NATAL LIFE: INFANCY, CHILDHOOD, AND PUBERTY

The decreasing trend in the whole hypothalamic–pituitary–testicular axis activity is reflected in low perinatal levels of all hormones (**Figure 2**) (9). Thereafter, an increase in circulating levels is observed in the neonate already by the end of the first week for gonadotropins, and from the second to fourth weeks for AMH, inhibin B, and testosterone (15, 16). It should be noted for testosterone that serum samples must be extracted to avoid interferences that artificially overestimate results (**Figure 2**). LH drives testosterone and INSL3 to peak levels during the third month; thereafter, they all decline and attain very low or undetectable levels after the sixth month (**Figure 1**) (16–18). Assays for INSL3 are now commercially available, with sufficient sensitivity to be used in patients during childhood (19), although an hCG test may be needed.

On the other hand, FSH continues to induce Sertoli cell proliferation resulting in a continuous increase in testis volume. Androgens may also exert a proliferative effect on Sertoli cells (20), but the effect should be indirect since the androgen receptor is still not expressed in Sertoli cells during early infancy [reviewed in Ref. (12)]. It should be noted that the absolute volume increment described in this period of life is modest (<1.5 mL) and cannot be clinically evidenced by palpation (21). AMH and inhibin B secretion is also enhanced: the levels of both hormones increase progressively through infancy (**Figure 1**)

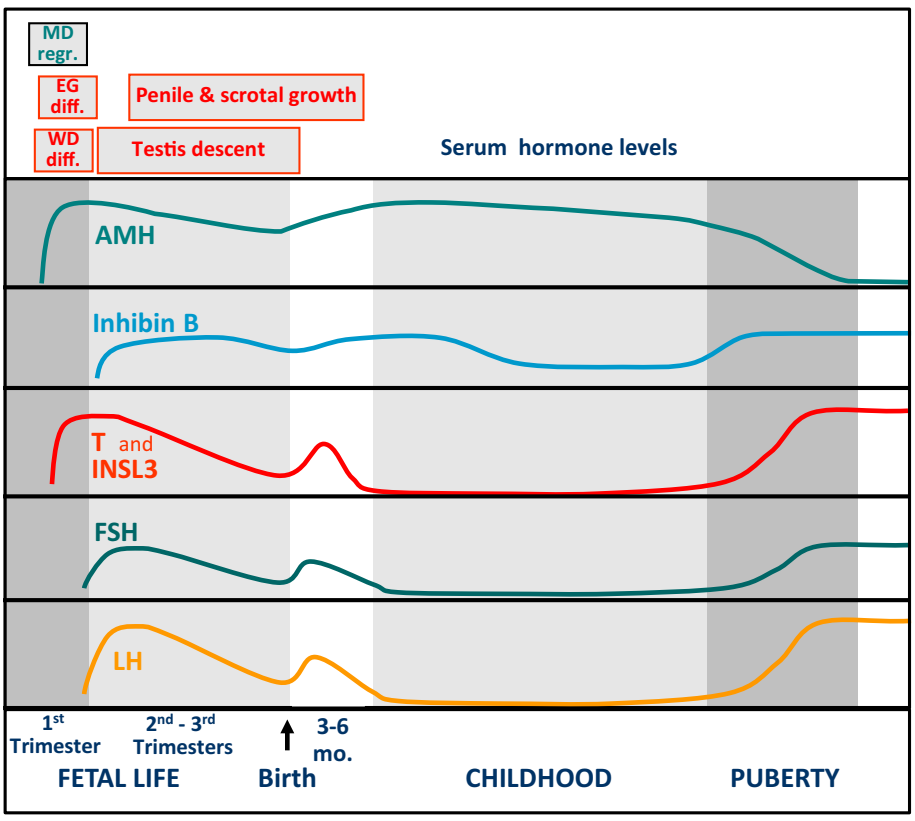

FIGURE 1 | Schematic representation of the pituitary–testicular axis hormone levels and of sexual differentiation and development of male internal and external genitalia. WD diff., Wolffian duct differentiation; MD regr., Müllerian duct regression; EG diff., differentiation of the external genitalia.

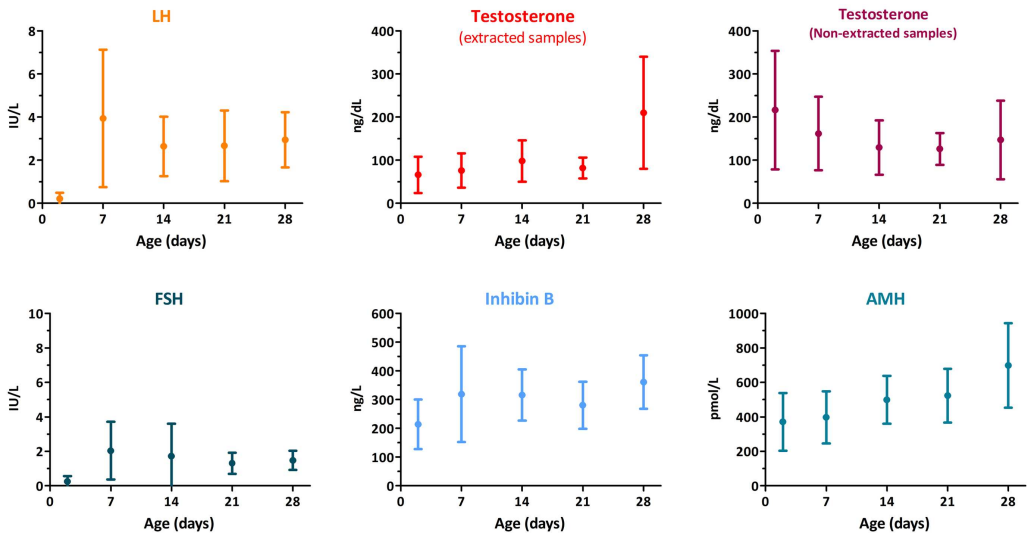

FIGURE 2 | Serum levels of gonadotropins and testicular hormones in male newborns. Data obtained from Ref. (15).

(15, 22, 23). The increase observed during the first months of life may be linked to the marked proliferation of Sertoli cells that occurs after mid-gestation with a further increment after birth (24), probably enhanced by the post-natal gonadotrophic surge. Serum inhibin B levels are as high as those observed in pubertal boys during the first 6 months of age; thereafter, a progressive fall occurs until the age of 4–6 years, but serum concentrations remain considerable, since they are above the lowest limit of normal adult range (23), and can be readily detected with the commercially available new generation assays (25). Serum AMH peaks during the second year and then remains fairly stable during childhood (22, 26). Altogether, these data clearly indicate that Sertoli cells are functionally active during infancy and childhood.

Testosterone and inhibin B are the most relevant physiological factors involved in gonadotropin negative feedback in the adult. A possible role for inhibin B in FSH negative feedback before puberty is still a matter of debate. Higher FSH than LH levels observed in boys with no functional gonadal tissue (27–29), the inverse correlation between FSH and inhibin B levels observed in cryptorchid boys (30), and the suppression of FSH secretion observed in prepubertal patients with Sertoli cell neoplastic proliferations and increased inhibin B (31) support the hypothesis of the active role that inhibin B has in regulating FSH. However, the decrease in LH and FSH levels during normal male childhood is not fully dependent on these testicular hormones, since it also occurs in a considerable proportion of boys with gonadal dysgenesis (27) or anorchia (**Figure 3**) (29).

A progressive increase in gonadotropin pulse amplitude and frequency occurring between 9 and 14 years of age triggers testicular pubertal maturation. LH induces Leydig cells androgen production again: intratesticular testosterone concentration increases and acts on Sertoli cells, which now express the androgen receptor. Consequently, they acquire a mature phenotype characterized by the development of the blood–testis barrier and a down-regulation of AMH production [reviewed in Ref. (12)]. The rise in serum testosterone occurs 1–2 years later (32, 33). Germ cells, hitherto limited to spermatogonia, enter meiosis and go through the complete spermatogenic process giving rise to spermatozoa. Spermatogenic development is responsible for the remarkable increase of testis volume during puberty. FSH and germ cells induce an increase in inhibin B. Serum levels of inhibin B increase concomitantly with testicular volume, and attains adult levels as early as pubertal stage II (23, 34, 35). INSL3 secretion also increases during puberty (36); in adult, the production and secretion of INSL3 is maintained by the long-term trophic effect of LH on Leydig cell structure and function and independent of the acute steroidogenic effect of LH (16).

DEFINITION AND CLASSIFICATION OF CONGENITAL MALE HYPOGONADISM

From the comprehension of the changes occurring in the normal physiology of the pituitary–testicular axis during pre- and postnatal life, it emerges clearly that male hypogonadism cannot be limited to hypoandrogenism. The definition should be extended to all situations characterized by a decreased testicular function, as compared to what is expected for age, involving an impaired hormone secretion by Leydig cells (androgens, INSL3) and/or Sertoli cells (AMH, inhibin B) and/or a disorder of spermatogenesis (**Table 1**).

It should also be considered that the clinical manifestations of male hypogonadism will vary according to: (a) the level of the hypothalamic–pituitary–testicular axis primarily affected, (b) the testicular cell population initially impaired, and (c) the period of life when the condition is established (37).

LEVEL OF THE AXIS PRIMARILY AFFECTED: CENTRAL, PRIMARY, OR COMBINED HYPOGONADISM

In central (or hypothalamic–pituitary) hypogonadism, testicular failure is secondary to a disorder affecting the secretion of GnRH

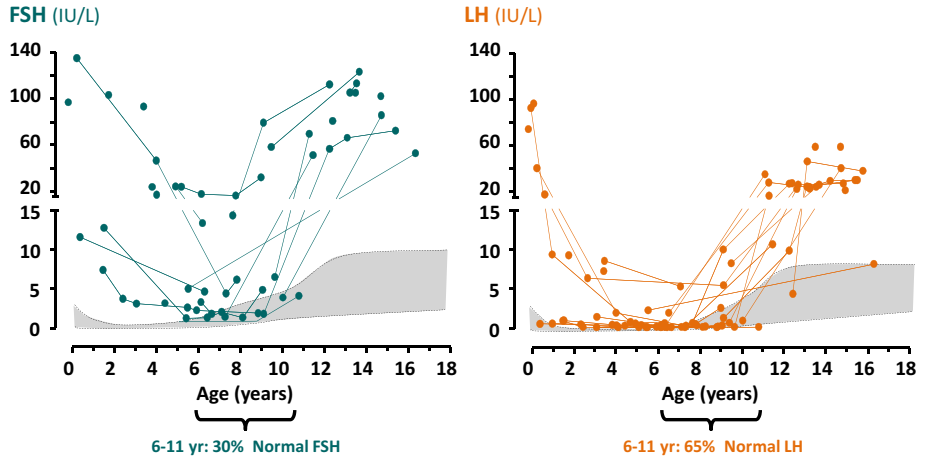

FIGURE 3 | Serum levels of gonadotropins in anorchid boys. Reproduced from Ref. (29), ©2012 Blackwell Publishing Ltd., with permission from Blackwell Publishing Ltd., John Wiley and Sons.

Table 1 | Classification of fetal-onset male hypogonadism.

	Whole gonadal dysfunction	Cell-specific gonadal dysfunction
PRIMARY HYPOGONADISM		
First trimester	Gonadal dysgenesis	Leydig cells LHCG-R mutation Steroidogenic protein defects Sertoli cells AMH mutation
Second – third trimesters	Testicular regression syndrome Testicular torsion Endocrine disruptors	Leydig cells INSL3 mutation Sertoli cells FSH-R mutation
CENTRAL HYPOGONADISM		
Second – third trimesters	Multiple pituitary hormone deficiency Isolated hypogonadotropic hypogonadism (IHH)	Leydig cells LHβ-subunit gene mutation Neurokinin defects Sertoli cells FSHβ-subunit gene mutation
COMBINED HYPOGONADISM		
First trimester	DAX1 gene mutations	None
Second – third trimesters	Prader–Willi syndrome	None

or gonadotropins. It is usually characterized by an impaired production of both LH and FSH, and thus called hypogonadotropic hypogonadism; however, as discussed later, this nomenclature is not always applicable, and some cases of central hypogonadism may present with normal or even increased levels of one gonadotropin.

In primary hypogonadism, the testis is the primarily affected organ. This may lead to an impaired production of testicular hormones and a disruption of the negative feedback to the hypothalamic–gonadotrope axis, which results in an elevation of FSH and/or LH. In adult endocrinology, primary hypogonadism is usually identified as hypergonadotropic; however, as discussed in the previous section, during childhood, primary hypogonadism – or even agonadism – may present with normal gonadotropin levels (27–29).

In certain disorders, both the hypothalamic–gonadotrope axis and the testis are affected concomitantly, e.g., in *DAX1* mutations or in oncologic patients exposed to cranial radiotherapy and chemotherapy. These "dual" conditions are characterized by a lack of gonadotropin elevation during puberty or adulthood in spite of the low testicular secretion of androgens and/or inhibin B [reviewed in Ref. (37)].

WHOLE TESTICULAR VERSUS SPERMATOGENIC, LEYDIG CELL-SPECIFIC OR SERTOLI CELL-SPECIFIC FAILURE

Whole testicular failure or hypogonadism reflects the concomitant impairment of all testicular cell populations. On the contrary, the disorder may primarily involve only one testicular cell population; for instance, spermatogenic-specific failure results from Yq chromosome deletions, steroidogenic failure from defects in LH, its receptor or steroidogenic enzymes, and Sertoli cell-specific hypogonadism from defects in FSH or its receptor or in the AMH gene, as we discuss more in detail below.

ONSET OF MALE HYPOGONADISM: FETAL VERSUS POST-NATAL LIFE

Male hypogonadism can be congenital, i.e., fetal-onset hypogonadism, or result from a condition acquired during post-natal life. The clinical presentation depends on the period of life in which testicular failure is established. In adulthood, androgen deficiency leads to decreased libido, impotence, fatigue, loss of bone and muscle mass, increased fat mass and metabolic disorders, and spermatogenic failure results in oligo- or azoospermia. At pubertal age, male hypogonadism results in the absence or the arrest

of pubertal development. Because the hypothalamic–pituitary–steroidogenic function is normally low during childhood – as explained above – male hypogonadism remains clinically unapparent when established in this period of life unless suspected and actively sought for by measuring serum AMH or inhibin B in basal conditions, or testosterone or INSL3 after stimulation with hCG [reviewed in Ref. (37)]. Fetal-onset hypogonadism may lead to a variety of clinical presentations, which are discussed in detail below.

PATHOPHYSIOLOGY OF FETAL-ONSET MALE HYPOGONADISM

The clinical consequences of fetal-onset male hypogonadism can be deduced from the understanding of the normal ontogeny of the male reproductive axis during fetal life described above. When established in the first trimester, the lack or insufficient levels of testis hormones during the critical window of male sex differentiation (weeks 8–13) lead to disorders of sex development (DSD) presenting with female or ambiguous genitalia. Because Leydig cell androgen production is essentially under placental hCG – not fetal LH – control in the first trimester, central hypogonadism does not result in DSD. Primary hypogonadism established in the second half of gestation and central hypogonadism lead to a decreased number of Sertoli cells and also to an impaired testicular output of androgens and INSL3. The clinical consequences are microorchidism, micropenis, and cryptorchidism.

PRIMARY HYPOGONADISM ESTABLISHED IN THE FIRST TRIMESTER

Whole testicular dysfunction: gonadal dysgenesis

Gonadal dysgenesis may result from chromosomal aberrations or mutations affecting genes controlling testicular differentiation (**Table 2**). Chromosome aberrations involving the short arm of the Y chromosome cause gonadal dysgenesis affecting all cell populations. Similarly, deletions of the short arm of chromosome 9 – where *DMRT1* and *DMRT2* map (38) – and duplications of Xp21.3-p21.2 – where *DAX1* gene maps (39) and of 1p31-p35 – where *WNT4* maps (40) – result in testicular dysgenesis. 46,XY patients with mutations in *SRY* (41) or *MAMLD1* (42) also present with gonadal dysgenesis. Mutations in other genes associate testicular dysgenesis with dysfunctions of other organs (**Table 2**). *SF1* mutations may associate gonadal dysgenesis with adrenal failure (43), yet isolated testicular dysfunction can be observed (44). Mutations in *WT1* result in gonadal dysgenesis associated with degenerative renal disease, resulting in Denys–Drash syndrome or in Frasier syndrome (45). Haploinsufficiency of *SOX9* leads to a polymalformative syndrome including gonadal dysgenesis, bowing and angulation of long bones (known as campomelic dysplasia), hip dislocation, hypoplastic scapula, small thoracic cage, macrocephaly, facial dysmorphism, and cardiac and renal defects (40, 46). Homozygous mutations of *DHH* gene result in the association of gonadal dysgenesis and minifascicular neuropathy (47, 48). Mutations in *XH2* gene cause the ATRX syndrome, characterized by α-thalassemia, mental retardation, facial dysmorphism and gonadal dysgenesis (49). Recently, *MAP3K1* mutations have been identified as another cause of partial or complete gonadal dysgenesis (50). Finally, mutations in *TSPYL1* have been found in patients with gonadal dysgenesis and sudden death (51). However, the vast majority of dysgenetic DSD cases remain unexplained, which suggests that several other gene defects may be the underlying cause.

Exposure to environmental disruptors *in utero* has also been implicated as the underlying cause for interlinked reproductive disorders like cryptorchidism, hypospadias, infertility and testicular cancer, which seem to show an increasing trend. This association is known as the testicular dysgenesis syndrome (52).

When the gonadal dysgenesis is complete, internal and external genitalia differentiate along the female pathway since the streak gonads do not secrete any androgens or AMH. These 46,XY girls are apparently normal and do not seek medical attention until pubertal age when they present with absence of telarche and menarche. Only in the case of contradiction between a karyotype performed during gestation and the lack of virilization, does the case present to the specialist immediately after birth.

In partial forms of testicular dysgenesis, the degree of undervirilization depends on the amount of functional gonadal tissue the patient has. The external genitalia may be more or less ambiguous, testes do not descend and Wolffian derivatives are more or less atrophic as signs of insufficient androgen secretion, reflecting Leydig cell dysfunction. The persistence of müllerian derivatives reflects defective AMH production as a sign of Sertoli cell dysfunction.

In both complete and partial forms, the androgen and inhibin B feedback mechanisms are insufficient and the gonadotrope secretion of gonadotropin is exaggerated.

Leydig cell-specific dysfunction: isolated fetal hypoandrogenism

When only Leydig cell development and/or function are primarily disturbed in the first trimester of fetal life, insufficient androgen production results in undervirilisation and cryptorchidism. On the contrary, Sertoli cells are normally active and secrete AMH which induces full regression of Müllerian ducts. Therefore, this apparently normal girl has no uterus and a short blind-end vagina. Similar to complete gonadal dysgenesis, these patients seek medical attention at pubertal age because of the absence of telarche and primary amenorrhea. In the cases of a partial defect, androgen secretion is insufficient to virilize the fetus adequately: the newborn has ambiguous external genitalia and hypotrophic Wolffian duct derivatives. The degree of virilization is commensurate with the residual steroidogenic activity of the gonads. The gonadotrope secretes excessive gonadotropins with an increased LH:FSH ratio, because FSH is negatively regulated by inhibin B.

Leydig cell aplasia is a rare form of isolated fetal hypoandrogenism leading to a DSD due to inactivating mutations of the LHCG-R (**Table 3**) [reviewed in Ref. (53)]. Defective androgen production by the testis can also result from mutations in one of the five enzymatic activities necessary for the synthesis of testosterone from cholesterol (**Table 3**). Three of these are common to adrenal and gonadal steroidogenesis: cholesterol side-chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), and 17α-hydroxylase (P450c17). A deficiency in any of these in 46,XY individuals results in testicular hypoandrogenism leading to genital ambiguity and adrenal insufficiency leading to congenital adrenal hyperplasia. Two steroidogenic steps – 17,20-lyase (activity contained in P450c17) and 17β-hydroxysteroid dehydrogenase

Table 2 | Clinical features in male patients with fetal-onset primary hypogonadism with whole gonadal dysfunction.

Affected chromosome	Gene	OMIM	Associated clinical features
9p24 deletion	*DMRT1* and *DMRT2*	#154230	Dysgenetic DSD Mental retardation, microcephaly, facial malformations, short stature Digestive or bronchial malformations
Xp21 duplication	*DAX1 = NR0B1* and other genes	#300018	Dysgenetic DSD
1p31-p35 duplication	*WNT4* and other genes	*603490	Dysgenetic DSD
Yp11.31	*SRY*	*48000	Dysgenetic DSD
Xq28	*MAMLD1*	*300120	Dysgenetic DSD
9q33.3	*SF1 = NR5A1*	+184757	Dysgenetic DSD Adrenal insufficiency
11p13	*WT1*	#136680 #194072 #194080	Dysgenetic DSD Renal dysgenesis/tumor (Denys–Drash, Frasier and WAGR syndromes)
17q24.3	*SOX9*	#114290	Dysgenetic DSD Campomelic dysplasia
12q13.12	*DHH*	#233420	Dysgenetic DSD Minifascicular neuropathy
Xq21.1	*ATRX = XH2*	#301040	Dysgenetic DSD Mental retardation, α-thalassemia
5q11.2	*MAP3K1*	#613762	Dysgenetic DSD
6q22.1	*TSPYL1*	#608800	Dysgenetic DSD Sudden infant death

DSD, disorder of sex development; OMIM, Online Mendelian Inheritance in Man locus, gene and phenotype numbers (http://www.ncbi.nlm.nih.gov/omim).

(17β-HSD) – are required only for gonadal steroidogenesis; therefore, their defects result only in hypovirilization without adrenal insufficiency [reviewed in Ref. (53)].

Sertoli cell-specific dysfunction: AMH deficiency

The persistent Müllerian duct syndrome (PMDS) is a rare form of DSD characterized by persistence of Müllerian derivatives in otherwise normally virilized 46,XY individuals. Regression of Müllerian ducts normally occurs between 8 and 10 weeks of fetal development, under the influence of AMH produced by fetal Sertoli cells. If active AMH is not produced, owing to *AMH* gene mutations, Müllerian ducts develop into uterus, fallopian tubes, and upper vagina notwithstanding normal virilization of external genitalia and urogenital sinus. PMDS can also be consecutive to mutations of the AMH receptor type II gene (*AMHR2*), but in this case testicular function is normal [reviewed in Ref. (54)]. PMDS should not be considered in patients with defects in the virilization of external genitalia. Gonadotrope activity is not affected during fetal life.

PRIMARY HYPOGONADISM ESTABLISHED IN THE SECOND AND THIRD TRIMESTERS

Whole testicular dysfunction: testicular regression syndrome

The existence of fully virilized external genitalia, i.e., completely fused scrotum and a urethral opening at the tip of the penis, is indicative of the existence of functional testes in the first trimester of gestation. However, the gonads may undergo regression (vanishing testes) due to torsion of the spermatic cord or to other unknown situations, resulting in a deficient or completely absent exposure to testicular hormones until the end of fetal life. The hypoandrogenism leads to scrotal hypotrophy and micropenis. Androgen and inhibin B insufficiency results in an exaggerated gonadotrope activity.

Leydig cell-specific dysfunction: INSL3 deficiency

Mutations in *INSL3* lead to a rare form of Leydig cell-specific dysfunction without hypoandrogenism. Newborns are normally virilized but present with cryptorchidism, reflecting the defect in testicular descent [reviewed in Ref. (55)]. Because INSL3 has no effect on the gonadotrope, LH and FSH secretion are not disturbed in these individuals during fetal life.

Sertoli cell-specific dysfunction: FSH receptor mutations

As already discussed, Sertoli cell differentiation in early fetal life is not dependent on FSH; therefore, male fetuses with FSH receptor mutations secrete sufficient amounts of AMH to induce Müllerian duct regression. On the contrary, since FSH is an important Sertoli cell mitogen, FSH-R mutations lead to Sertoli cell hypoplasia and small testes. Adults have low sperm count, low inhibin B, and moderately elevated FSH (56).

Table 3 | Clinical features in male patients with fetal-onset primary hypogonadism with Leydig cell-specific (steroidogenic) dysfunction.

Gene	Protein	OMIM	Hormone levels	Associated clinical features
LHCG-R	LH/CG receptor	#238320	↓↓ All steroids	None
STAR	StAR	#201710	↓↓ All steroids	Lipoid congenital adrenal hyperplasia
CYP11A1	P450scc	#613743	↓↓ All steroids	Adrenal insufficiency
CYP17A1	P450c17 (17α-hydroxylase activity)	#202110	↑ Pregnenolone ↑ Progesterone	Adrenal insufficiency Hypertension
CYP17A1	P450c17 (17,20-lyase activity)	#202110	↑ 17OH-pregnenolone ↑ 17OH-progesterone ↑ Pregnenolone ↑ Progesterone	Adrenal insufficiency
POR	P450 oxidoreductase	#613571	↑ Progesterone ↑ 17OH-progesterone	Antley–Bixler syndrome
HSD3B2	3β-HSD type 2	#201810	↑ DHEA ↑ 17OH-pregnenolone ↑ Pregnenolone	Adrenal insufficiency
HSD17B3	17β-HSD type 3	#264300	↑ Androstenedione ↑ DHEA ↑ 17OH-progesterone ↑ 17OH-pregnenolone	None

OMIM, Online Mendelian Inheritance in Man locus, gene and phenotype numbers (http://www.ncbi.nlm.nih.gov/omim).

CENTRAL HYPOGONADISM ESTABLISHED IN THE SECOND AND THIRD TRIMESTERS

Whole testicular dysfunction: hypogonadotropic hypogonadism

As already discussed, deficient LH and FSH production by the fetal pituitary has no effect on sexual differentiation occurring in the ninth to thirteenth weeks of gestation, but do impact on genital development dependent on testicular function in the second and third trimesters of fetal life. Gonadotropin deficiency may result from an impaired differentiation of the gonadotrope in the context of a defective development of the pituitary primordium, and is therefore associated with multiple pituitary hormone deficiency. Alternatively, the defect may be restricted to the gonadotrope axis as a consequence of an impaired development, migration or function of the GnRH neurons, or of an impaired function of the gonadotrope. The lack of gonadotropin stimulus in this period of fetal development may result in small testes due to FSH deficiency, micropenis reflecting hypoandrogenism due to LH deficiency, and cryptorchidism as a sign of androgen and INSL3 insufficiency secondary to LH deficiency.

Multiple pituitary hormone deficiency. Congenital hypopituitarism occurs in approximately 1:4,000–1:10,000 newborns, with a 7:3 male-to-female ratio (57), and involves multiple pituitary cell lineages in approximately 80% of the cases. Mutations in genes involved in early pituitary differentiation and development usually result in multiple pituitary hormone deficiency including hypogonadotropic hypogonadism, usually due to pituitary hypoplasia. Although variable, there are a few clinical signs that may help in the identification of the underlying cause (**Table 4**) [reviewed in Ref. (58, 59)]. For instance, the association of congenital multiple pituitary hormone deficiency with septo-optic dysplasia (midline neural defects and optic nerve hypoplasia) has been observed in patients with mutations in *HESX1*, *SOX2* and *SOX3*. Midline defects, coloboma and polydactyly are also present in *HESX1* patients, anophthalmia or microphthalmia and esophageal atresia in *SOX2* cases, and X-linked mental retardation in *SOX3* mutations. *LHX3* defects are present in patients with rigid and short cervical spine; *LHX4* mutations can be found in individuals with abnormalities in the central skull base; *GLI2* in patients with holoprosencephaly; *PITX2* in patients with Axenfeld–Rieger syndrome (anomalies of anterior eye chamber, dental hypoplasia, craniofacial dysmorphism, and protuberant umbilicus); *SIX6* in patients with absent optic chiasm and brain cortical atrophy, and *OTX2* in patients with microphthalmia. Defects in late development factors, like *PROP1* are present in non-syndromic patients with multiple pituitary hormone deficiencies. Currently, only <15% of the etiologies of congenital hypopituitarism have been identified (60).

Isolated hypogonadotropic hypogonadism. Congenital isolated central hypogonadism can present as the only manifestation of the disorder (normosmic hypogonadotropic hypogonadism), or be associated with partial or complete loss of olfaction (Kallmann syndrome or anosmic hypogonadotropic hypogonadism), usually associated with other anatomical and/or neurological defects [reviewed in Ref. (61)].

Hyposmic/anosmic hypogonadotropic hypogonadism with or without other syndromic features results from mutations in the genes involved in the development and migration of the GnRH

Table 4 | Clinical features in male patients with fetal-onset central hypogonadism associated with multiple pituitary hormone deficiency.

Gene	OMIM	Other pituitary lineages affected	Associated clinical features
HESX1	#182230	Somatotrope Lactotrope Thyrotrope Corticotrope	Septo-optic dysplasia Midline defects Coloboma Polydactyly
SOX2	#206900	Somatotrope	Septo-optic dysplasia Anopthalmia/microphthalmia Sensorineural defects Esophageal atresia
SOX3	#312000	Somatotrope Thyrotrope Corticotrope	Septo-optic dysplasia
LHX3	#221750	Somatotrope Lactotrope Thyrotrope	Rigid and short cervical spine Limited head rotation
LHX4	#262700	Somatotrope Thyrotrope Corticotrope	Hindbrain defects Abnormality of central skull base
GLI2	#610829	Somatotrope Lactotrope Thyrotrope Corticotrope	Holoprosencephaly
PITX2	#180500	Somatotrope Thyrotrope	Axenfeld–Rieger syndrome (anomalies of anterior eye chamber, dental hypoplasia, craniofacial dysmorphism and protuberant umbilicus)
SIX6	#212550	Somatotrope	Anophthalmia Brain cortical atrophy Brachiootorenal syndrome Oculoauriculovertebral spectrum
OTX2	#613986	Somatotrope Thyrotrope Corticotrope	Microphthalmia/anophthalmia Cleft palate Developmental delay
PROP1	#262600	Somatotrope Thyrotrope Corticotrope	Intra- and extra-sellar cell mass, which may degenerate leading to empty sella later in life

OMIM, Online Mendelian Inheritance in Man locus, gene and phenotype numbers (http://www.ncbi.nlm.nih.gov/omim).

neurons from the olfactory placode to the hypothalamus [reviewed in Ref. (61)]. The insufficient GnRH production is associated with olfactory bulb hypoplasia or aplasia in magnetic resonance imaging. Associated clinical manifestations may change according to the defective gene: *KAL1*, *FGF8* and its receptor *FGFR1*, *PROK2* and its receptor *PROKR2*, *CHD7*, *NELF*, *HS6ST1*, *WDR11*, *SEMA3A* (**Table 5**) [reviewed in Ref. (4)].

Normosmic isolated hypogonadotropic hypogonadism is the consequence of defects in genes involved in the regulation and function of the GnRH neuron or the gonadotrope. Impaired GnRH production may result from mutations in the *GNRH1* gene or from defective regulation of the GnRH neuron by kisspeptin, neurokinin, or leptin signaling via their respective receptors. Mutations in the *GNRHR* gene, encoding the GnRH receptor present in the gonadotrope, are responsible for an impaired pituitary response to GnRH. In all the cases, except for defects in the neurokinin system, the secretion of both LH and FSH is impaired.

Cell-specific dysfunction: dissociated hypogonadism

Isolated LH deficiency. Congenital isolated LH deficiency with normal or high FSH production results from mutations in the *LHB* gene encoding the β subunit of LH (62, 63), and from defects in the neurokinin system responsible for the regulation of GnRH pulses. Neurokinin is a neuropeptide encoded by *TAC3*, which signals via the neurokinin receptor encoded by *TACR3* (64, 65). Micropenis and cryptorchidism may be observed, as a consequence of the fetal hypoandrogenism during the second and

Table 5 | Associated clinical features in male patients with fetal-onset isolated central hypogonadism due to defects in the migration of the GnRH neuron.

Gene	OMIM	Associated clinical features
KAL1	#308700	Bimanual synkinesia, unilateral renal agenesis Less frequently: palate defects (cleft lip/palate), dental agenesis, ataxia, nystagmus, ear anomalies, hearing loss, visual defects, abnormal ocular movements
FGF8/ *FGFR1*	#612702 #147950	Cleft lip/palate, bone anomalies (syndactilia), dental agenesis Less frequently: hearing loss, bimanual synkinesia, ear anomalies, midline facial defects, choanal atresia, cardiac defects, coloboma
PROK2/ *PROKR2*	#610628 #244200	Sleep disorder, high-arched palate, bimanual synkinesia, hearing loss, pectus excavatum, hypodontia, obesity, nystagmus
CHD7	#612370	Coloboma, heart defects, choanal atresia, retardation of growth, genital anomalies, and ear abnormalities
NELF	#614838	None
HS6ST1	#614880	Cleft lip/palate, clinodactyly
WDR11	#614858	No
SEMA3A	#614897	No

OMIM, Online Mendelian Inheritance in Man locus, gene and phenotype numbers (http:// www.ncbi.nlm.nih.gov/ omim).

third trimesters, but there is normal testes volume in the newborn and child, because FSH levels are adequate. A mild form of isolated LH deficiency is the underlying pathophysiology of the "fertile eunuch" syndrome, characterized by the absence of signs of hypoandrogenism until puberty, when eunuchoid proportions become apparent in males with normal testis volume and sperm production. Mutations in *GNRHR* (66) and *LHB* (67) genes have been described.

Isolated FSH deficiency. Male fetuses with insufficient FSH may develop small testes during the second and third trimesters owing to Sertoli cell hypoplasia. External genitalia do not show signs of hypoandrogenism since LH production is normal or elevated (68).

COMBINED OR DUAL (PRIMARY AND CENTRAL) HYPOGONADISM

DAX1 is a transcription factor encoded by *NR0B1* mapping to the short arm of the X chromosome. It has essential functions at several levels of the pituitary–gonadal and adrenal axes. DAX1 mutations result in a disorder characterized by adrenal hypoplasia and combined hypogonadism (**Table 1**). Testicular Sertoli and Leydig cell function is primarily affected resulting in moderately low hormone production; however, since the hypothalamic–pituitary axis is also defective, the gonadotrope is unable to increase LH and FSH production, despite the absence of an effective negative feedback loop.

Prader–Willi syndrome is another form of combined central and primary hypogonadism. This condition results from the lack of the paternally inherited chromosome 15 region q11-q13; this can be due to deletions in the paternal chromosome, to maternal disomy of 15q11-q13, or to a defective imprinting that silences the paternal chromosome 15. Several genes expressed exclusively from the paternal chromosome are believed to be involved in this syndrome (including *MAGEL2*, *MKRN3*, *NDN*, *SNURF-SNRPN*, and the *HBII* genes), although their underlying mechanism is not well understood (69). Hypogonadism is reflected in signs such as micropenis, cryptorchidism, scrotal hypoplasia, and microorchidism (70). However, the pathophysiology seems to be heterogeneous, and hypogonadism may be observed earlier or later in life, with a diverse participation of the hypothalamic–pituitary axis (19, 71–74).

DIAGNOSTIC ASSESSMENT OF FETAL-ONSET MALE HYPOGONADISM

The clinical and laboratory assessment of boys with suspected hypogonadism shows a wide spectrum and varies according to the etiology of the condition. Signs of hypoandrogenism are common to all; however, as already discussed, these signs will vary according to the period of fetal life in which hypogonadism is established. On the other hand, the evaluation of the other testicular hormones, and of any associated non-reproductive phenotype, may be extremely helpful in the diagnostic assessment of these boys.

PATIENTS WITH AMBIGUOUS OR UNDERVIRILIZED EXTERNAL GENITALIA

If fetal hypogonadism is the underlying cause for the existence of a DSD presenting with ambiguous or insufficiently virilized genitalia (i.e., hypospadias, bifid scrotum), the condition can only be due to primary gonadal failure. The need for a differential diagnosis between testicular dysgenesis (i.e., whole gonadal dysfunction) and a specific steroidogenic failure emerges. A few clinical signs can be helpful in certain cases: the existence of two palpable gonads >1 mL is highly indicative of non-dysgenetic DSD (75), whereas the association of syndromic phenotypes – like skeletal dysplasia, macro/microcephaly, cardiac or renal defects, thalassemia, mental retardation, or minifascicular neuropathy orientate to gonadal dysgenesis (**Table 2**). Skeletal dysmorphisms may be present in patients with POR deficiency associated with the Antley–Bixler syndrome. Association with adrenal insufficiency is indicative of a non-dysgenetic steroidogenic defect (StAR, P450scc, P450c17, POR, 3β-HSD), although mutations in SF1 resulting in gonadal dysgenesis are also a possible cause.

Results from hormonal laboratory assessment in the newborn and infant should be interpreted according to reference values for age. In DSD patients, this is particularly relevant in the first month of life (**Figure 2**) (15), when patients are studied for diagnosis. During the first 3–6 months after birth, basal hormone level determinations may be helpful (**Figure 4**). The existence of normal levels of testosterone, AMH, and inhibin B rule out testicular dysfunction, and other etiologies of DSD should be sought (76, 77). When all testicular hormones are low and gonadotropins are elevated, gonadal dysgenesis is most likely [reviewed in Ref. (77)].

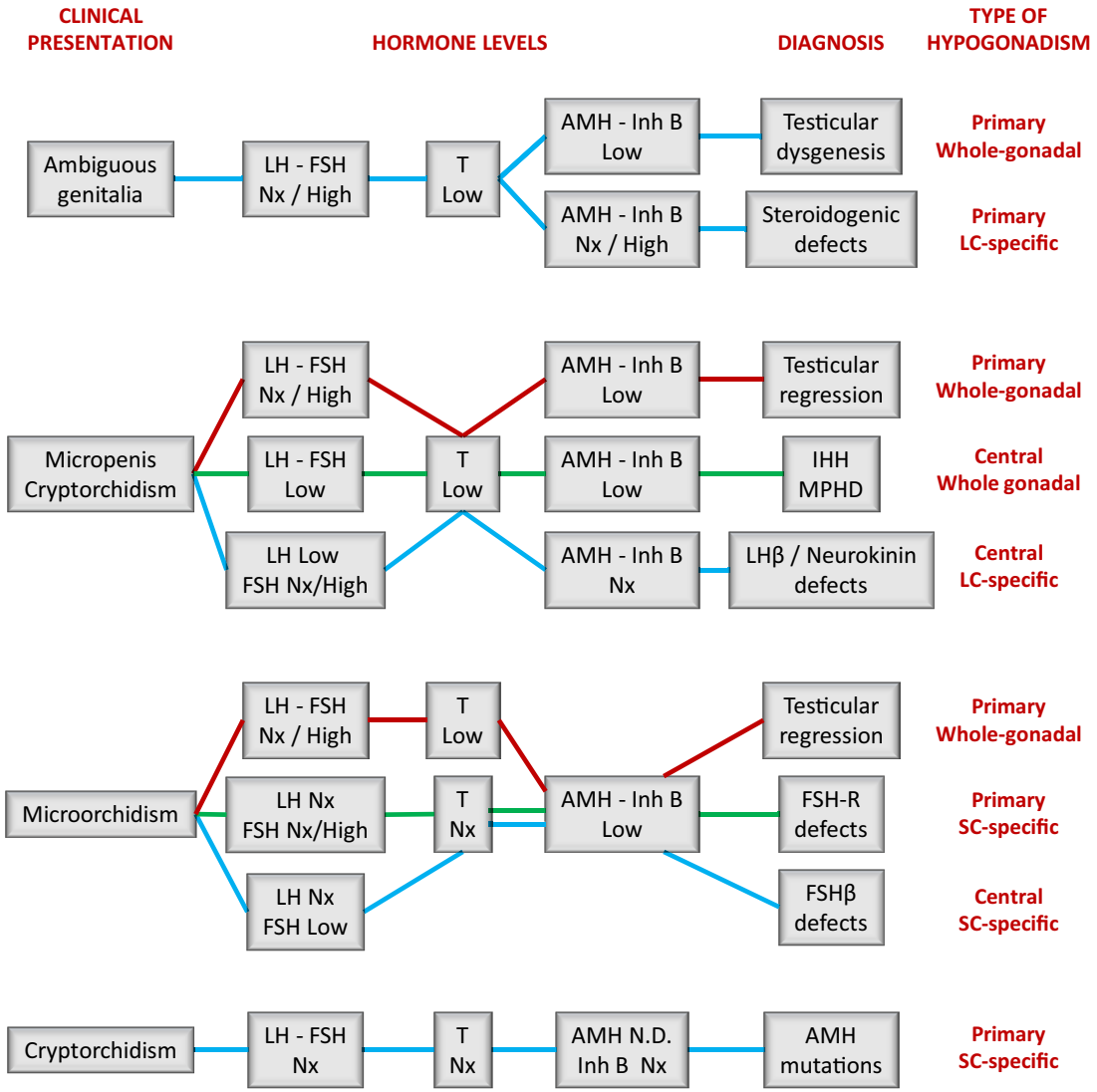

FIGURE 4 | A schematic guide for the interpretation of serum hormone levels in patients with fetal-onset male hypogonadism. IHH, isolated hypogonadotropic hypogonadism; Inh B, inhibin B; MPHD, multiple pituitary hormone deficiency; Nx, normal. Levels are considered as high, normal, or low as compared to the reference levels for newborns or prepubertal boys.

Low testosterone (53) with normal or elevated AMH (78) is characteristic of Leydig cell-specific hypogonadism. A prolonged hCG test (six IM injections every other day) and an ACTH test are necessary to distinguish between LHCG-R, StAR, and steroidogenic enzyme defects (**Table 3**). Gonadotropin levels may be somewhat elevated in the first months of life but they are usually normal during childhood in patients with steroidogenic defects (53). This is another example where primary hypogonadism is not hypergonadotropic in pediatric patients.

PATIENTS WITH MALE GENITALIA

The existence of normal male external genitalia rules out a fetal primary hypogonadism established in the first trimester, except for the rare form of Sertoli cell dysfunction due to *AMH* mutations leading to PMDS (54). PMDS patients were most frequently present with bilateral cryptorchidism; serum AMH is undetectable but the other reproductive hormones are within the normal range for age.

Fetal-onset central hypogonadism and primary hypogonadism established in the second or third trimester have clinical signs of hypoandrogenism as a common feature: small penis and undescended gonads. Microorchidism can be indicative of insufficient FSH stimulus – i.e., central hypogonadism – or of a testicular regression syndrome – i.e., a primary hypogonadism that can progress to anorchism. Because the hypothalamic–pituitary–testicular axis remains active for 3–6 months after birth (17, 18), this period represents a window of opportunity to establish the diagnosis of hypogonadism (1). However, the diagnosis can still be suspected and confirmed during the rest of infancy and childhood.

In some cases, the clinical presentation with cholestasis and/or hypoglycemia in the newborn or failure to thrive in infants can orientate the diagnosis to multiple pituitary hormone deficiency. Associated malformations in cerebral and hypothalamic-pituitary regions found on magnetic resonance imaging can be of further help (**Table 4**). A familial history of anosmia/hyposmia is suggestive of the diagnosis of isolated central hypogonadism, which could be reinforced by some anatomical or neurodevelopmental features in the infant or child (**Table 5**). Associated primary adrenal failure could orientate to adrenal hypoplasia congenital due to DAX1 mutations, whereas neonatal hypotonia and developmental delay may be indicative of Prader–Willi syndrome.

In childhood, primary hypogonadism does not equate to hypergonadotropic hypogonadism

The endocrine laboratory is necessary to certify the diagnosis of male hypogonadism. Basal gonadotropins, testosterone, and INSL3 are useful until the age of 3–6 months; thereafter dynamic stimulation tests are necessary to assess them. On the contrary, the Sertoli cell markers, AMH and inhibin B, are informative all through infancy and childhood without the need for stimulation tests. As discussed earlier, the occurrence of micropenis and non-palpable gonads prompts the differential diagnosis between central hypogonadism and testicular regression after the first trimester (**Figure 4**). If the patient is <3–6 months old, low levels of gonadotropins and Leydig and Sertoli cell hormones are suggestive of central hypogonadism (16, 79, 80), whereas high gonadotropins associated with low/undetectable testicular hormones are diagnostic of primary hypogonadism. After the age of 6 months, basal testosterone and INSL3 are no longer informative because they are normally low/undetectable during the rest of infancy and childhood. Low gonadotropins also lose usefulness. Undetectable AMH (81–83) and inhibin B (83, 84) are diagnostic of anorchia. The elevated levels of LH and FSH observed in these boys during the first years of life can subsequently decline to normal levels; therefore, serum gonadotropins within the reference range for age may not be informative during childhood (29). This is another clear example in pediatrics where primary hypogonadism is not hypergonadotropic.

Central hypogonadism is not always hypogonadotropic

The presence of micropenis, cryptorchidism, and microorchidism should prompt an early diagnosis of central hypogonadism, from which two main benefits may derive: first is to orientate the diagnosis of multiple pituitary hormone deficiency, favoring the opportune hormone replacement treatment (thyroid hormone, hydrocortisone, growth hormone). Second, as it has been postulated that the neonatal gonadotropic surge is physiologically important for testicular activity later in puberty and adulthood (85), early treatment with recombinant FSH and LH or hCG could be beneficial (79, 80). This also applies to isolated central hypogonadism. Analogously to the usefulness of testosterone and INSL3 to monitor Leydig cell response to LH/hCG (16), AMH (86) and inhibin B (87) are excellent markers of Sertoli cell response to FSH. In patients with a suspicion of central hypogonadism, AMH and inhibin B levels are suggestive if low but do not rule out the diagnosis if normal (88).

The hypoandrogenic states leading to micropenis and cryptorchidism – resulting from isolated LH deficiency due to mutations in the LHβ subunit or in the neurokinin system – are characterized by low LH and testosterone, but normal or elevated FSH. Interestingly, this central form of hypogonadism can even be hypergonadotropic, as observed in a young patient with delayed puberty, who had a functionally inactive but immunoreactive LH resulting in elevated serum levels associated with low testosterone (89).

Conversely, congenital isolated FSHβ deficiency, which presents with microorchidism but normal penile size and scrotal testes, has undetectable FSH and low inhibin B in adults with normal androgen with high LH after puberty (89, 90). No reports exist in childhood.

CONCLUDING REMARKS

Fetal hypogonadism of the first trimester is primary and results in dysgenetic or cell-specific forms of DSD. In the second and third trimesters, primary and central hypogonadism share signs of hypoandrogenism and defective INSL secretion – i.e., micropenis, hypoplastic scrotum and cryptorchidism – and of Sertoli cell hypoplasia – i.e., microorchidism. In prepubertal patients, classical serum markers, like gonadotropins and testosterone, are helpful essentially during the first 3–6 months of life. With the advent of AMH and inhibin B, a biochemical diagnosis can also be envisaged during the rest of childhood. Clinical findings may also help in the diagnosis beyond early infancy. Finally, the pediatrician should not expect elevated gonadotropin levels during childhood to foresee a primary hypogonadism.

AUTHOR CONTRIBUTIONS

All authors contributed to manuscript writing and approved the final version.

REFERENCES

1. Grumbach MM. A window of opportunity: the diagnosis of gonadotropin deficiency in the male infant. *J Clin Endocrinol Metab* (2005) **90**:3122–7. doi:10.1210/jc.2004-2465
2. Schwanzel-Fukuda M, Pfaff DW. Origin of luteinizing hormone-releasing hormone neurons. *Nature* (1989) **338**:161–4. doi:10.1038/338161a0
3. Crowley WF. The developmental biology of the GnRH neurons. *Mol Cell Endocrinol* (2011) **346**:1–3. doi:10.1016/j.mce.2011.06.023
4. Karges B, Neulen J, de Roux N, Karges W. Genetics of isolated hypogonadotropic hypogonadism: role of GnRH receptor and other genes. *Int J Endocrinol* (2012) **2012**:147893. doi:10.1155/2012/147893
5. Romero CJ, Pine-Twaddell E, Radovick S. Novel mutations associated with combined pituitary hormone deficiency. *J Mol Endocrinol* (2011) **46**:R93–102. doi:10.1530/JME-10-0133
6. Asa SL, Kovacs K, Singer W. Human fetal adenohypophysis: morphologic and functional analysis in vitro. *Neuroendocrinology* (1991) **53**:562–72. doi:10.1159/000125775
7. Kaplan SL, Grumbach MM. The ontogenesis of human foetal hormones. II. Luteinizing hormone (LH) and follicle stimulating hormone (FSH). *Acta Endocrinol* (1976) **81**:808–29.
8. Debieve F, Beerlandt S, Hubinont C, Thomas K. Gonadotropins, prolactin, inhibin A, inhibin B, and activin A in human fetal serum from midpregnancy and term pregnancy. *J Clin Endocrinol Metab* (2000) **85**:270–4. doi:10.1210/jcem.85.1.6249
9. Kuijper EA, Ket JC, Caanen MR, Lambalk CB. Reproductive hormone concentrations in pregnancy and neonates: a systematic review. *Reprod Biomed Online* (2013) **27**:33–63. doi:10.1016/j.rbmo.2013.03.009

10. Lasala C, Carré-Eusèbe D, Picard JY, Rey R. Subcellular and molecular mechanisms regulating anti-Mullerian hormone gene expression in mammalian and nonmammalian species. *DNA Cell Biol* (2004) **23**:572–85. doi:10.1089/1044549041939232
11. Lasala C, Schteingart HF, Arouche N, Bedecarrás P, Grinspon RP, Picard JY, et al. SOX9 and SF1 are involved in cyclic AMP-mediated upregulation of anti-Müllerian gene expression in the testicular prepubertal Sertoli cell line SMAT1. *Am J Physiol Endocrinol Metab* (2011) **301**:E539–47. doi:10.1152/ajpendo.00187.2011
12. Rey RA, Musse M, Venara M, Chemes HE. Ontogeny of the androgen receptor expression in the fetal and postnatal testis: its relevance on Sertoli cell maturation and the onset of adult spermatogenesis. *Microsc Res Tech* (2009) **72**:787–95. doi:10.1002/jemt.20754
13. Cole LA. Biological functions of hCG and hCG-related molecules. *Reprod Biol Endocrinol* (2010) **8**:102. doi:10.1186/1477-7827-8-102
14. Bay K, Main KM, Toppari J, Skakkebaek NE. Testicular descent: INSL3, testosterone, genes and the intrauterine milieu. *Nat Rev Urol* (2011) **8**:187–96. doi:10.1038/nrurol.2011.23
15. Bergadá I, Milani C, Bedecarrás P, Andreone L, Ropelato MG, Gottlieb S, et al. Time course of the serum gonadotropin surge, inhibins, and anti-Mullerian hormone in normal newborn males during the first month of life. *J Clin Endocrinol Metab* (2006) **91**:4092–8. doi:10.1210/jc.2006-1079
16. Bay K, Andersson AM. Human testicular insulin-like factor 3: in relation to development, reproductive hormones and andrological disorders. *Int J Androl* (2011) **34**:97–109. doi:10.1111/j.1365-2605.2010.01074.x
17. Forest MG, Sizonenko PC, Cathiard AM, Bertrand J. Hypophyso-gonadal function in humans during the first year of life. 1. Evidence for testicular activity in early infancy. *J Clin Invest* (1974) **53**:819–28. doi:10.1172/JCI107621
18. Kuiri-Hanninen T, Seuri R, Tyrvainen E, Turpeinen U, Hamalainen E, Stenman UH, et al. Increased activity of the hypothalamic-pituitary-testicular axis in infancy results in increased androgen action in premature boys. *J Clin Endocrinol Metab* (2011) **96**:98–105. doi:10.1210/jc.2010-1359
19. Hirsch HJ, Eldar-Geva T, Gross-Tsur V, Benarroch F, Roger M, Lahlou N. Normal insulin-like peptide-3 levels despite low testosterone in adult males with Prader–Willi syndrome: variations in Leydig cell function from infancy through adulthood. *J Clin Endocrinol Metab* (2013) **98**:E135–43. doi:10.1210/jc.2012-2171
20. Scott HM, Hutchison GR, Mahood IK, Hallmark N, Welsh M, De Gendt K, et al. Role of androgens in fetal testis development and dysgenesis. *Endocrinology* (2007) **148**:2027–36. doi:10.1210/en.2006-1622
21. Müller J, Skakkebæk NE. Quantification of germ cells and seminiferous tubules by stereological examination of testicles from 50 boys who suffered from sudden death. *Int J Androl* (1983) **6**:143–56. doi:10.1111/j.1365-2605.1983.tb00333.x
22. Grinspon RP, Bedecarrás P, Ballerini MG, Iñíguez G, Rocha A, Mantovani Rodrigues Resende EA, et al. Early onset of primary hypogonadism revealed by serum anti-Müllerian hormone determination during infancy and childhood in trisomy 21. *Int J Androl* (2011) **34**:e487–98. doi:10.1111/j.1365-2605.2011.01210.x
23. Bergadá I, Rojas G, Ropelato MG, Ayuso S, Bergadá C, Campo SM. Sexual dimorphism in circulating monomeric and dimeric inhibins in normal boys and girls from birth to puberty. *Clin Endocrinol* (1999) **51**:455–60. doi:10.1046/j.1365-2265.1999.00814.x
24. Berensztein EB, Sciara MI, Rivarola MA, Belgorosky A. Apoptosis and proliferation of human testicular somatic and germ cells during prepuberty: high rate of testicular growth in newborns mediated by decreased apoptosis. *J Clin Endocrinol Metab* (2002) **87**:5113–8. doi:10.1210/jc.2002-020032
25. Kalra B, Kumar A, Patel K, Patel A, Khosravi MJ. Development of a second generation inhibin B ELISA. *J Immunol Methods* (2010) **362**:22–31. doi:10.1016/j.jim.2010.08.002
26. Aksglæde L, Sorensen K, Boas M, Mouritsen A, Hagen CP, Jensen RB, et al. Changes in anti-Mullerian hormone (AMH) throughout the life span: a population-based study of 1027 healthy males from birth (cord blood) to the age of 69 years. *J Clin Endocrinol Metab* (2010) **95**:5357–64. doi:10.1210/jc.2010-1207
27. Ropelato MG, Escobar ME, Gottlieb S, Bergadá C. Gonadotropin secretion in prepubertal normal and agonadal children evaluated by ultrasensitive time-resolved immunofluorometric assays. *Horm Res* (1997) **48**:164–72. doi:10.1159/000185508
28. Conte FA, Grumbach MM, Kaplan SL. A diphasic pattern of gonadotropin secretion in patients with the syndrome of gonadal dysgenesis. *J Clin Endocrinol Metab* (1975) **40**:670–4. doi:10.1210/jcem-40-4-670
29. Grinspon RP, Ropelato MG, Bedecarrás P, Loreti N, Ballerini MG, Gottlieb S, et al. Gonadotrophin secretion pattern in anorchid boys from birth to pubertal age: pathophysiological aspects and diagnostic usefulness. *Clin Endocrinol* (2012) **76**:698–705. doi:10.1111/j.1365-2265.2011.04297.x
30. Raivio T, Dunkel L. Inverse relationship between serum inhibin B and FSH levels in prepubertal boys with cryptorchidism. *Pediatr Res* (1999) **46**:496–500. doi:10.1203/00006450-199911000-00002
31. Bergadá I, Del Toro K, Katz O, Chemes H, Campo SM. Serum inhibin B concentration in a prepubertal boy with gynecomastia and Peutz-Jeghers syndrome. *J Pediatr Endocrinol Metab* (2000) **13**:101–3. doi:10.1515/JPEM.2000.13.1.101
32. Pasqualini T, Chemes H, Rivarola MA. Testicular testosterone levels during puberty in cryptorchidism. *Clin Endocrinol* (1981) **15**:545–54. doi:10.1111/j.1365-2265.1981.tb00700.x
33. Page ST. Physiologic role and regulation of intratesticular sex steroids. *Curr Opin Endocrinol Diabetes Obes* (2011) **18**:217–23. doi:10.1097/MED.0b013e328345d50e
34. Raivio T, Perheentupa A, McNeilly AS, Groome NP, Anttila R, Siimes MA, et al. Biphasic increase in serum inhibin B during puberty: a longitudinal study of healthy Finnish boys. *Pediatr Res* (1998) **44**:552–6. doi:10.1203/00006450-199810000-00014
35. Crofton PM, Illingworth PJ, Groome NP, Stirling HF, Swanston I, Gow S, et al. Changes in dimeric inhibin A and B during normal early puberty in boys and girls. *Clin Endocrinol* (1997) **46**:109–14. doi:10.1046/j.1365-2265.1997.d01-1744.x
36. Ferlin A, Garolla A, Rigon F, Rasi CL, Lenzi A, Foresta C. Changes in serum insulin-like factor 3 during normal male puberty. *J Clin Endocrinol Metab* (2006) **91**:3426–31. doi:10.1210/jc.2006-0821
37. Rey RA, Grinspon RP, Gottlieb S, Pasqualini T, Knoblovits P, Aszpis S, et al. Male hypogonadism: an extended classification based on a developmental, endocrine physiology-based approach. *Andrology* (2013) **1**:3–16. doi:10.1111/j.2047-2927.2012.00008.x
38. Ottolenghi C, McElreavey K. Deletions of 9p and the quest for a conserved mechanism of sex determination. *Mol Genet Metab* (2000) **71**:397–404. doi:10.1006/mgme.2000.3060
39. Bardoni B, Zanaria E, Guioli S, Floridia G, Worley KC, Tonini G, et al. A dosage sensitive locus at chromosome Xp21 is involved in male to female sex reversal. *Nat Genet* (1994) **7**:497–501. doi:10.1038/ng0894-497
40. Biason-Lauber A. Control of sex development. *Best Pract Res Clin Endocrinol Metab* (2010) **24**:163–86. doi:10.1016/j.beem.2009.12.002
41. Veitia R, Ion A, Barbaux S, Jobling MA, Souleyreau N, Ennis K, et al. Mutations and sequence variants in the testis-determining region of the Y chromosome in individuals with a 46,XY female phenotype. *Hum Genet* (1997) **99**:648–52. doi:10.1007/s004390050422
42. Fukami M, Wada Y, Miyabayashi K, Nishino I, Hasegawa T, Nordenskjöld A, et al. CXorf6 is a causative gene for hypospadias. *Nat Genet* (2006) **38**:1369–71. doi:10.1038/ng1900
43. Kohler B, Lin L, Mazen I, Cetindag C, Biebermann H, Akkurt I, et al. The spectrum of phenotypes associated with mutations in steroidogenic factor 1 (SF-1, NR5A1, Ad4BP) includes severe penoscrotal hypospadias in 46,XY males without adrenal insufficiency. *Eur J Endocrinol* (2009) **161**:237–42. doi:10.1530/EJE-09-0067
44. Allali S, Muller JB, Brauner R, Lourenco D, Boudjenah R, Karageorgou V, et al. Mutation analysis of NR5A1 encoding steroidogenic factor 1 in 77 patients with 46, XY disorders of sex development (DSD) including hypospadias. *PLoS One* (2011) **6**:e24117. doi:10.1371/journal.pone.0024117
45. Fallat ME, Donahoe PK. Intersex genetic anomalies with malignant potential. *Curr Opin Pediatr* (2006) **18**:305–11. doi:10.1097/01.mop.0000193316.60580.d7
46. Haldin CE, LaBonne C. SoxE factors as multifunctional neural crest regulatory factors. *Int J Biochem Cell Biol* (2010) **42**:441–4. doi:10.1016/j.biocel.2009.11.014
47. Umehara F, Tate G, Itoh K, Yamaguchi N, Douchi T, Mitsuya T, et al. A novel mutation of desert hedgehog in a patient with 46,XY partial gonadal dysgenesis accompanied by minifascicular neuropathy. *Am J Hum Genet* (2000) **67**:1302–5. doi:10.1016/S0002-9297(07)62958-9

48. Canto P, Söderlund D, Reyes E, Méndez JP. Mutations in the desert hedgehog (DHH) gene in patients with 46,XY complete pure gonadal dysgenesis. *J Clin Endocrinol Metab* (2004) **89**:4480–3. doi:10.1210/jc.2004-0863
49. Reardon W, Gibbons RJ, Winter RM, Baraitser M. Male pseudohermaphroditism in sibs with the alpha-thalassemia/mental retardation (ATR-X) syndrome. *Am J Med Genet* (1995) **55**:285–7. doi:10.1002/ajmg.1320550308
50. Pearlman A, Loke J, Le CC, White S, Chin L, Friedman A, et al. Mutations in MAP3K1 cause 46,XY disorders of sex development and implicate a common signal transduction pathway in human testis determination. *Am J Hum Genet* (2010) **87**:898–904. doi:10.1016/j.ajhg.2010.11.003
51. Puffenberger EG, Hu-Lince D, Parod JM, Craig DW, Dobrin SE, Conway AR, et al. Mapping of sudden infant death with dysgenesis of the testes syndrome (SIDDT) by a SNP genome scan and identification of TSPYL loss of function. *Proc Natl Acad Sci U S A* (2004) **101**:11689–94. doi:10.1073/pnas.0401194101
52. Main KM, Skakkebæk NE, Virtanen HE, Toppari J. Genital anomalies in boys and the environment. *Best Pract Res Clin Endocrinol Metab* (2010) **24**:279–89. doi:10.1016/j.beem.2009.10.003
53. Mendonça BB, Costa EM, Belgorosky A, Rivarola MA, Domenice S. 46,XY DSD due to impaired androgen production. *Best Pract Res Clin Endocrinol Metab* (2010) **24**:243–62. doi:10.1016/j.beem.2009.11.003
54. Josso N, Rey R, Picard JY. Testicular anti-Mullerian hormone: clinical applications in DSD. *Semin Reprod Med* (2012) **30**:364–73. doi:10.1055/s-0032-1324719
55. Ivell R, Anand-Ivell R. Biological role and clinical significance of insulin-like peptide 3. *Curr Opin Endocrinol Diabetes Obes* (2011) **18**:210–6. doi:10.1097/MED.0b013e3283453fe6
56. Tapanainen JS, Aittomaki K, Min J, Vaskivuo T, Huhtaniemi IT. Men homozygous for an inactivating mutation of the follicle-stimulating hormone (FSH) receptor gene present variable suppression of spermatogenesis and fertility. *Nat Genet* (1997) **15**:205–6. doi:10.1038/ng0297-205
57. van Tijn DA, de Vijlder JJ, Verbeeten B Jr, Verkerk PH, Vulsma T. Neonatal detection of congenital hypothyroidism of central origin. *J Clin Endocrinol Metab* (2005) **90**:3350–9. doi:10.1210/jc.2004-2444
58. Romero CJ, Nesi-França S, Radovick S. The molecular basis of hypopituitarism. *Trends Endocrinol Metab* (2009) **20**:506–16. doi:10.1016/j.tem.2009.06.005
59. Davis SW, Castinetti F, Carvalho LR, Ellsworth BS, Potok MA, Lyons RH, et al. Molecular mechanisms of pituitary organogenesis: in search of novel regulatory genes. *Mol Cell Endocrinol* (2010) **323**:4–19. doi:10.1016/j.mce.2009.12.012
60. Castinetti F, Reynaud R, Saveanu A, Barlier A, Brue T. Genetic causes of combined pituitary hormone deficiencies in humans. *Ann Endocrinol (Paris)* (2012) **73**:53–5. doi:10.1016/j.ando.2012.03.025
61. Balasubramanian R, Crowley WF Jr. Isolated GnRH deficiency: a disease model serving as a unique prism into the systems biology of the GnRH neuronal network. *Mol Cell Endocrinol* (2011) **346**:4–12. doi:10.1016/j.mce.2011.07.012
62. Valdes-Socin H, Salvi R, Daly AF, Gaillard RC, Quatresooz P, Tebeu PM, et al. Hypogonadism in a patient with a mutation in the luteinizing hormone beta-subunit gene. *N Engl J Med* (2004) **351**:2619–25. doi:10.1056/NEJMoa040326
63. Lofrano-Porto A, Barra GB, Giacomini LA, Nascimento PP, Latronico AC, Casulari LA, et al. Luteinizing hormone beta mutation and hypogonadism in men and women. *N Engl J Med* (2007) **357**:897–904. doi:10.1056/NEJMoa071999
64. Topaloglu AK, Reimann F, Guclu M, Yalin AS, Kotan LD, Porter KM, et al. TAC3 and TACR3 mutations in familial hypogonadotropic hypogonadism reveal a key role for neurokinin B in the central control of reproduction. *Nat Genet* (2009) **41**:354–8. doi:10.1038/ng.306
65. Brioude F, Bouligand J, Trabado S, Francou B, Salenave S, Kamenicky P, et al. Non-syndromic congenital hypogonadotropic hypogonadism: clinical presentation and genotype-phenotype relationships. *Eur J Endocrinol* (2010) **162**:835–51. doi:10.1530/EJE-10-0083
66. Pitteloud N, Boepple PA, DeCruz S, Valkenburgh SB, Crowley WF Jr, Hayes FJ. The fertile eunuch variant of idiopathic hypogonadotropic hypogonadism: spontaneous reversal associated with a homozygous mutation in the gonadotropin-releasing hormone receptor. *J Clin Endocrinol Metab* (2001) **86**:2470–5. doi:10.1210/jc.86.6.2470
67. Shiraishi K, Naito K. Fertile eunuch syndrome with the mutations (Trp8Arg and Ile15Thr) in the beta subunit of luteinizing hormone. *Endocr J* (2003) **50**:733–7. doi:10.1507/endocrj.50.733
68. Layman LC, Porto AL, Xie J, da Motta LA, da Motta LD, Weiser W, et al. FSH beta gene mutations in a female with partial breast development and a male sibling with normal puberty and azoospermia. *J Clin Endocrinol Metab* (2002) **87**:3702–7. doi:10.1210/jc.87.8.3702
69. Shao XY, Zhang R, Hu C, Wang CR, Lu JY, Qin W, et al. Precise microdeletion detection of Prader–Willi syndrome with array comparative genome hybridization. *Biomed Environ Sci* (2010) **23**:194–8. doi:10.1016/S0895-3988(10)60052-9
70. Eiholzer U, L'Allemand D, Rousson V, Schlumpf M, Gasser T, Girard J, et al. Hypothalamic and gonadal components of hypogonadism in boys with Prader–Labhart–Willi syndrome. *J Clin Endocrinol Metab* (2006) **91**:892–8. doi:10.1210/jc.2005-0902
71. Fillion M, Deal CL, Van Vliet G. Normal minipuberty of infancy in boys with Prader–Willi syndrome. *J Pediatr* (2006) **149**:874–6. doi:10.1016/j.jpeds.2006.08.077
72. Hirsch HJ, Eldar-Geva T, Benarroch F, Rubinstein O, Gross-Tsur V. Primary testicular dysfunction is a major contributor to abnormal pubertal development in males with Prader–Willi syndrome. *J Clin Endocrinol Metab* (2009) **94**:2262–8. doi:10.1210/jc.2008-2760
73. Radicioni AF, Di GG, Grugni G, Cuttini M, Losacco V, Anzuini A, et al. Multiple forms of hypogonadism of central, peripheral or combined origin in males with Prader–Willi syndrome. *Clin Endocrinol* (2012) **76**:72–7. doi:10.1111/j.1365-2265.2011.04161.x
74. Siemensma EP, de Lind van Wijngaarden RF, Otten BJ, de Jong FH, Hokken-Koelega AC. Testicular failure in boys with Prader–Willi syndrome: longitudinal studies of reproductive hormones. *J Clin Endocrinol Metab* (2012) **97**:E452–9. doi:10.1210/jc.2011-1954
75. Arcari AJ, Bergadá I, Rey RA, Gottlieb S. Predictive value of anatomical findings and karyotype analysis in the diagnosis of patients with disorders of sexual development. *Sex Dev* (2007) **1**:222–9. doi:10.1159/000104772
76. Rey RA, Codner E, Iñíguez G, Bedecarrás P, Trigo R, Okuma C, et al. Low risk of impaired testicular Sertoli and Leydig cell functions in boys with isolated hypospadias. *J Clin Endocrinol Metab* (2005) **90**:6035–40. doi:10.1210/jc.2005-1306
77. Rey RA, Grinspon RP. Normal male sexual differentiation and aetiology of disorders of sex development. *Best Pract Res Clin Endocrinol Metab* (2011) **25**:221–38. doi:10.1016/j.beem.2010.08.013
78. Rey RA, Belville C, Nihoul-Fékété C, Michel-Calemard L, Forest MG, Lahlou N, et al. Evaluation of gonadal function in 107 intersex patients by means of serum anti-Müllerian hormone measurement. *J Clin Endocrinol Metab* (1999) **84**:627–31. doi:10.1210/jc.84.2.627
79. Bouvattier C, Maione L, Bouligand J, Dodé C, Guiochon-Mantel A, Young J. Neonatal gonadotropin therapy in male congenital hypogonadotropic hypogonadism. *Nat Rev Endocrinol* (2012) **8**:172–82. doi:10.1038/nrendo.2011.164
80. Bougnères P, François M, Pantalone L, Rodrigue D, Bouvattier C, Demesteere E, et al. Effects of an early postnatal treatment of hypogonadotropic hypogonadism with a continuous subcutaneous infusion of recombinant follicle-stimulating hormone and luteinizing hormone. *J Clin Endocrinol Metab* (2008) **93**:2202–5. doi:10.1210/jc.2008-0121
81. Josso N. Anti-Müllerian hormone and Sertoli cell function. *Horm Res* (1992) **38**(Suppl 2):72–6. doi:10.1159/000182602
82. Lee MM, Donahoe PK, Silverman BL, Hasegawa T, Hasegawa Y, Gustafson ML, et al. Measurements of serum Müllerian inhibiting substance in the evaluation of children with nonpalpable gonads. *N Engl J Med* (1997) **336**:1480–6. doi:10.1056/NEJM199705223362102
83. Brauner R, Neve M, Allali S, Trivin C, Lottmann H, Bashamboo A, et al. Clinical, biological and genetic analysis of anorchia in 26 boys. *PLoS One* (2011) **6**:e23292. doi:10.1371/journal.pone.0023292
84. Kubini K, Zachmann M, Albers N, Hiort O, Bettendorf M, Wolfle J, et al. Basal inhibin B and the testosterone response to human chorionic gonadotropin correlate in prepubertal boys. *J Clin Endocrinol Metab* (2000) **85**:134–8. doi:10.1210/jcem.85.1.6285
85. Hadziselimovic F, Zivkovic D, Bica DT, Emmons LR. The importance of minipuberty for fertility in cryptorchidism. *J Urol* (2005) **174**:1536–9. doi:10.1097/01.ju.0000181506.97839.b0
86. Young J, Chanson P, Salenave S, Noël M, Brailly S, O'Flaherty M, et al. Testicular anti-Mullerian hormone secretion is stimulated by recombinant human FSH

in patients with congenital hypogonadotropic hypogonadism. *J Clin Endocrinol Metab* (2005) **90**:724–8. doi:10.1210/jc.2004-0542
87. Raivio T, Toppari J, Perheentupa A, McNeilly AS, Dunkel L. Treatment of prepubertal gonadotrophin-deficient boys with recombinant human follicle-stimulating hormone. *Lancet* (1997) **350**:263–4. doi:10.1016/S0140-6736(05)62227-1
88. Vizeneux A, Hilfiger A, Bouligand J, Pouillot M, Brailly-Tabard S, Bashamboo A, et al. Congenital hypogonadotropic hypogonadism during childhood: presentation and genetic analyses in 46 boys. *PLoS One* (2013) **8**:e77827. doi:10.1371/journal.pone.0077827
89. Huhtaniemi IT. The role of mutations affecting gonadotrophin secretion and action in disorders of pubertal development. *Best Pract Res Clin Endocrinol Metab* (2002) **16**:123–38. doi:10.1053/beem.2002.0185
90. Lindstedt G, Nyström E, Matthews C, Ernest I, Janson PO, Chatterjee K. Follitropin (FSH) deficiency in an infertile male due to FSHβ gene mutation. A syndrome of normal puberty and virilization but under-developed testicles with azoospermia, low FSH but high lutropin and normal serum testosterone concentrations. *Clin Chem Lab Med* (1998) **36**:663–5. doi:10.1515/CCLM.1998.118

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Mechanisms of prenatal programing: identifying and distinguishing the impact of steroid hormones

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Developmental programing is gaining considerable leverage as a conceptual framework for understanding individual variability in human behavioral and somatic health. The current mini-review examines some of the key conceptual and methodological challenges for developmental programing research focused on fetal sex steroid exposure and physical, behavioral, physiological, and health outcomes. Specifically, we consider the bases for focusing on sex steroids, methods for assessing prenatal steroid hormone exposure, confounding factors, and the most relevant postnatal outcomes. We conclude with a brief consideration, based on current knowledge, of the applications of the existing findings for further research and practice.

Keywords: developmental programing, sex steroids, prenatal maternal distress, child developmental outcomes, methodology

The developmental origins of health and disease hypothesis and the programing mechanisms that underlie it are a major focus of current basic science and clinical health research. A common theme is that stress (or in humans, anxiety) experienced by the mother is transmitted to the fetus and the ensuing developmental changes may have long-lasting effects on offspring biology and behavior. The hypothalamic–pituitary–adrenal (HPA) axis – and particularly glucocorticoid exposure – has attracted attention as a likely mediating mechanism (1–3). However, it may be worthwhile to look beyond the HPA axis. In this mini-review, we consider the role that sex steroids may play in prenatal programing and identify strategies for managing some of the methodological challenges that have arisen.

PROGRAMING EFFECTS FROM PRENATAL STRESS/ANXIETY: SEX STEROIDS

It is natural that HPA axis-mediated programing mechanisms have attracted substantial research attention given the dominant role of prenatal stress as a "risk phenotype" in the animal and, more recently, human research. The mediating role of glucocorticoids in programing fetal physiology may extend beyond prenatal psychosocial stress to also include exposure to other types of stressors, such as nutritional deprivation. Moreover, there are equally compelling reasons why an exclusive focus on a glucocorticoid-mediated mechanism will be inadequate and consideration of complementary mechanisms, notably sex steroid hormones, could be informative. For example, the HPA and hypothalamic–pituitary–gonadal (HPG) axes show considerable overlap and interaction (4); just as stress may affect sex steroid hormone production, so sex steroids may act on the stress response system.

One way of evaluating the likely importance of sex steroids in developmental programing is to examine sex differences in the associations between prenatal stress and outcomes in offspring. For instance, in animal models, male and female offsprings often show different patterns of developmental programing (5). In some cases, only one sex appears to be affected by prenatal stress (6), but in other research, the sexes show opposite directions of effect. For example, in a rat model, prenatal restraint stress was associated with increased anxiety in males but decreased anxiety in females compared to controls (7).

Whether or not there are sex differences in human studies of the effects of prenatal stress/anxiety on child outcomes is far from clear. Some examples have been reported for behavioral and cognitive development (8, 9) and the evidence for cardiovascular outcomes is strong (10). However, for several of the most widely researched stress-related outcomes, such as behavioral and emotional problems and HPA axis function, few, if any, robust and consistent sex differences have been found (11, 12). This inconsistency across human studies raises several critical issues. First, there is no reason to expect that responses to prenatal stress will differ by sex across all traits, so carefully selecting only those phenotypes of most relevance is important. Second, it is plausible that focusing on HPA axis-mediated mechanisms, to the exclusion of other mediators, may have limited our ability to detect and understand sex differences.

Two lines of study provide evidence of sex steroid involvement in stress-related developmental programing. In animal models, prenatal stress is associated with changes in anogenital distance (AGD), a marker of prenatal androgen exposure; these associations have now been noted in humans (13–15). Critically, the direction of effects differs by sex: prenatal stressed males show demasculinization and females show masculinization of AGD. Interestingly, in contrast to the literature on prenatal nutritional stress, in which males tend to be more affected postnatally (16), the effects of psychosocial stress on reproductive development appear

to be stronger in females (13, 17). Although research on prenatal stress and reproductive health and development in humans is limited, the fact that AGD appears to be stable over time and has been linked to adult reproductive outcomes (18, 19) suggests the potential importance of this line of research. A related line of work suggests prenatal programing from sex steroids from testosterone in amniotic fluid on autistic behaviors (20, 21) as well as temperament and play behavior (9, 22) in childhood. These findings point to the need for further clinical research on developmental programing of sex steroids and their effects on human health and development.

METHODOLOGICAL CHALLENGES FOR INVESTIGATING PRENATAL PROGRAMING EFFECTS OF SEX STEROIDS IN HUMAN STUDIES

There are several methodological challenges for future research on the possible programing role of prenatal sex steroid exposure; we consider several in this mini-review.

ASSESSING FETAL EXPOSURE

The first, most basic question is how to assess fetal exposure to sex steroid hormones. Several studies have inferred fetal exposure to sex steroids from prenatal maternal distress (13, 23), although direct evidence of an association has not been found, e.g., Ref. (24). Directly measuring fetal exposure to sex steroids remains a major challenge. In the past, many studies were able to measure hormones in amniotic fluid when there was clinical indication; however, amniocentesis is losing favor as a clinical diagnostic tool and is unlikely to be a viable measurement strategy moving forward. Less direct measures of fetal exposure may be obtained from the mother; saliva, serum, and hair have been suggested as potential candidates. Circulating maternal hormone levels may represent production by the fetus, placenta, and the mother herself, with the relative proportions differing by hormone and stage of gestation (25–27). Circulating maternal testosterone levels could be a useful index, but it is likely that most fetal androgens are aromatized by the placenta before reaching maternal circulation (28). Finally, if circulating maternal sex steroids are correlated with fetal exposure, it still remains unclear which maternal medium is most appropriate for measurement, given that there may be inconsistencies across media (29). In any event, there is good reason to suspect that peripheral maternal levels of sex steroids may have minimal influence on fetal exposure. One potentially valuable strategy to index fetal exposure is to examine placental gene expression (see below).

Indexing fetal exposure to sex steroids *in utero* is an essential but difficult task for research. The availability of non-intrusive and reliable estimates of fetal exposure has proved difficult and is a central challenge to overcome in future research.

DISTINGUISHING BETWEEN CORRELATED STEROID HORMONE EXPOSURES

Distinguishing the impact of sex steroid hormones from other sources of programing is another major challenge for research. For example, cortisol and testosterone are positively correlated in fetal blood (30) and in amniotic fluid (31). Given that, it may be difficult to separate programing effects attributable to sex steroids from those attributable to glucocorticoids. One approach in past research is to examine natural experiments in which a medical condition, such as congenital adrenal hyperplasia (CAH), alters the fetal hormonal milieu in a well-characterized manner. Findings from studies of girls with CAH, for example, have helped to establish that prenatal exposure to sex steroids may program postnatal outcomes ranging from play behavior, to motor development, to personality (32–34). Interestingly, females with non-classical CAH have "female-typical" genitalia at birth (with evidence of impaired fertility later in life) (35), however, this does not rule out the possibility of subtly masculinized AGD, which would not be evident to a casual observer.

On the other hand, it is unclear if the findings can be generalized to non-clinical populations with fetal hormonal exposures within the normal range of variation. Other means of differentiating between glucocorticoid and sex steroid-based programing within healthy populations are needed. It is an interesting possibility that the developmental programing studies associated with maternal prenatal distress may have over-attributed effects to stress hormones because they have (largely) ignored sex steroids.

ROLE OF PLACENTAL STRUCTURE AND FUNCTION

A novel and potentially promising approach to indexing fetal steroid hormone exposure is to examine placental gene expression and epigenetic changes. As the main maternal–fetal interface, the placenta is of inarguable importance for understanding developmental programing. To date, research has been influenced by a glucocorticoid-mediated model. For instance, there has been great interest in the effects of stress on placental production of 11-β-hydroxysteroid dehydrogenase 2 (11BHSD2), an enzyme which shields the fetus from maternal cortisol by converting it to inactive cortisone (36). However, the placenta's endocrine production and regulation clearly extend far beyond 11BHSD2; prenatal distress may affect other placental steroid hormone pathways, impacting sex steroidogenesis and activity. At the same time, remarkably few studies on developmental programing have looked forward from placental structure and function to clinical phenotypes in the offspring; exploratory work on this subject is needed.

There are obviously substantial hurdles to assessing placental gene expression; it is a methodology that poses significant collection, cost, and laboratory demands. In healthy pregnancies, moreover, we are limited to looking at placental morphology and physiology at birth rather than at critical periods earlier in gestation. Nevertheless, examining placental variation, particularly in steroidogenesis pathway activity, in relation to prenatal exposures and postnatal outcomes requires attention given the (other) inherent challenges of estimating fetal exposure to sex steroids in a non-intrusive, reliable way.

IDENTIFYING RELEVANT POSTNATAL PHENOTYPES

A final challenge to be considered is the selection of relevant postnatal phenotypes. Traits with notable sex differences may be the most fruitful starting points. Autism spectrum disorders and associated traits, for instance, differ quite notably in prevalence and presentation between the sexes (37). Play behavior is

another strong candidate given the extensive evidence that from early childhood onward, males and females show clear preferences for sex-typical toys (38). Both autism and sexually dimorphic play behaviors have been associated with exposure to prenatal stress (17, 23), although further work is clearly needed. Other phenotypes that do not show consistent sex differences, such as temperament, may be less relevant to consider in this context.

Levels of circulating sex hormones are extremely low from shortly after birth until puberty. Nevertheless, even in infancy there appears to be sex differences in neurodevelopmental traits (39), suggesting that there may be prenatal, organizational effects of exposure of sex steroids. Other sex differences in development (in brain development, for instance), emerge later in childhood, but prior to the peripubertal increase in sex hormones and may plausibly be the product of *in utero* sex steroid programing. Studies showing sex differences in infancy are interesting because there are minimally detectable levels of sex hormones in circulation; that means that these infant sex differences may be induced by prenatal sex steroid exposure. There are other well-known differences between the sexes that are evident early in development, including physical growth and brain development, e.g., Ref. (40). A challenge for future research is to examine if these – and perhaps other – early-emerging sex differences in biology and behavior can be attributable in part to prenatal programing of sex steroid exposure.

CONCLUSION

Research on human health and development is just beginning to translate the animal work on developmental programing effects of sex steroids. Further studies are needed to substantiate this emerging line of investigation and to provide a broader biological context in which to interpret the sizable research based on developmental programing associated with prenatal stress and HPA axis mechanisms, a literature which has begun to influence practice and policy, e.g., Ref. (41). Progress in the area of research will require the consideration and surmounting of several methodological challenges, which we have highlighted.

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REFERENCES

1. Gluckman PD, Hanson MA, Beedle AS, Raubenheimer D. Fetal and neonatal pathways to obesity. *Front Horm Res* (2008) **36**:61–72. doi:10.1159/000115337
2. Glover V, O'Connor TG, O'Donnell K. Prenatal stress and the programming of the HPA axis. *Neurosci Biobehav Rev* (2010) **35**:17–22. doi:10.1016/j.neubiorev.2009.11.008
3. Cottrell EC, Seckl JR. Prenatal stress, glucocorticoids and the programming of adult disease. *Front Behav Neurosci* (2009) **3**:19. doi:10.3389/neuro.08.019.2009
4. Lovejoy DA, Barsyte-Lovejoy D. Systems approaches to genomic and epigenetic inter-regulation of peptide hormones in stress and reproduction. *Prog Biophys Mol Biol* (2013) **113**(3):375–86. doi:10.1016/j.pbiomolbio.2013.02.002
5. Bale TL. Sex differences in prenatal epigenetic programming of stress pathways. *Stress* (2011) **14**:348–56. doi:10.3109/10253890.2011.586447
6. Howerton CL, Morgan CP, Fischer DB, Bale TL. O-GlcNAc transferase (OGT) as a placental biomarker of maternal stress and reprogramming of CNS gene transcription in development. *Proc Natl Acad Sci U S A* (2013) **110**:5169–74. doi:10.1073/pnas.1300065110
7. Zuena AR, Mairesse J, Casolini P, Cinque C, Alemà GS, Morley-Fletcher S, et al. Prenatal restraint stress generates two distinct behavioral and neurochemical profiles in male and female rats. *PLoS One* (2008) **3**:e2170. doi:10.1371/journal.pone.0002170
8. Sandman CA, Glynn LM, Davis EP. Is there a viability-vulnerability tradeoff? Sex differences in fetal programming. *J Psychosom Res* (2013) **75**:327–35. doi:10.1016/j.jpsychores.2013.07.009
9. Bergman K, Glover V, Sarkar P, Abbott DH, O'Connor TG. In utero cortisol and testosterone exposure and fear reactivity in infancy. *Horm Behav* (2010) **57**:306–12. doi:10.1016/j.yhbeh.2009.12.012
10. Grigore D, Ojeda NB, Alexander BT. Sex differences in the fetal programming of hypertension. *Gend Med* (2008) **5**(Suppl A):S121–32. doi:10.1016/j.genm.2008.03.012
11. O'Donnell KJ, Glover V, Barker ED, O'Connor TG. The persisting effect of maternal mood in pregnancy on childhood psychopathology. *Dev Psychoppathol* (2014) (in press).
12. O'Donnell KJ, Glover V, Jenkins J, Browne D, Ben-Shlomo Y, Golding J, et al. Prenatal maternal mood is associated with altered diurnal cortisol in adolescence. *Psychoneuroendocrinology* (2013) **38**:1630–8. doi:10.1016/j.psyneuen.2013.01.008
13. Barrett ES, Parlett LE, Sathyanarayana S, Liu F, Redmon JB, Wang C, et al. Prenatal exposure to stressful life events is associated with masculinized anogenital distance (AGD) in female infants. *Physiol Behav* (2013) **11**(4–115):14–20. doi:10.1016/j.physbeh.2013.03.004
14. vom Saal FS, Quadagno DM, Even MD, Keisler LW, Keisler DH, Khan S. Paradoxical effects of maternal stress on fetal steroids and postnatal reproductive traits in female mice from different intrauterine positions. *Biol Reprod* (1990) **43**:751–61. doi:10.1095/biolreprod43.5.751
15. Ward IL, Weisz J. Maternal stress alters plasma testosterone in fetal males. *Science* (1980) **207**:328–9. doi:10.1126/science.7188648
16. Begum G, Davies A, Stevens A, Oliver M, Jaquiery A, Challis J, et al. Maternal undernutrition programs tissue-specific epigenetic changes in the glucocorticoid receptor in adult offspring. *Endocrinology* (2013) **154**:4560–9. doi:10.1210/en.2013-1693
17. Barrett ES, Redmon JB, Wang C, Sparks A, Swan SH. Exposure to prenatal life events stress is associated with masculinized play behavior in girls. *Neurotoxicology* (2014) **41C**:20–7. doi:10.1016/j.neuro.2013.12.011
18. Eisenberg ML, Hsieh MH, Walters RC, Krasnow R, Lipshultz LI. The relationship between anogenital distance, fatherhood, and fertility in adult men. *PLoS One* (2011) **6**:e18973. doi:10.1371/journal.pone.0018973
19. Mendiola J, Roca M, Mínguez-Alarcón L, Mira-Escolano MP, López-Espín JJ, Barrett ES, et al. Anogenital distance is related to ovarian follicular number in young Spanish women: a cross-sectional study. *Environ Health* (2012) **11**:90. doi:10.1186/1476-069X-11-90
20. Knickmeyer RC, Baron-Cohen S, Auyeung B, Ashwin E. How to test the extreme male brain theory of autism in terms of foetal androgens? *J Autism Dev Disord* (2008) **38**:995–6; author reply 997–8. doi:10.1007/s10803-008-0553-y
21. Auyeung B, Taylor K, Hackett G, Baron-Cohen S. Foetal testosterone and autistic traits in 18 to 24-month-old children. *Mol Autism* (2010) **1**:11. doi:10.1186/2040-2392-1-11
22. Auyeung B, Baron-Cohen S, Ashwin E, Knickmeyer R, Taylor K, Hackett G, et al. Fetal testosterone predicts sexually differentiated childhood behavior in girls and in boys. *Psychol Sci* (2009) **20**:144–8. doi:10.1111/j.1467-9280.2009.02279.x
23. Hines M, Johnston KJ, Golombok S, Rust J, Stevens M, Golding J, et al. Prenatal stress and gender role behavior in girls and boys: a longitudinal, population study. *Horm Behav* (2002) **42**:126–34. doi:10.1006/hbeh.2002.1814
24. Sarkar P, Bergman K, O'Connor TG, Glover V. Maternal antenatal anxiety and amniotic fluid cortisol and testosterone: possible implications for foetal programming. *J Neuroendocrinol* (2008) **20**:489–96. doi:10.1111/j.1365-2826.2008.01659.x
25. Tulchinsky D, Hobel CJ, Yeager E, Marshall JR. Plasma estrone, estradiol, estriol, progesterone, and 17-hydroxyprogesterone in human pregnancy. I. Normal pregnancy. *Am J Obstet Gynecol* (1972) **112**:1095–100.
26. Speroff L, Fritz MA. *Clinical Gynecologic Endocrinology and Infertility*. 7th ed. Philadelphia: Lippincott Williams and Wilkins (2005).
27. Strauss JF III. *Yen and Jaffe's Reproductive Endocrinology: Physiology, Pathophysiology, and Clinical Management*. 6th ed. Philadelphia: Saunders (2009).
28. Toriola AT, Vääräsmäki M, Lehtinen M, Zeleniuch-Jacquotte A, Lundin E, Rodgers KG, et al. Determinants of maternal sex steroids during the first half of pregnancy. *Obstet Gynecol* (2011) **118**:1029–36. doi:10.1097/AOG.0b013e3182342b7f

29. Hampson E, Phillips SD, Soares CN, Steiner M. Steroid concentrations in antepartum and postpartum saliva: normative values in women and correlations with serum. *Biol Sex Differ* (2013) **4**:7. doi:10.1186/2042-6410-4-7
30. Gitau R, Adams D, Fisk NM, Glover V. Fetal plasma testosterone correlates positively with cortisol. *Arch Dis Child Fetal Neonatal Ed* (2005) **90**:F166–9. doi:10.1136/adc.2004.049320
31. Sarkar P, Bergman K, Fisk NM, O'Connor TG, Glover V. Amniotic fluid testosterone: relationship with cortisol and gestational age. *Clin Endocrinol (Oxf)* (2007) **67**:743–7. doi:10.1111/j.1365-2265.2007.02955.x
32. Collaer ML, Brook C, Conway GS, Hindmarsh PC, Hines M. Motor development in individuals with congenital adrenal hyperplasia: strength, targeting, and fine motor skill. *Psychoneuroendocrinology* (2009) **34**:249–58. doi:10.1016/j.psyneuen.2008.09.007
33. Mathews GA, Fane BA, Conway GS, Brook CGD, Hines M. Personality and congenital adrenal hyperplasia: possible effects of prenatal androgen exposure. *Horm Behav* (2009) **55**:285–91. doi:10.1016/j.yhbeh.2008.11.007
34. Pasterski V, Geffner ME, Brain C, Hindmarsh P, Brook C, Hines M. Prenatal hormones and childhood sex-segregation: playmate and play style preferences in girls with congenital adrenal hyperplasia. *Horm Behav* (2011) **59**:549–55. doi:10.1016/j.yhbeh.2011.02.007
35. Witchel SF, Azziz R. Nonclassic congenital adrenal hyperplasia. *Int J Pediatr Endocrinol* (2010) **2010**:625105. doi:10.1155/2010/625105
36. O'Donnell KJ, Bugge Jensen A, Freeman L, Khalife N, O'Connor TG, Glover V. Maternal prenatal anxiety and downregulation of placental 11beta-HSD2. *Psychoneuroendocrinology* (2012) **37**:818–26. doi:10.1016/j.psyneuen.2011.09.014
37. Lai MC, Lombardo MV, Suckling J, Ruigrok AN, Chakrabarti B, Ecker C, et al. Biological sex affects the neurobiology of autism. *Brain* (2013) **136**:2799–815. doi:10.1093/brain/awt216
38. Berenbaum SA, Hines M. Early androgens are related to childhood sex-types toy preferences. *Psychol Sci* (1992) **3**:203–6. doi:10.1111/j.1467-9280.1992.tb00028.x
39. Quinn PC, Liben LS. A sex difference in mental rotation in young infants. *Psychol Sci* (2008) **19**:1067–70. doi:10.1111/j.1467-9280.2008.02201.x
40. Lenroot RK, Gogtay N, Greenstein DK, Wells EM, Wallace GL, Clasen LS, et al. Sexual dimorphism of brain developmental trajectories during childhood and adolescence. *Neuroimage* (2007) **36**:1065–73. doi:10.1016/j.neuroimage.2007.03.053
41. O'Connor TG, Monk C, Fitelson EM. Practitioner review: maternal mood in pregnancy and child development – implications for child psychology and psychiatry. *J Child Psychol Psychiatry* (2014) **55**:99–111. doi:10.1111/jcpp.12153

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Postnatal testosterone concentrations and male social development

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Converging evidence from over 40 years of behavioral research indicates that higher testicular androgens in prenatal life and at puberty contribute to the masculinization of human behavior. However, the behavioral significance of the transient activation of the hypothalamic–pituitary–gonadal (HPG) axis in early postnatal life remains largely unknown. Although early research on non-human primates indicated that suppression of the postnatal surge in testicular androgens had no measurable effects on the later expression of the male behavioral phenotype, recent research from our laboratory suggests that postnatal testosterone concentrations influence male infant preferences for larger social groups and temperament characteristics associated with the later development of aggression. In later assessment of gender-linked behavior in the second year of life, concentrations of testosterone at 3–4 months of age were unrelated to toy choices and activity levels during toy play. However, higher concentrations of testosterone predicted less vocalization in toddlers and higher parental ratings on an established screening measure for autism spectrum disorder. These findings suggest a role of the transient activation of the HPG axis in the development of typical and atypical male social relations and suggest that it may be useful in future research on the exaggerated rise in testosterone secretion in preterm infants or exposure to hormone disruptors in early postnatal life to include assessment of gender-relevant behavioral outcomes, including childhood disorders with sex-biased prevalence rates.

Keywords: postnatal testosterone, social development, infancy, sex differences, autism spectrum disorder

Testicular androgens have a central role in human male development. In prenatal life, increased testicular androgens around 4–6 weeks gestation masculinize the genitalia and initiate the sexual differentiation of the brain through hormonally dependent, sex-specific changes in the ultrastructure of the developing central nervous system (e.g., cell proliferation, cell death, patterns of cell migration, dendritic branching) (1). After a childhood of relative testicular quiescence, rising androgens at puberty activate the development of secondary sex characteristics and the maturation of the reproductive system (2). Converging evidence from approximately 40 years of research on human development indicates these hormonal changes also contribute to the masculinization of behavior (3, 4). However, the behavioral significance of another related surge in testicular androgens that results from a transient activation of the hypothalamic–pituitary–gonadal (HPG) axis in early postnatal life remains largely unexplored. It is significant, therefore, that recent findings from our laboratory suggest the postnatal endocrine surge may contribute to gender-linked typical and atypical social development.

POSTNATAL TESTOSTERONE SURGE AND EARLY THEORETICAL CHALLENGES TO BEHAVIORAL INVESTIGATION

In infant boys, serum testosterone (T) concentrations that are gonadal in origin increase to pubertal concentrations between 1 and 3 months of age and fall to prepubertal values around 6 months of age (5–7). Salivary measures of bioavailable T in male neonates show concentrations well below those in men (8–10). Nonetheless, the results of a mammalian cell bio-assay provide direct evidence of biologically active androgens in male infants at 3 months of age (11) consistent with other findings that the postnatal surge contributes to the normal growth of male-external genitalia and sperm production (12). In a large prospective study of male infants, for example, higher concentrations of free T at 3 months of age predicted greater penile growth across the first 3 years of development (13).

In view of evidence indicating the postnatal endocrine surge is necessary to establish normal adult male reproductive function, a reasonable question is whether it is also necessary to establish male behaviors that facilitate reproductive success. An early perceptive commentary (14) suggested that the transient activation of the HPG axis may contribute to the sexual differentiation of primate behavior and expressed related concerns that the exaggerated rise in T secretion in preterm infants (15) or exposure to hormone disruptors in early postnatal life (16) may have adverse consequences for male primate reproductive development. Yet, 15 years passed before tests of the postnatal developmental programing hypothesis appeared in the human hormone-behavior literature.

The long delay in examining the postnatal developmental programing hypothesis in humans is understandable given the paucity of supporting evidence from studies in non-human primates. Investigations using a variety of mammalian species had

established that higher androgen concentrations in prenatal life promoted a male behavioral phenotype in juveniles (17), including higher frequencies of rough-and-tumble play and mounting behavior in non-human primates (18). In contrast, independent laboratories reported that transient suppression of T in early postnatal life (19–21) had no measurable effects on the later expression of primate behaviors described as the "fundamental sexually dimorphic behavioral characteristics of the male" [(20), p. 334]. Suppression of T in males was reported in one investigation to decrease male juvenile independence from the mother, as measured by increased (i.e., more female-typical) proximity seeking relative to controls (20). However, whereas the prenatal period as a critical time in the sexual differentiation of primate behavior was supported by extensive experimental evidence, a similar role for the postnatal period was not.

Additionally, pioneering researchers of human hormone–behavior relations argued that the effects of androgens would be most apparent on behaviors showing large sex differences in expression, for example play preferences in childhood (17). Research applying this reasoning established that girls exposed to higher concentrations of prenatal androgens because of congenital adrenal hyperplasia (CAH) showed masculinized play relative to unaffected relatives, as measured by their stronger preference for toys typically preferred by boys (e.g., vehicles, balls) and weaker preference for toys typically preferred by girls (e.g., dolls) (22). Subsequently, masculinizing effects were found to extend to other behaviors showing large sex differences: girls with CAH showed enhanced targeting ability (23), increased levels of aggression (24, 25), and reported elevated activity levels (25).

Thus, studies of hormone–behavior relations in early postnatal life were likely further delayed because infants lack the physical and cognitive maturity to express known androgen-sensitive behaviors, such as aggression or toy play. In addition, few investigations specifically examined sex differences in infant behavior (26, 27). Limited research targeting relevant variables, such as activity levels (28), found small sex differences in infant behavior suggestive of small or absent hormone effects at this time in development. Finally, early gender socialization, apparent in infants' gender-specific clothes and toys (29), raised the possibility that small sex differences in infancy may be attributable to the differential treatment of males and females and care-giver expectations of gender-congruent behavior. Supporting the role of gender expectancies, researchers reported that the assignment of a gender label resulted in differences in the perceived emotional responsiveness of an infant, such that a negative emotion was labeled "anger" if the infant was thought to be a boy, and "fear" if the infant was thought to be a girl (30). Significantly, although these findings have been cited over 130 times, the reported effects of gender labels on adult perceptions of infant behavior were not replicable (31, 32). Moreover, adults' subjective perceptions of infant girls as smaller softer, and finer featured than boys (33) are supported by objective measures showing that compared to boys at birth, girls have higher length-to-weight ratios (34), a less prominent chin, and anteriorly narrower dental arcade (35).

Although other research has shown that gender labels influence adult response to infants (36, 37), we suggest that the differential treatment of male and female infants results from a bi-directional relationship between biological and social factors evident from birth: infants categorized as male and female on the basis of their genitalia, differ in other ways that support expectations based on gender labels. For instance, consistent with known sex differences in adult personality, meta-analyses of gender differences in infant temperament found that compared to girls, boys are less able to inhibit responses, and show less sensitivity to environmental changes, less fearfulness, and higher activity levels (38). Boys shortly after birth show stronger visual preferences for a mechanical mobile than for a face (39) and infant boys compared to girls show less orientation to a face or voice (39, 40) and shorter eye contact with an experimenter (41). Similar to the sex difference in adult emotional processing, infant boys also show less discrimination of emotional expression than their female counterparts (42) and appear to show a weaker propensity to empathic reactions, as indicated by shorter crying responses to recordings of a cry from a female infant (43, 44). Overall, male infants compared to female infants appear to differ in their responsiveness to stimuli with cues and characteristics associated with an animate form. Greater responsiveness to such cues in females may support social behaviors that enhance their reproductive success, such as interest in infants and "tend and befriend" responses to threat (45). As such, sex differences in infancy may indicate a biological preparedness for gender roles that for full expression requires the subsequent coupling of these early sex differences with social experiences imposed by contemporary gender socialization (46, 47).

POSTNATAL SALIVARY TESTOSTERONE AND THE EMERGENCE OF GENDER-LINKED SOCIAL RELATIONS

Small sex differences in infant temperament may represent essential structures supporting the development of adult sex differences in socio-emotional behavior (27) and recent evidence suggests the transient activation of the HPG axis may influence this process. Language researchers examining brain functional asymmetries first documented an association between serum T concentrations in infants at 4 weeks of age and developing language systems (48). In that research, female infants showed an advantage over males on a phoneme discrimination task that elicited bilateral activation of brain hemispheres, as measured by EEG. Male infants with low postnatal serum T compared to high T male infants showed phoneme discrimination with only left hemisphere activation. In contrast, male infants with higher postnatal serum T showed no discrimination effect, suggesting a relationship between postnatal androgen concentrations and developing brain systems that may contribute to later sex differences in language processing.

At the same time, studies on gender-linked toy preferences in infancy were ongoing in our lab using eye-tracking technology to measure infant interests (8, 46). Although toys are cultural artifacts linked to domestic and non-domestic activities that define traditional gender roles (49), biological influences on children's toy choices are indicated by similar sex-linked object preferences in two non-human primate species (50, 51) and findings noted above that higher prenatal androgen concentrations in girls are associated with masculinized toy preferences (22). We examined toy preferences in 30 infants at 3–8 months of age by measuring the number of visual fixations on a truck or doll presented

FIGURE 1 | Male infants with higher postnatal T show stronger visual preferences for the group of figures.

simultaneously in a puppet theater for two brief time intervals (10 s), counterbalancing for side of presentation. Across both trials, infant girls compared to boys directed more visual interest to the doll, whereas infant boys compared to girls directed more visual interest to the truck. These findings are clearly consistent with the hypothesized greater social sensitivity in females relative to males. They also imply that the well-documented emergence of gender-linked toy play in the second year of life (52–54) may build on inborn preferences in males and females for the perceptual features that define the conceptual categories of "masculine" and "feminine" toys.

In a second eye-tracking study of 41 infants at 3–4 months of age, we examined whether hormonal factors might contribute to early visual preferences by determining whether the ratio of the lengths of the second and fourth digits of the right hand (a putative marker of prenatal androgen action) (55) and salivary concentrations of T measured at 3–4 months of age would predict infants' interest toward two categories of gender-linked stimuli: pictures of toys differentially preferred by older children (i.e., vehicle vs. doll) and a brief animation of a solitary figure and a group of figures (see **Figure 1**). The second set of stimuli were selected on the basis of previous research indicating innate sex differences in primate social organization, such that males compared to females prefer interactions with larger numbers of individuals (56). In male infants, smaller digit ratios suggestive of greater androgen action in prenatal life predicted greater visual attention to the male-typical toys, consistent with the earlier research indicating higher prenatal androgens result in stronger preferences for male-typical toys (57). Contrary to our predictions, salivary T concentrations measured in early postnatal life were unrelated to visual interest directed to gender-linked toys. However, whereas digit ratios were unrelated to interest directed to the animated figures, higher salivary T concentrations in male infants predicted greater visual attention to the group of figures. To our knowledge, this finding represents the first documented association between postnatal T and gender-linked preferences in infancy.

Interpretation of the association between higher salivary T in male infants and visual attention to a group of figures representing male-typical social organization may be informed by our other finding that higher salivary T in male infants at 3–4 months of age also predicted maternal ratings of greater negative affectivity on a well-established measure of infant temperament (58). Further analyses showed that the effect on negative affectivity was attributable to a strong association between higher T concentrations in male infants and infant frustration, as defined by fussing, crying, or showing distress when confined or unable to perform a desired action. Frustration in infancy is a temperament variable thought to contribute to the development of activity and aggression (59), personality traits with adaptive significance for males. Consistent with past speculation that a reduced independence from the mother following suppression of postnatal T may indicate neonatal T organizes the dynamics of primate social interactions (20), we hypothesize these recent findings from our research in human infants suggest postnatal T may be necessary for developmental programing of male social relations.

It is notable that although, we found associations between biological factors and gender-relevant behavior in our infant research, there were no sex differences in infant behavior at 3–4 months of age. For that reason, we describe behavior measured at 3–4 months of age as "pre-emergent" sex-linked behavior (8). We explain associations between digit ratios and visual attention to male-typical toys by suggesting that prenatal androgen concentrations may influence the incentive value of social stimuli in very early postnatal life and thereby promote the well-documented sex differences in object preferences in later life. Similarly, we suggest that postnatal androgens may encourage preferences for groups and associated male personality traits, such as higher rates of aggression and increased activity levels, and thereby influence the development of male-typical social structures.

Evidence of any organizational effects of postnatal T on behavior, however, requires establishing associations between postnatal concentrations of T and behavioral outcomes beyond the infantile period of transient HPG activation. For that reason, we tested whether postnatal T concentrations measured at 3–4 months of age would predict gender-typical play behavior and activity levels in children in the second year of life (60). Play preferences were videotaped and children's interactions with male-typical, female-typical, and gender-neutral toys were coded later using behavioral coding software (Noldus Observer XT). In addition, children wore small accelerometers (i.e., actigraphs) that provided direct recording of movement during the play sessions. Actigraph data has been validated against other physiological measures of energy expenditure in children (61) and used widely as a measure of the cumulative intensity and frequency of movement in studies of activity related dysfunctions in childhood (e.g., ADHD) (62). Consistent with evidence from studies of girls with CAH, more male-typical digit ratios in girls predicted higher activity counts during play and weaker preferences for female-typical toys. In contrast, in both sexes, salivary T concentrations measured at 3–4 months of age were unrelated to our two measures of gender-linked behavior in young children.

Sex differences in social relations include verbal ability and emotional processing. Therefore, it is noteworthy that in contrast to the absence of associations between postnatal T concentrations and toddler toy choices summarized above, higher T concentrations at 3–4 months of age predicted higher scores on the autism spectrum disorder (ASD) scale of the Brief Infant-Toddler Social and Emotional Assessment (63) and lower verbalization in 84 children at 18 months of age (64). Postnatal T was unrelated to behavioral aggression and eye contact coded during toy play with the care-giver (65), which argues against our proposal that

postnatal T may facilitate personality traits such as aggression. However, our measure of aggression was limited to behavior during toy play and a stronger test of the association between postnatal T and these aspects of sex-linked social behavior would come from an examination of children's response to peers in an unstructured setting or levels of aggression following puberty.

Our findings in toddlers are consistent with those from our earlier research on infants. Associations between digit ratios and interest in gender-linked toys in the first and second year of life suggest prenatal androgens influence pre-emergent and emergent sex-linked object interests. Additionally, our findings of no association between T concentrations in the early postnatal period and toy interests suggest the postnatal developmental programing hypothesis may not extend to all aspects of gender-linked behavior. Findings from another lab (66) appear inconsistent with this conclusion. Urinary T concentrations collected seven times across the first 6 months of life correlated positively with parental reports of more male-typical preschool activities in boys but not girls at 14 months of age. The different results reported in this investigation may be attributable to the different hormone measurement. In addition, the parental report measure includes a range of gender-linked activities, including play with sex-typed toys, engagement in sex-typed activities (e.g., ballgames, cooking/cleaning play), and sex-typed child characteristics (e.g., interested in snakes, likes pretty things). Whether all three components contributed to the observed association between T in postnatal life and the global score on the parental report measure is unknown. Further, the observed correlations between T and children's toy preferences are difficult to interpret. Boys with higher concentrations of postnatal T played less with a male-typical toy (a truck) and a female-typical toy (a baby doll) and more with a gender-neutral toy (soft book). Girls with higher concentrations of postnatal T played more with one male-typical toy (train) – but postnatal concentrations of T were unrelated to time girls spent playing with another male-typical toys (truck) or female-typical toys (tea set, dolls). The inconsistent behavioral data strengthen the possibility that aspects of the parental reports sensitive to postnatal T concentrations in boys are those involving activities that recruit social organization preferences (group vs. solitary) and are influenced by temperament.

FUTURE DIRECTIONS

Our research indicating androgen concentrations in early postnatal life may influence risk for ASD is interesting given a need to better understand the mechanisms that contribute to the sex-biased prevalence rate and the deficits in communication and social interactions that characterize ASD. Indeed, ASD, a neurodevelopmental disorder diagnosed more frequently in boys, is associated with impairments in social interactions, restricted and stereotypical behaviors, and communication delays (67). Because typically developing boys relative to girls show decreased empathy and emotional processing (42), more frequent stereotyped interests (68), and decreased verbal abilities (69), behavioral features of ASD appear consistent with a "hypermasculinization" of the brain suggesting higher prenatal androgens in males may be a risk factor for the disorder (70). Consistent with this possibility, measures of higher prenatal T in typically developing children are associated with relative deficiencies in social interactions (e.g., eye contact and empathy) (71, 72) and language development (73). Girls exposed to higher prenatal androgens because of an endocrine disorder report more autistic traits than unaffected relatives (72) and digit ratios (a putative proxy for prenatal androgen action) (55) are smaller (i.e., more masculine) in children with ASD than unaffected, unrelated children (74). Our findings add to this literature by indicating a possible role for concentrations of T in the postnatal period. Interestingly, in a longitudinal investigation of over 800 children, umbilical cord blood concentrations of bioavailable T measured at birth were not associated with significant negative behavioral outcomes in either sex. Indeed, boys with higher concentrations of bioavailable T in cord blood at birth showed lower scores on a measure of attention problems at 5–10 years of age (75). In sum, these findings suggest that it may be informative in future studies of the postnatal programing hypothesis to investigate the possible interaction between antenatal and postnatal concentrations of T on positive and negative behavioral outcomes by including measurement of T concentrations in cord blood and during the postnatal surge at 1–3 months of age.

Finally, we have shown one measure of salivary T at 3–4 months of age can predict aspects of infant and toddler behavior. In this relatively uncharted research area, these findings clearly require independent replication. Nonetheless, the general results of this growing body of research support the earlier speculation (14) that the exaggerated rise in T secretion in preterm infants (15) or exposure to hormone disruptors in early postnatal life (16) may have adverse consequences for male primate reproductive development. Today, it is known that higher postnatal salivary T concentrations in very low birth weight babies are associated with greater health problems, including delays in growth and longer hospitalizations (76) and infants are widely exposed to substances in formula, food, breast-milk that can disrupt normal hormonal processes in postnatal life (77). Our research findings suggest that future research in these areas may inform understanding of hormone–behavior relations in early development by including measurement of gender-relevant behavioral outcomes.

REFERENCES

1. MacLusky NJ, Bowlby DA, Brown TJ, Peterson RE, Hochberg RB. Sex and the developing brain: suppression of neuronal estrogen sensitivity by developmental androgen exposure. *Neurochem Res* (1997) **22**:1395–414. doi:10.1023/A:1022027408234
2. Marcell AV, Wibbelsman C, Seigel WM. Adolescence, male adolescent sexual and reproductive health care. *Pediatrics* (2011) **128**:e1658–78. doi:10.1542/peds.2011-2384
3. Berenbaum S, Beltz AM. Sexual differentiation of human behavior: effects of prenatal and pubertal organizational hormones. *Front Neuroendocrinol* (2011) **32**:183–200. doi:10.1016/j.yfrne.2011.03.001
4. Peper JS, Dahl RE. The teenage brain: surging hormones – brain-behavior interactions during puberty. *Curr Dir Psychol Sci* (2013) **22**:134–9. doi:10.1177/0963721412473755
5. Forest MG, Sizonenko PC, Cathiard AM, Bertrand J. Hypophysogonadal function in humans during the first year of life: 1. Evidence for testicular activity in early infancy. *J Clin Invest* (1974) **53**:819–28. doi:10.1172/JCI107621
6. Forest MG, Deperetii E, Bertrand J. Hypothalamic-pituitary-gonadal relationships in man from birth to puberty. *Clin Endocrinol* (1976) **5**:551–69. doi:10.1111/j.1365-2265.1976.tb01985.x
7. Andersson AM, Toppari J, Haavisto AM, Petersen JH, Simell T, Simell O, et al. Longitudinal reproductive hormone profiles in infants: peak of inhibit B levels

in infant boys exceeds levels in adult men. *J Clin Endocrinol Metab* (1998) **83**:675–81. doi:10.1210/jc.83.2.675
8. Alexander GM, Wilcox T, Farmer M-B. Hormone-behavior associations in early infancy. *Horm Behav* (2009) **56**:498–502. doi:10.1016/j.yhbeh.2009.08.003
9. Huhtaniemi I, Dunkel L, Perheentupa J. Transient increase in postnatal testicular activity is not revealed by longitudinal measurements of salivary testosterone. *Pediatr Res* (1986) **20**:1324–7. doi:10.1203/00006450-198612000-00028
10. Knickmeyer RC, Woolson W, Hamer RM, Konneker T, Gilmore JH. 2D:4D ratios in the first 2 years of life: stability and relation to testosterone exposure and sensitivity. *Horm Behav* (2011) **60**:256–63. doi:10.1016/j.yhbeh.2011.05.009
11. Raivio T, Toppari J, Kaleva M, Virtanen HE, Haavisto A, Janne OA. Serum androgen bioactivity in cryptorchid and noncryptorchid boys during the postnatal reproductive hormone surge. *J Clin Endocrinol Metab* (2003) **88**:2597–9. doi:10.1210/jc.2002-021676
12. Main KM, Schmidt IM, Skakkebaek NE. A possible role for reproductive hormones in newborn boys: progressive hypogonadism with the postnatal testosterone peak. *J Clin Endocrinol Metab* (2005) **85**:4905–7. doi:10.1210/jcem.85.12.7058
13. Boas M, Boisen KA, Virtanen HE, Kaleva M, Suomi A-M, Schmidt IM, et al. Postnatal penile length and growth rate correlate to serum testosterone levels: a longitudinal study of 1962 normal boys. *Eur J Endocrinol* (2006) **154**:125–9. doi:10.1530/eje.1.02066
14. Mann DR, Fraser HM. The neonatal period: a critical interval in male primate development. *J Endocrinol* (1996) **149**:191–7. doi:10.1677/joe.0.1490191
15. Kuiri-Hanninen T, Seuri R, Tyrvainen E, Turpeinen U, Hamalainen E, Stenman UH, et al. Increased activity of the hypothalamic-pituitary-testicular axis in infancy results in increased androgen action in premature boys. *J Clin Endocrinol Metab* (2011) **96**:98–105. doi:10.1210/jc.2010-1359
16. Weiss B. The intersection of neurotoxicolgy and endocrine disruption. *Neurotoxicology* (2012) **33**:1410–9. doi:10.1016/j.neuro.2012.05.014
17. Hines M. Prenatal gonadal hormones and sex differences in human behavior. *Psychol Bull* (1982) **92**:56–80. doi:10.1037/0033-2909.92.1.56
18. Goy RW, McEwen BS. *Sexual Differentiation of the Brain.* Cambridge, MA: MIT Press (1980).
19. Brown GR, Dixson AF. Investigation of the role of postnatal testosterone in the expression of sex differences in behavior in infant rhesus macaques (*Macaca mulatta*). *Hormones Behav* (1999) **35**:186–94. doi:10.1006/hbeh.1999.1512
20. Wallen K, Maestripieri D, Mann DR. Effects of neonatal testicular suppression with a GnRH anatonist on social behavior in group-living juvenile Rhesus monkeys. *Horm Behav* (1995) **29**:322–37. doi:10.1006/hbeh.1995.1023
21. Nevison CM, Brown GR, Dixson AF. Effects of altering testosterone in early infancy on social behaviour in captive yearling rhesus monkeys. *Physiol Behav* (1997) **62**:1397–403. doi:10.1016/S0031-9384(97)00209-6
22. Berenbaum SA, Hines M. Early androgens are related to childhood sex-typed toy preferences. *Psychol Sci* (1992) **3**:203–6. doi:10.1111/j.1467-9280.1992.tb00028.x
23. Collaer ML, Brook CGD, Conway GS, Hindmarsh PC, Hines M. Motor development in individuals with congenital adrenal hyperplasia: strength, targeting, and fine motor skill. *Psychoneuroendocrinology* (2009) **34**:249–58. doi:10.1016/j.psyneuen.2008.09.007
24. Mathews GA, Fane BA, Conway GS, Brook CGD, Hines M. Personality and congenital hyperplasia: possible effects of prenatal androgen exposure. *Horm Behav* (2009) **55**:285–91. doi:10.1016/j.yhbeh.2008.11.007
25. Pasterski VL, Hindmarsch P, Geffner M, Brook C, Brain C, Hines M. Increased aggression and activity level in 3- to 11-year-old girls with congenital adrenal hyperplasia (CAH). *Horm Behav* (2007) **52**:368–74. doi:10.1016/j.yhbeh.2007.05.015
26. Weinberg MK, Tronick EZ, Cohn JF, Olson KL. Gender differences in emotional expressivity and self-regulation during early infancy. *Dev Psychol* (1999) **35**:175–88. doi:10.1037/0012-1649.35.1.175
27. Alexander GM, Wilcox T. Sex differences in early infancy. *Child Dev Perspect* (2012) **6**:400–6. doi:10.1111/j.1750-8606.2012.00247.x
28. Eaton WO, Enns LR. Sex differences in human motor activity level. *Psychol Bull* (1986) **100**:19–28. doi:10.1037/0033-2909.100.1.19
29. Pomerleau A, Bolduc D, Malcuit G, Cossette L. Pink or blue: environmental gender stereotypes in the first two years of life. *Sex Roles* (1990) **22**:359–67. doi:10.1007/BF00288339
30. Condry J, Condry S. Sex differences: a study of the eye of the beholder. *Child Dev* (1976) **47**:812–9. doi:10.1111/j.1467-8624.1976.tb02248.x
31. Plant EA, Hyde JS, Keltner D, Devine PG. The gender stereotyping of emotions. *Psychol Women Q* (2000) **24**:81–92. doi:10.1111/j.1471-6402.2000.tb01024.x
32. Steuer FB, Bode BC, Rada KA, Hittner JB. Gender label and perceived infant emotionality: a partial replication of a classic study. *Psychol Rep* (2010) **107**:139–44. doi:10.2466/07.10.17.PR0.107.4.139-144
33. Rubin J, Provenzano R, Luria Z. The eye of beholder – parents views on sex of newborns. *Am J Orthopsychiatry* (1974) **44**:512–9. doi:10.1111/j.1939-0025.1974.tb00905.x
34. Thomas P, Peabody J, Turnier V, Clark RH. A new look at intrauterine growth and the impact of race, altitude and gender. *Pediatrics* (2000) **106**:e21. doi:10.1542/peds.106.2.e21
35. Schutkowski H. Sex determination of infant and juvenile skeletons: I. Morphognostic features. *Am J Phys Anthropol* (1993) **90**:199–205. doi:10.1002/ajpa.1330900206
36. Lewis M. State as an infant-environment interaction: an analysis of mother-infant interaction as a function of sex. *Merrill Palmer Q* (1972) **18**:95–121.
37. Greenes DS, Wigotsky M, Schutzman SA. Gender differences in rates of unintentional head injury in the first 3 months of life. *Ambul Pediatr* (2001) **1**:178–80. doi:10.1367/1539-4409(2001)001<0178:GDIROU>2.0.CO;2
38. Else-Quest NM, Hyde JS, Goldsmith HH, Van Hulle CA. Gender differences in temperament: a meta-analysis. *Psychol Bull* (2006) **132**:33–72. doi:10.1037/0033-2909.132.1.33
39. Connellan J, Baron-Cohen S, Wheelwright S, Batki A, Ahluwalia J. Sex differences in human neonatal social perception. *Infant Behav Dev* (2000) **23**:113–8. doi:10.1016/S0163-6383(00)00032-1
40. Osofsky JD, O'Connell EJ. Patterning of newborn behavior in an urban population. *Child Dev* (1977) **48**:532–6. doi:10.2307/1128650
41. Hittelman JH, Dickes R. Sex differences in neonatal eye contact time. *Merrill Palmer Q* (1979) **25**:171–84.
42. McClure EB. A meta-analytic review of sex differences in facial expression processing and their development in infants, children, and adolescents. *Psychol Bull* (2000) **126**:424–53. doi:10.1037/0033-2909.126.3.424
43. Sagi A, Hoffman ML. Empathic distress in the newborn. *Dev Psychol* (1976) **12**:175–6. doi:10.1037/0012-1649.12.2.175
44. Simner ML. Newborn's response to the cry of another infant. *Dev Psychol* (1971) **5**:136–50. doi:10.1037/h0031066
45. Taylor SE. Tend and befriend: biobehavioral bases of affiliation under stress. *Curr Dir Psychol Sci* (2006) **15**:273–7. doi:10.1111/j.1467-8721.2006.00451.x
46. Alexander GM, Wilcox T, Woods R. Sex differences in infants' visual interest in toys. *Arch Sex Behav* (2009) **38**:427–33. doi:10.1007/s10508-008-9430-1
47. Alexander GM. An evolutionary perspective of sex-typed toy preferences: pink, blue and the brain. *Arch Sex Behav* (2003) **32**:7–14. doi:10.1023/A:1021833110722
48. Friederici AD, Pannekamp A, Partsch C-J, Ulmen U, Oehler K, Schmutzler R, et al. Sex hormone testosterone affects language organization in the infant brain. *Neuroreport* (2008) **19**:283–6. doi:10.1097/WNR.0b013e3282f5105a
49. Crabb PB, Bielawski D. The social representation of material culture and gender in chidlren's books. *Sex Roles* (1994) **30**:69–79. doi:10.1007/BF01420740
50. Alexander GM, Hines M. Sex differences in responses to children's toys in a non-human primate (*Cercopithecus aethiops* sabaeus). *Evol Hum Behav* (2002) **23**:467–79. doi:10.1016/S1090-5138(02)00107-1
51. Hassett JM, Siebert ER, Wallen K. Sex differences in rhesus monkey toy preferences parallel those of children. *Horm Behav* (2008) **54**:359–64. doi:10.1016/j.yhbeh.2008.03.008
52. Fagot BI. Consequences of moderate cross-gender behavior in preschool children. *Child Dev* (1977) **48**:902–7. doi:10.2307/1128339
53. Fagot BI, Leinbach MD, Hagan R. Gender labeling and the adoption of sex-typed behaviors. *Dev Psychol* (1986) **22**:440–3. doi:10.1037/0012-1649.22.4.440
54. O'Brien M, Huston AC. Development of sex-typed play behavior in toddlers. *Dev Psychol* (1985) **21**:866–71. doi:10.1037/0012-1649.21.5.866
55. Manning JT, Scott D, Wilson J, Lewis-Jones DI. The ratio of 2nd to 4th digit length: a predictor of sperm numbers and concentrations of testosterone, luteinizing hormone and oestrogen. *Hum Reprod* (1998) **13**:3000–4. doi:10.1093/humrep/13.11.3000
56. Benenson JF, Markovits H, Muller I, Challen A, Carder HP. Explaining sex differences in infants' preferences for groups. *Infant Behav Dev* (2007) **30**:587–95. doi:10.1016/j.infbeh.2007.03.010

57. Hines M. Prenatal testosterone and gender-related behaviour. *Eur J Endocrinol* (2006) **155**:S115–21. doi:10.1530/eje.1.02236
58. Alexander GM, Saenz J. Postnatal testosterone levels and temperament in early infancy. *Arch Sex Behav* (2011) **40**:1287–92. doi:10.1007/s10508-010-9701-5
59. Crockenberg SC, Leerkes EM, Barrig Jo PS. Predicting aggressive behavior in the third year from infant reactivity and regulation as moderated by maternal behavior. *Dev Psychopathol* (2008) **20**:37–54. doi:10.1017/S0954579408000023
60. Alexander GM, Saenz J. Early androgens, activity levels and toy choices of children in the second year of life. *Horm Behav* (2012) **2012**:500–4. doi:10.1016/j.yhbeh.2012.08.008
61. Pfeiffer KA, McIver KI, Dowda M, Almeida MJ, Pate RR. Validation and calibration of an accelerometer in preschool children. *Med Sci Sports Exer* (2006) **38**:152–7. doi:10.1249/01.mss.0000183219.44127.e7
62. Inoue K, Nadaoka T, Oiji A, Morioka Y, Totsuka S, Kanbayashi T, et al. Clinical evaluation of attention-deficit hyperactivity disorder by objective quantitative measures. *Child Psychiatry Hum Dev* (1998) **28**:179–88. doi:10.1023/A:1022885827086
63. Carter AS, Briggs-Gowan MJ, Jones SM, Little TD. The Infant-Toddler social and emotional assessment (ITSEA): factor structure, reliability, and validity. *J Abnorm Child Psychol* (2003) **31**:495–514. doi:10.1023/A:1025449031360
64. Saenz J, Alexander GM. Postnatal testosterone levels and disorder relevant behavior in the second year of life. *Biol Psychol* (2013) **94**:152–9. doi:10.1016/j.biopsycho.2013.05.011
65. Saenz J, Alexander GM. Digit ratios (2D:4D), postnatal testosterone and eye contact in toddlers. *Biol Psychol* (2013) **94**:106–8. doi:10.1016/j.biopsycho.2013.05.010
66. Lamminmaki A, Hines M, Kuiri-Hanninen T, Kilpelainen L, Dunkel L, Sankilampi U. Testosterone measured in infancy predicts subsequent sex-typed behavior in boys and in girls. *Horm Behav* (2012) **61**:611–6. doi:10.1016/j.yhbeh.2012.02.013
67. American Phychiatric Association. *Diagnostic and Statistical Manual of Mental Disorder (Fourth Edition, Text Revision)*. Washington, DC: American Phychiatric Association (2000).
68. DeLoache JS, Simcock G, Macari S. Planes, trains, automobiles – and tea sets: extremely intense interests in very young children. *Dev Psychol* (2007) **43**:1579–86. doi:10.1037/0012-1649.43.6.1579
69. Hyde JS, Linn MC. Gender differences in verbal ability: a meta-analysis. *Psychol Bull* (1988) **104**:doi:10.1037/0033-2909.104.1.53
70. Baron-Cohen S, Hammer J. Is autism an extreme form of the male brain? *Adv Infancy Res* (1997) **11**:193–217.
71. Chapman E, Baron-Cohen S, Auyeung B, Knickmeyer R, Taylor K, Hackett G. Fetal testosterone and empathy: evidence from the empathy quotient (EQ) and the "reading the mind n the eyes" test. *Soc Neurosci* (2006) **1**:135–48. doi:10.1080/17470910600992239
72. Knickmeyer RC, Baron-Cohen S, Fane BA, Wheelwright S, Mathews GA, Conway GS, et al. Androgens and autistic traits: a study of individuals with congenital adrenal hyperplasia. *Horm Behav* (2006) **50**:148–53. doi:10.1016/j.yhbeh.2006.02.006
73. Lutchmaya S, Baron-Cohen S, Raggatt P. Foetal testosterone and vocabulary size in 18- and 24-month-old infants. *Infant Behav Dev* (2002) **24**:418–24. doi:10.1016/S0163-6383(02)00087-5
74. Manning JT, Baron-Cohen S, Wheelwright S, Sanders G. The 2nd to 4th digit ratio and autism. *Dev Med Child Neurol* (2001) **43**:160–4. doi:10.1111/j.1469-8749.2001.tb00181.x
75. Robinson M, Whitehouse AJO, Jacoby P, Mattes E, Sawyer MG, Keelan JA, et al. Umbilical cord blood testosterone and childhood internalizing and externalizing behavior: a prospective study. *PLoS One* (2013) **8**:e59991. doi:10.1371/journal.pone.0059991
76. Cho JI, Carlo W, Su X, McCormick KL. Associations between salivary testosterone and cortisol levels and neonatal health and growth outcomes. *Early Hum Dev* (2012) **88**:789–94. doi:10.1016/j.earlhumdev.2012.05.002
77. Behr M, Oehlmann J, Wagner M. Estrogens in the daily diet: in vitro analysis indicates that estrogenic activity is omnipresent in foodstuff and infant formula. *Food and Chemical Toxicology* (2011) **49**:2681–8.

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Environmental and genetic contributors to salivary testosterone levels in infants

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Transient activation of the hypothalamic–pituitary–gonadal axis in early infancy plays an important role in male genital development and sexual differentiation of the brain, but factors contributing to individual variation in testosterone levels during this period are poorly understood. We measured salivary testosterone levels in 222 infants (119 males, 103 females, 108 singletons, 114 twins) between 2.70 and 4.80 months of age. We tested 16 major demographic and medical history variables for effects on inter-individual variation in salivary testosterone. Using the subset of twins, we estimated genetic and environmental contributions to salivary testosterone levels. Finally, we tested single nucleotide polymorphisms (SNPs) within ±5 kb of genes involved in testosterone synthesis, transport, signaling, and metabolism for associations with salivary testosterone using univariate tests and random forest (RF) analysis. We report an association between 5 min APGAR scores and salivary testosterone levels in males. Twin modeling indicated that individual variability in testosterone levels was primarily explained by environmental factors. Regarding genetic variation, univariate tests did not reveal any variants significantly associated with salivary testosterone after adjusting for false discovery rate. The top hit in males was rs10923844, an SNP of unknown function located downstream of *HSD3B1* and *HSD3B2*. The top hits in females were two SNPs located upstream of *ESR1* (rs3407085 and rs2295190). RF analysis, which reflects joint and conditional effects of multiple variants, indicated that genes involved in regulation of reproductive function, particularly *LHCGR*, are related to salivary testosterone levels in male infants, as are genes involved in cholesterol production, transport, and removal, while genes involved in estrogen signaling are related to salivary testosterone levels in female infants.

Keywords: testosterone, twins, infancy, APGAR, *LHCGR*, hypothalamic–pituitary–gonadal axis, minipuberty, neonate

INTRODUCTION

Transient activation of the hypothalamic–pituitary–gonadal (HPG) axis in the early post-natal period results in elevated levels of gonadotropins and testosterone in human males, a phenomenon known as the "minipuberty" or "neonatal surge" (1–5). The consequences of this activation are not fully understood, but it likely plays an important role in genital development (6–9) and has been linked to future fertility (10). There is also an increasing body of evidence that this is a critical period for the development of sexually dimorphic behavior and psychopathology (11–16). Understanding the causes of individual variation in testosterone levels during the minipuberty is thus of considerable theoretical and clinical interest.

Levels of pubertal and post-pubertal testosterone in males are determined, in part, genetically, with heritability estimates around 40–70% (17–23) for both salivary and plasma measures. Studies in females are less consistent with some reporting minimal heritable variation (23) and others reporting heritabilities similar to those seen in males (17, 20, 22). Recently, genome-wide association studies (GWAS) have been used to identify single nucleotide polymorphisms (SNPs) associated with serum levels of testosterone in adults. Ohlsson et al. (24) identified two loci that met genome-wide significance for serum testosterone levels in men of European ancestry, one in the gene for sex hormone-binding globulin (*SHBG*, lead SNPs rs12150660 and rs6258) and one near *family with sequence similarity 9, member B* (*FAM9B*) on Xp22 (lead SNP rs5934505). Jin et al. (25) confirmed both loci in an independent study, although the lead SNP in the *SHBG* locus differed (rs727428). They also reported a new hit at rs10822184, which is located in *receptor accessory protein 3* (*REEP3*). Chen et al. (26) reported a significant association between rs2075230 in the *SHBG* locus and serum testosterone in a sample of Chinese men. A GWAS of 1600 post-menopausal women failed to find any genome-wide significant associations with testosterone (27).

There is evidence to suggest that distinct genetic mechanisms influence testosterone levels across developmental time (17), so it is theoretically possible that the relative importance of genetic factors and the specific variants involved may be different in the minipuberty. Indeed, a study of salivary testosterone in twins between

4 and 8 months of age reported that variation was completely explained by common and unique environmental factors (28). This result is extremely intriguing, but the age range studied is slightly beyond the minipuberty as classically defined. Most studies suggest that the post-natal surge in testosterone peaks in males between 1 and 3 months of age and declines significantly by 6 months of age (4, 5, 29, 30). The gonads are the main source of testosterone in males during the minipuberty, but after 6 months the adrenal becomes the primary source of testosterone (31) as a consequence of the regression and degeneration of neonatal Leydig cells (32). Complementary studies at earlier ages are therefore needed as are studies looking at the impact of specific environmental factors.

The current study takes advantage of an ongoing GWAS of infant brain development being carried out at the University of North Carolina (UNC) at Chapel Hill. The study includes both twins and singletons. A subset of participating children donated saliva samples for the assessment of salivary testosterone around 3 months of age [see Ref. (33)]. We used this sample to estimate genetic and environmental contributions to salivary testosterone levels during the minipuberty. We also examined the impact of 16 major demographic and medical history variables on inter-individual variation in salivary testosterone. In addition, we tested SNPs within ±5 kb of genes involved in testosterone synthesis, transport, signaling, and metabolism for their relationship with salivary testosterone. We also tested SNPs in *REEP3* and the Xp22 loci identified in GWAS studies of serum testosterone in adult males to see if these were predictive in infants. Finally, to address the issue of potentially small effect sizes for individual SNPs, as well as non-additive effects, we used random forest (RF) methods to identify combinations of SNPs contributing to variation in salivary testosterone in infancy.

MATERIALS AND METHODS

SUBJECTS

Two-hundred twenty-two infants (119 males, 103 females) between 2.70 and 4.80 months of age (mean age 3.34 months post-date of birth ± 0.36) with high quality genetic information and saliva samples suitable for hormone assay are included in this analysis. The sample includes 108 singletons and 114 twins (27 same-sex DZ pairs, 27 same-sex MZ pairs, 2 opposite-sex DZ pairs, and 2 unpaired twins). All children were participating in prospective longitudinal studies of early brain development for which the senior author is a co-investigator. Mothers were recruited during the second trimester of pregnancy from the outpatient obstetrics and gynecology clinics at UNC hospitals. Exclusion criteria at enrollment were the presence of abnormalities on fetal ultrasound or major medical illness in the mother. Demographic variables (maternal age, paternal age, maternal education, paternal education, maternal ethnicity, paternal ethnicity, maternal psychiatric history, paternal psychiatric history, and total household income) were collected via maternal report at the time of enrollment (see Supplementary Material). For the purpose of the current study, maternal psychiatric history and paternal psychiatric history were treated as binary variables. Individuals were counted as positive for psychiatric history if they had received any psychiatric diagnosis. Medical history variables (birth weight, gestational age at birth, 5 min APGAR scores, stay in neonatal intensive care unit over 24 h, gestation number, and delivery method) were collected from maternity and pediatric medical records shortly after birth (see Supplementary Material). Maternal smoking during pregnancy was collected via maternal report at two timepoints during pregnancy and shortly after birth. Demographic and medical history data are summarized in **Table 1**. Experiments were undertaken with the understanding and written consent of each subject's mother or father, with the approval of the Institutional Review Board of the UNC School of Medicine.

Table 1 | Demographic and medical history information.

	Male ($N = 119$)	Female ($N = 103$)
CONTINUOUS VARIABLES Mean (SD)		
Age since DOB[a] (months)	3.32 (0.36)	3.36 (0.37)
Gestational age at birth (weeks)	37.3 (3.0)	37.7 (2.3)
Birth weight (g)	2886 (816)	2910 (563)
5-Min APGAR Score	8.7 (0.7)	8.6 (0.7)
Maternal age (years)	29.7 (6.1)	30.3 (5.4)
Paternal age (years)	31.5 (5.9)	32.5 (6.1)
Maternal education (years)	15.7 (3.3)	15.7 (3.0)
Paternal education (years)	15.7 (3.1)	15.0 (3.0)
CATEGORICAL VARIABLES NO. (%)		
Gestation number	Twin 65 (55%) Singleton 54 (45%)	Twin 49 (48%) Singleton 54 (52%)
NICU stay >24 h	No 98 (82%) Yes 21 (18%)	No 86 (83%) Yes 17 (17%)
Caesarian section	No 60 (50%) Yes 59 (50%)	No 56 (54%) Yes 47 (46%)
Maternal ethnicity	White 89 (75%) Black 24 (20%) Asian 4 (3%) Other 2 (1%)	White 82 (80%) Black 20 (19%) Asian 1 (1%)
Paternal ethnicity	White 86 (72%) Black 26 (22%) Asian 7 (6%)	White 81 (79%) Black 19 (18%) Asian 3 (3%)
Maternal psychiatric history	No 93 (78%) Yes 26 (22%)	No 81 (79%) Yes 22 (21%)
Paternal psychiatric history	No 103 (87%) Yes 16 (13%)	No 94 (91%) Yes 9 (9%)
Income[b]	High (29%) Middle (39%) Low (30%) Missing (2%)	High (33%) Middle (37%) Low (28%) Missing (2%)
Maternal smoking	No 107 (90%) Yes 12 (10%)	No 93 (90%) Yes 10 (10%)

[a] *DOB = date of birth,*

[b] *low income: at or below 200% of federal poverty level (FPL), middle income: between 200 and 400% of FPL, high income: above 400% of FPL.*

SALIVARY ASSAYS

Saliva samples were collected during visits to participants' homes. Visits were scheduled for 9:00 a.m. Mean ± SD for collection start time was 9:14 a.m. ± 16 min. Parents were advised not to feed their children for at least 15 min prior to collection. One milliliter of passive drool was collected from each participant using a suction catheter (Centurion Healthcare Products, Howell, MO, USA). Mean ± SD for duration of collection was 4 ± 2 min. All samples were frozen within 5 h and stored in a −80 °C freezer. Mean ± SD for time until storage was 60 ± 31 min.

Salivary testosterone levels were measured by enzyme immunoassay using a commercially available kit (Salimetrics, State College, PA, USA). The intra-assay precision for samples with low testosterone levels (mean 18.12 pg/ml) is 6.7%; for high testosterone levels (mean 188.83 pg/ml) it is 2.5%. Inter-assay precision for samples with low testosterone levels (mean 19.6 pg/ml) is 14.05%; for high testosterone levels (mean 199.08 pg/ml) it is 5.6%. Percent recovery for this assay varies from 92 to 111.4%. The minimal concentration of testosterone that can be distinguished from 0 is <1.0 pg/ml. Only one sample had a concentration below the detection limit. This sample was coded as 0 pg/ml in subsequent analyses. We also evaluated all samples for blood contamination using the Salimetrics Salivary Blood Contamination Enzyme Immunoassay kit, which quantitatively measures transferrin, a large protein, which is present in abundance in blood, but that is normally present in only trace amounts in saliva. Intra-assay precision for samples with high (3.88 mg/dl) transferrin levels is 10.2%, for samples with low (0.42 mg/dl) transferrin levels it is 4.9%. Inter-assay precision is 7.1% for low (1.02 mg/dl) and 7.2% for high (4.93 mg/dl) transferrin levels. Percent recovery varies from 91.9 to 101.5%. The minimal concentration of transferrin that can be distinguished from zero is 0.08 mg/dl.

GENOTYPING

DNA was extracted from buccal cells using standard methods as described in the Puregene® DNA Purification Kit (Gentra Systems) or using phenol/chloroform. After extraction, samples were stored in a −80 °C freezer until analysis. Genotyping was carried out at the Bionomics Research and Technology Center at Rutgers (Piscataway, NJ, USA) using Affymetrix Axiom Genome-Wide LAT and Exome arrays. Samples were randomized across 96-well plates. Each plate contained a common control sample. Genotype calling was performed with the Affymetrix Genotyping Console. Rigorous quality control procedures were carried out prior to analysis. In brief, we excluded samples with low DishQC (<0.82 for LAT array and <0.79 for Exome array), low call rates (<95%), outliers for homozygosity, sex, or zygosity from genotypes inconsistent with reported phenotypes, ancestry outliers, excessive relatedness, and unexpected relatedness. We also removed individual SNPs that deviated from Hardy–Weinberg equilibrium ($P_{HWE} < 1 \times 10^{-8}$), had low call rate (<95%), high deviation of allele frequency from 1000G EUR/AFR founders, and that did not match 1000G EUR/AFR founders. Population stratification was assessed using principal component analysis (PCA) (34, 35). Population stratification is the presence of systematic differences in allele frequencies between subpopulations with different genetic ancestry and can lead to spurious results in genetic association studies. We first pruned the genotyped SNPs using the pruning tool in PLINK (36). We then created a subset of our total GWAS sample without related subjects by randomly selecting one individual from twin and sibling pairs (all non-twins/non-sibs were also included). Smartpca in EIGENSOFT was applied to compute the principal components (PCs or eigenvectors). The first three PCs explain 6.8% of the variability in genetic variables. See **Figures 1** and **2** for a scree plot and 3D scatter plots of the first three PCs color-coded by maternal and paternal reported ethnicity. Strong clustering of subjects with similar reported ethnicity confirms that the first three PCs index genetic ancestry. Imputation was then performed with MaCH-Admix using 1000G reference panel (phase1_release_v3.20101123) followed by post-imputation quality control based on imputation quality score.

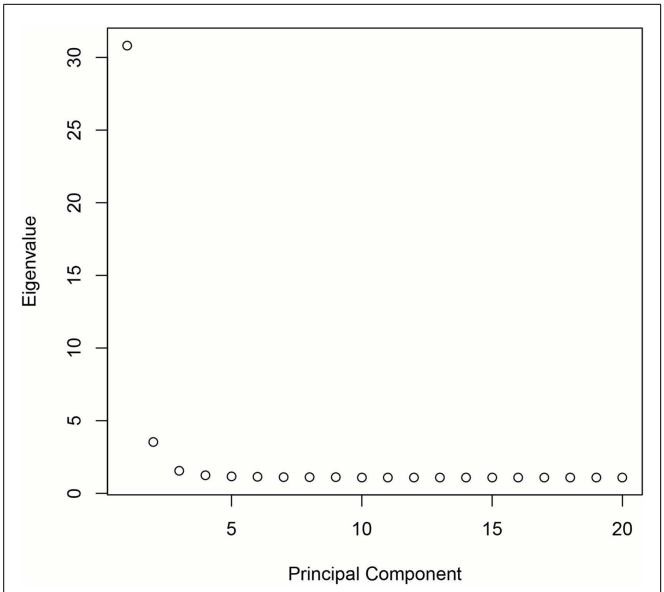

FIGURE 1 | Scree plot demonstrating that the majority of genetic variation is explained by the first three PCs.

For the current analysis, we extracted all genotyped SNPs within ±5 kb of genes involved in testosterone synthesis, transport, signaling, and metabolism (see **Table 2**). We also included genotyped SNPs in/near *REEP3* and the Xp22 loci flagged in GWAS studies of serum testosterone in adult males. Three of the top GWAS hits in *SHBG* were not genotyped by either array, but were imputed with good quality (rs727428, rs12150660, and rs2075230). The imputed SNPs were used in the current analysis. rs6258 was directly genotyped, but was excluded due to low minor-allele frequency (MAF). Top hits in *REEP3* and the Xp22 loci were also imputed (rs10822184 and rs5934505). Additional imputed SNPs near the Xp22 loci were also included due to the low number of genotyped SNPs in this region. SNPs with MAF <0.05 were not included in our analyses. In total, 512 SNPs were examined in males and 473 SNPs were examined in females.

ENVIRONMENTAL ASSOCIATION ANALYSIS

In order to determine the potential impact of major demographic and medical history variables on inter-individual variation in

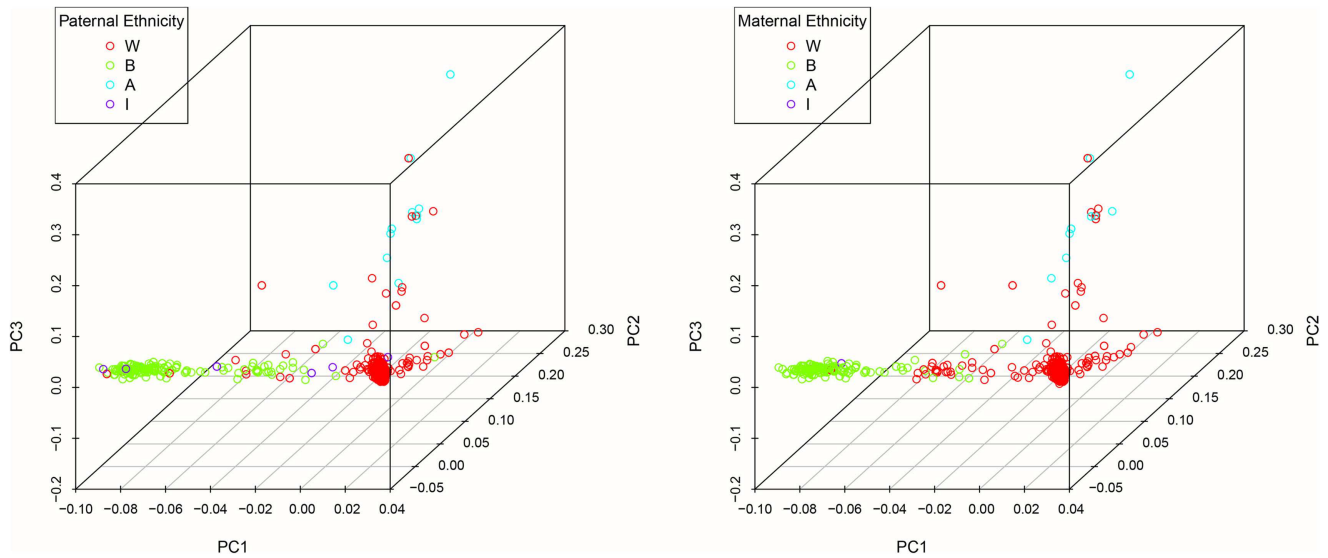

FIGURE 2 | 3D scatter plots of the first three PCs color-coded by maternal and paternal reported ethnicity.

salivary testosterone, we used a moment-based method to select fixed effects in linear mixed effects models (37). Twins are treated as repeated measures. For fixed effects selection, we applied an adaptive Lasso penalty using the feasible generalized least squares estimator as an initial. In the model, we always include log(10) transferrin and age since DOB as predictors. We focused on selecting a list of predictors including maternal age, paternal age, maternal education, paternal education, maternal ethnicity, paternal ethnicity, maternal psychiatric history, paternal psychiatric history, total household income, maternal smoking during pregnancy, birth weight, gestational age at birth, 5 min APGAR scores, stay in neonatal intensive care unit over 24 h, gestation number, and delivery method. We used the BIC statistic to select the tuning parameter of the adaptive Lasso as in Section 4 of Ahn et al. (37). Before applying our variable selection method, we standardized all covariates and centered the response variable (testosterone). We also applied bootstrap methods 1000 times to assess the stability of our results.

After model selection, we ran a mixed effect model using the selected variables for significance testing and to estimate r^2 values. Mixed effect models were also run including all variables for comparison. Males and females were analyzed separately for this and all subsequent analyses. We separated the sexes because the source of testosterone is primarily gonadal in males and adrenal in females during the minipuberty (31). Studies in adults also suggest that different genetic factors influence testosterone concentrations in men and women (17). Variables were considered significant if they were selected in the Lasso model and survived Bonferroni correction in the mixed effect model including all variables.

INTRACLASS CORRELATION COEFFICIENT AND HERITABILITY ESTIMATION

The intraclass correlation coefficient (ICCs) and their confidence intervals were estimated separately for MZM (monozygotic male), DZM (dizygotic male), MZF (monozygotic female), and DZF (dizygotic female) using R package called ICC. The heritability was estimated using linear mixed effect model (ACE model) while restricting variance of genetic, shared environment, and random error to be >0. One-tailed t-tests were used to test whether the estimates were significantly >0 (p-values <0.05 were considered significant). For males, log(10)transferrin, age since DOB, and 5 min APGAR score were included as covariates. For females, log(10)transferrin and age since DOB were included as covariates.

UNIVARIATE GENETIC ASSOCIATION ANALYSIS

Males and females were analyzed separately. For males, log(10)transferrin, age since DOB, and 5 min APGAR score were included as covariates. For females, log(10)transferrin and age since DOB were included as covariates. In addition, the first three principle components derived from all genotyped SNPs were included as covariates to control for possible population stratification. Association analysis was performed using mixed effect models with likelihood ratio tests. p-values were adjusted by false discovery rate (FDR); p-values <0.05 after FDR correction were considered significant.

Univariate genetic association analysis provides a straightforward approach to identifying genetic variants associated with phenotypes of interest, in this case salivary testosterone levels. However, given the large number of tests performed, this approach is underpowered for identifying SNPs with small effect sizes. In addition, it does not account for genetic interactions, i.e., causal effects that are only observed when specific combinations of mutations and/or non-mutations are jointly present. In order to address these limitations, we also carried out RF analysis to identify combinations of SNPs contributing to variation in salivary testosterone in infancy.

To gain a better understanding of the power of our univariate genetic association analysis, we generated power plots using different SNP MAFs and effect sizes (beta).

Table 2 | Genes probed in the current analysis.

Gene symbol	Gene name	General category
CYP11A	Cytochrome P450, family 11, subfamily A, polypeptide 1	Synthesis and metabolism
CYP11B1	Cytochrome P450, family 11, subfamily B, polypeptide 1	Synthesis and metabolism
CYP17A1	Cytochrome P450, family 17, subfamily A, polypeptide 1	Synthesis and metabolism
CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1 (Aromatase)	Synthesis and metabolism
CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1	Synthesis and metabolism
CYP21A2	Cytochrome P450, family 21, subfamily A, polypeptide 2	Synthesis and metabolism
CYP3A4	Cytochrome P450, family 3, subfamily A, polypeptide 4	Synthesis and metabolism
CYP3A43	Cytochrome P450, family 3, subfamily A, polypeptide 43	Synthesis and metabolism
CYP3A5	Cytochrome P450, family 3, subfamily A, polypeptide 5	Synthesis and metabolism
CYP3A7	Cytochrome P450, family 3, subfamily A, polypeptide 7	Synthesis and metabolism
CYP7A1	Cytochrome P450, family 7, subfamily A, polypeptide 1	Synthesis and metabolism
DHCR7	7-dehydrocholesterol reductase	Synthesis and metabolism
HSD17B1	Hydroxysteroid (17-beta) dehydrogenase 1	Synthesis and metabolism
HSD17B2	Hydroxysteroid (17-beta) dehydrogenase 2	Synthesis and metabolism
HSD17B3	Hydroxysteroid (17-beta) dehydrogenase 3	Synthesis and metabolism
HSD17B6	Hydroxysteroid (17-beta) dehydrogenase 6	Synthesis and metabolism
HSD17B7	Hydroxysteroid (17-beta) dehydrogenase 7	Synthesis and metabolism
HSD17B8	Hydroxysteroid (17-beta) dehydrogenase 8	Synthesis and metabolism
HSD3B1	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	Synthesis and metabolism
HSD3B2	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2	Synthesis and metabolism
POR	P450 (cytochrome) oxidoreductase	Synthesis and metabolism
SRD5A1	Steroid-5-alpha-reductase, alpha polypeptide 2 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1)	Synthesis and metabolism
SRD5A2	Steroid-5-alpha-reductase, alpha polypeptide 2 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 2)	Synthesis and metabolism
SRD5A3	Steroid 5 alpha-reductase 3	Synthesis and metabolism
STAR	Steroidogenic acute regulatory protein	Synthesis and metabolism
STS	Steroid sulfatase (microsomal), isozyme S	Synthesis and metabolism
SULT2A1	Sulfotransferase family, cytosolic, 2a, dehydroepiandrosterone (DHEA)-preferring, member 1	Synthesis and metabolism
SHBG	Sex hormone-binding globulin	Transport
TSPO	Translocator protein (18 kDa)	Transport
ALB	Albumin	Transport
AR	Androgen receptor	Receptors
ESR1	Estrogen receptor alpha	Receptors
ESR2	Estrogen receptor beta	Receptors
CGA	Glycoprotein hormones, alpha polypeptide	Regulators of reproductive function
GNRH1	Gonadotropin-releasing hormone 1 (luteinizing-releasing hormone)	Regulators of reproductive function
LHB	Luteinizing hormone beta polypeptide	Regulators of reproductive function
GNRHR	Gonadotropin-releasing hormone receptor	Regulators of reproductive function
LHCGR	Luteinizing hormone/choriogonadotropin receptor	Regulators of reproductive function
REEP3	Receptor accessory protein 3	GWAS hit for serum testosterone
Xp22 near rs5934505	Closest gene is family with sequence similarity 9, member B	GWAS hit for serum testosterone

We used the same number of subjects (and proportion of MZs, DZs, and singletons) as our real data and similar scale of effect size, mean, variance, and additive genetic and shared environment effects estimated from the real data. Ten thousand simulations were done for each scenario and the significance level was set to 0.05/500, which is similar to our data after Bonferroni correction.

RANDOM FORESTS

Random forest analysis was implemented using R software. The result of RF analysis reflects joint and conditional effects of multiple variables. As variables with strong effects may mask weaker, yet important effects, we removed the effects of log(10)transferrin, age since DOB, 5 min APGAR score, and the first three principle components (for males) and log(10)transferrin, age since DOB, and the first three principle components (for females). Specifically, we (1) used the residual from the regression Testosterone ~log(10)transferrin + age since DOB + 5 min APGAR score (males only) + PC1 + PC2 + PC3 as the new response, and (2) used the residual from the regression SNPs ~log(10)transferrin + age since DOB + 5 min APGAR score (males only) + PC1 + PC2 + PC3 as the new predictor variables for the RF analysis. The number of randomly preselected variables (m_{try}) was set to the number of SNPs divided by 3 ($p/3$) as recommended by Liaw and Wiener (38). We also examined the error rate for $m_{try} = 2p/3, 0.5p/3$, and p. Error rate was highly similar across different m_{try}, but $p/3$ produced the lowest error rate. The number of trees (*ntree*) was set to 2000. RF analysis was run 50 times, each run using different seeds for random number generation (RNG). In each run, we recorded the 30 SNPs with the highest variable importance. After all 50 runs were completed, we identified those SNPs, which appeared in the "top 30 list" in every 1 of 50 runs. Because RF analysis is not set-up to treat twins as a repeated measure, we randomly picked one twin from each twin pair to ensure that no related subjects were included in the RF analysis.

RESULTS

Mean ± SD concentration of salivary testosterone was 40.39 ± 13.39 pg/ml in males and 39.70 ± 16.64 pg/ml in females and did not differ significantly ($p = 0.73$). This is likely a consequence of differing correlations between salivary testosterone and serum testosterone in males and females. According to Salimetrics, the relationship between serum and saliva for males as determined by linear regression is y (total serum testosterone in nanogram per milliliter) $= 0.2421 + 0.0496 \times x$ (salivary testosterone in picogram per milliliter). The linear regression equation for females is y (total serum testosterone in nanogram per milliliter) $= 0.1415 + 0.0055 \times x$ (salivary testosterone in picogram per milliliter). Assuming that the relationship between total serum testosterone and salivary testosterone in infants is similar to that seen in adults; we would estimate a mean serum level of 2.25 ng/ml for male infants and 0.36 ng/ml for female infants, which is comparable to published reports on serum testosterone levels in this age range (39, 40).

Mean ± SD for transferrin was 0.844 ± 0.93 mg/dl in males and 0.91 ± 0.98 mg/dl in females. A moderate correlation between transferrin and testosterone was observed in both males and females ($r = 0.28$, $p = 0.002$ and $r = 0.43$, $p < 0.001$, respectively). Transferrin levels were higher than expected, which raises the possibility of blood contamination. We addressed this issue in two ways. (1) In all our primary analyses, we included transferrin as a covariate. Specifically, we adjusted for log(10)transferrin as the untransformed variable showed high levels of skewness and kurtosis. (2) For the environmental association analysis, univariate genetic analyses, and RF analysis, we also performed sensitivity analyses in infants with transferrin levels <0.50 mg/dl [cut-off based on the recommendation of Granger et al. (41)]. For ICC and heritability estimation, we did not perform sensitivity analyses due to insufficient sample size.

ENVIRONMENTAL ASSOCIATION ANALYSIS

The final model for males using adaptive Lasso included 5 min APGAR score in addition to the fixed variables, log(10)transferrin, and age since DOB. Bootstrapping supported the importance of 5 min APGAR score in that it was selected in 837 out of 1000 tests (see **Table 3**). A linear mixed effect model including these variables explained approximately 32% of the variance in salivary testosterone. Examination of a linear mixed effect model including all predictors confirmed that 5 min APGAR score was the only major demographic or medical history variable examined which significantly predicted salivary testosterone in males after Bonferroni correction (0.05/29 predictors = 0.0017) and explained approximately 15% of the variance. In males with transferrin levels <0.50 mg/dl, a linear mixed model including age since DOB and 5 min APGAR score explained 22% of the variance in salivary

Table 3 | Bootstrapping results.

	Male	Female
Transferrin[a] (fixed)	1000	1000
Age since DOB (fixed)	1000	1000
NICU Stay >24 h	43	26
Birth weight	152	84
Gestational age birth	54	28
Maternal ethnicity (White vs. Black)	122	114
Maternal ethnicity (White vs. Asian)	132	3
Maternal ethnicity (White vs. American Indian)	83	
Paternal ethnicity (White vs. Black)	122	9
Paternal ethnicity (White vs. Asian)	8	22
Maternal education	49	23
Paternal education	92	17
Maternal age	110	12
Paternal age	118	19
Maternal psych history	111	6
Paternal psych history	184	11
Income (low vs. middle)	48	141
Income (low vs. high)	410	35
Income (low vs. missing)	39	379
5-Min APGAR	837	20
Gestation number	103	43
C-section	227	32
Smoking	126	5

[a] *Log(10)transferrin.*

Table 4 | Association of salivary testosterone with demographic and medical history variables in males.

Model	R^2	Predictors	Beta	Sig	r^2
Mixed effect model (selected variables)	0.32	Intercept	110.07		
		Transferrin[a]	17.36	<0.001	0.24
		Age since DOB	−5.84	0.08	0.02
		5 Min APGAR	−5.26	<0.001	0.08
Full mixed effect model	0.39	Intercept	183.68		
		Transferrin[a]	17.89	<0.001	0.23
		Age since DOB	−7.99	0.02	0.04
		NICU >24 h	−2.87	0.44	<0.01
		Birth weight	0.00	0.49	0.01
		Gestational age birth	−0.10	0.40	0.02
		Mat ethnicity		0.05	
		White	−14.60		0.20
		Black	−12.56		0.12
		American Indian	−25.34		0.05
		Pat ethnicity		0.51	
		White	2.99		<0.01
		Black	0.13		<0.01
		Mat education	−0.05	0.94	<0.01
		Pat education	−0.64	0.35	0.02
		Mat age	−0.24	0.55	<0.01
		Pat age	0.31	0.50	0.02
		Mat psych history	2.71	0.51	<0.01
		Pat psych history	−6.07	0.17	0.02
		Income		0.08	
		High	3.85		0.02
		Middle	−0.71		<0.01
		Missing	7.78		<0.01
		5-Min APGAR	−7.57	<0.001	0.15
		Gestation number	−3.18	0.38	0.01
		C-section	−3.34	0.18	0.01
		Mat smoking	2.53	0.60	<0.01
Reduced mixed effect model in infants with transferrin <0.5 ng/dl	0.22	Intercept	105.03		
		Age since DOB	−2.93	0.50	<0.01
		5-Min APGAR	−7.07	<0.001	0.22

[a] *Log(10)transferrin.*

Table 5 | Association of salivary testosterone with demographic and medical history variables in females.

Model	R^2	Predictors	Beta	Sig	r^2
Mixed effect model (selected variables)	0.18	Intercept	57.37		
		Transferrin[a]	14.24	<0.001	0.15
		Age since DOB	−4.86	0.28	0.01
Full mixed effect model	0.41	Intercept	70.37		
		Transferrin[a]	15.71	<0.001	0.16
		Age since DOB	−5.73	0.20	0.01
		NICU stay >24 h	−6.41	0.27	0.02
		Birth weight	−0.01	0.08	0.07
		Gestational age birth	0.06	0.72	<0.01
		Mat Ethnicity		0.24	
		White	−5.03		0.01
		Black	3.01		<0.01
		Pat ethnicity		0.16	
		White	14.02		0.11
		Black	8.37		0.03
		Mat education	0.43	0.58	<0.01
		Pat education	0.24	0.73	<0.01
		Mat age	0.16	0.74	<0.01
		Pat age	−0.21	0.62	<0.01
		Mat psych history	0.18	0.96	<0.01
		Pat psych history	−2.67	0.64	<0.01
		Income		0.004	
		High	−2.59		<0.01
		Middle	2.87		<0.01
		Missing	−28.57		0.05
		5-Min APGAR	−0.97	0.66	<0.01
		Gestation number	−6.03	0.23	0.03
		C-section	−0.26	0.94	<0.01
		Mat smoking	1.53	0.79	<0.01
Reduced mixed effect model in infants with transferrin <0.5 ng/dl	0.01	Intercept	17.89		
		Age since DOB	3.48	0.22	0.01

[a] *Log(10)transferrin.*

testosterone (see **Table 4**). The final model for females using adaptive Lasso only included the fixed variables, log(10)transferrin, and age since DOB. A mixed effect model including these variables explained approximately 18% of the variance in salivary testosterone. Examination of a mixed effect model including all predictors confirmed that none of the demographic or medical history variables examined significantly predicted salivary testosterone in females after Bonferroni correction (0.05/29 predictors = 0.0017). In females with transferrin levels <0.50 mg/dl, a mixed model including age since DOB explained 1% of the variance in salivary testosterone (see **Table 5**). Given the converging evidence for the importance of 5 min APGAR score on salivary testosterone in males, it was included as a covariate in subsequent analyses.

INTRACLASS CORRELATION COEFFICIENT AND HERITABILITY ESTIMATION

The intraclass correlations for MZ and DZ twins are shown in **Figure 3** and suggest a high environmental component and a low genetic component for both sexes. The ACE model confirmed that the majority of variation in salivary testosterone was explained by shared environmental factors in both sexes (see **Table 6**).

UNIVARIATE GENETIC ASSOCIATION ANALYSIS

In males, no SNPs were significantly associated with salivary testosterone levels after adjusting for FDR in the full sample. The most significant association was for rs10923844, a variant of unknown function located downstream of *HSD3B1* and *HSD3B2* (adjusted p-value = 0.07, unadjusted $p = 0.0002$). This same SNP had an

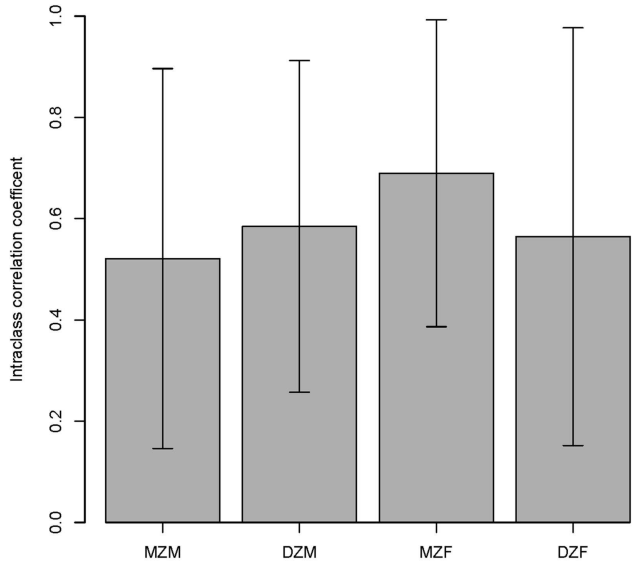

FIGURE 3 | Intraclass correlations within twin pairs are shown. MZM (monozygotic male), DZM (dizygotic male), MZF (monozygotic female), and DZF (dizygotic female).

Table 6 | Univariate genetic model.

	Variance explained (%)	*p*-value
Males		
A	0	0.81
C	62.7	<0.001
Females		
A	19.1	0.36
C	41.8	0.19

Percentages of phenotypic variation explained by additive genetic (A) and shared environmental (C) factors.

unadjusted *p*-value of 0.02 in the subsample of males with transferrin <0.50 mg/dl. Effect sizes for this variant were highly similar in the total male sample [adjusting for log(10)transferrin] and the subsample of males with transferrin <0.50 mg/dl (Beta = 6.26 and 6.02, respectively). Indeed, effect sizes for the full male sample are highly similar to the corresponding effect sizes in the subset of males with transferrin <0.50 mg/dl (see **Figure 4**). None of the previous GWAS hits for serum testosterone levels in adult males were significantly associated with salivary testosterone in infant males before or after adjusting for FDR in either the full sample or the subset of males with transferrin <0.50 mg/dl. In females, no SNPs were significantly associated with salivary testosterone levels after adjusting for FDR. The most significant associations were for two SNPs located upstream of *ESR1* (rs3407085 and rs2295190) within the intron region of *SYNE1* (*spectrin repeat containing, nuclear envelope (1)*; adjusted and unadjusted p-values were 0.09 and 0.0004 for both variants (these variants are in complete linkage disequilibrium within our sample). Both variants had an unadjusted p-value of 0.04 in the subsample of females

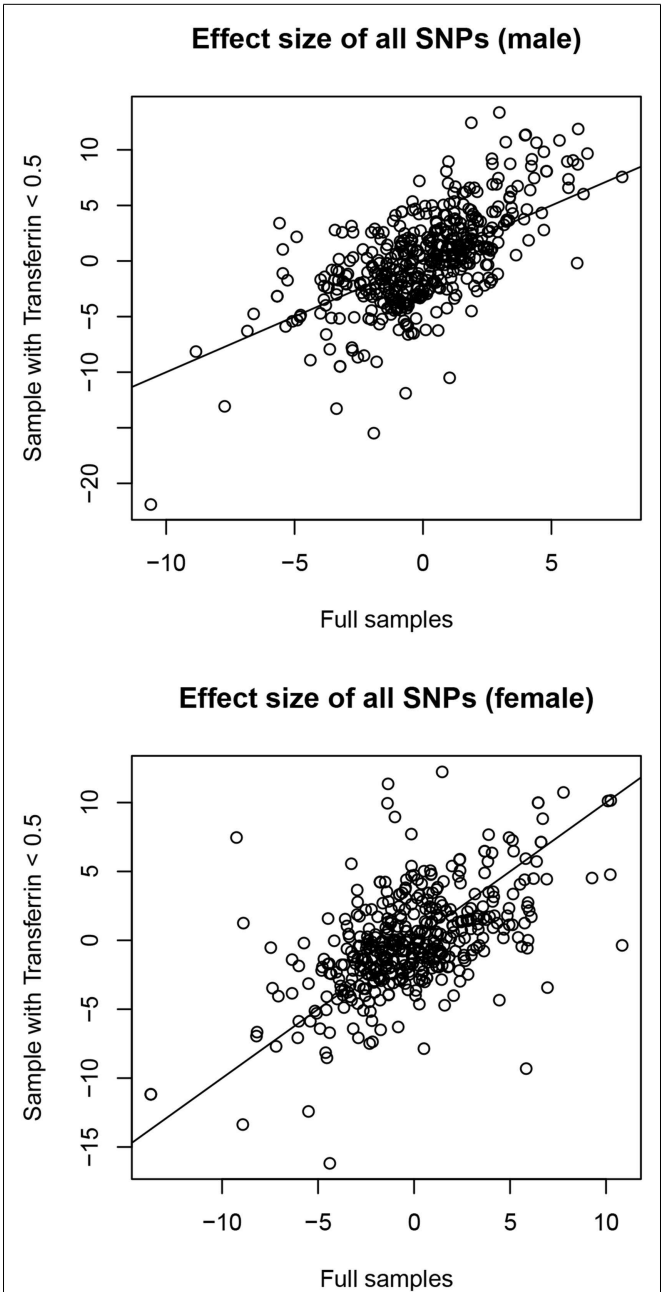

FIGURE 4 | Correlation between effect sizes in full samples [correcting for log(10)transferrin] and subsamples with transferrin levels <0.50 mg/dl.

with transferrin <0.50 mg/dl. Effect sizes were highly similar for the total female sample [adjusting for log(10)transferrin] and the subsample of females with transferrin <0.50 mg/dl (Beta = −13.7 and −11.2, respectively), though we note that effect sizes for the full female sample do not correspond as well to the effect sizes in the reduced sample as compared to males (See **Figure 4**). Full results are available as supplemental data. Power calculations indicate that theoretically, our analysis is well-powered to identify variants with Beta values >6 in the MAF range examined (see **Figure 5**).

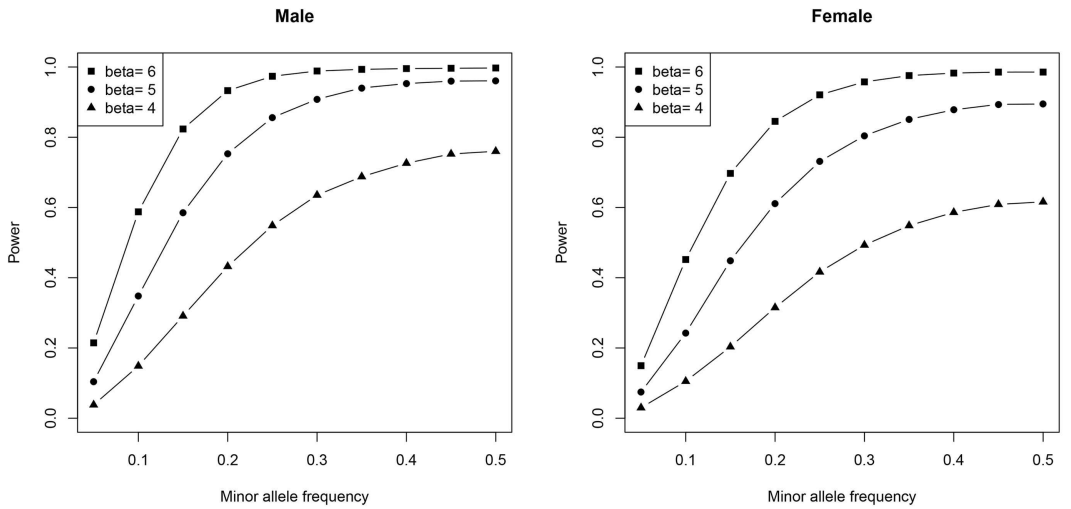

FIGURE 5 | Plots demonstrating the relationship between statistical power, minor-allele frequencies, and effect size are shown.

RANDOM FORESTS

Tables 7 and **8** display the top SNPs in males and females, respectively, defined as the intersection of the top 30 variables obtained from each of 50 RF runs. Also included in the table are the beta.hat, likelihood ratio, and unadjusted and FDR adjusted *p*-values from the univariate association tests. In males, regulators of reproductive function appear to play an important role. Specifically, multiple variants in or near *LHCGR* were consistently selected as high importance. Multiple SNPs in genes related to cholesterol production, transport, and removal were also selected. These genes included *DHCR7*, *TSPO*, *CYP7A1*, and *POR*. The top hit from the univariate analyses (rs10923844) was selected, as were two variants near the Xp22 loci identified in GWAS studies of serum testosterone in adult males. Analyses in the subset of males with transferrin <0.50 mg/dl supported the importance of regulators of reproductive function and cholesterol-related genes. Five SNPs were selected as high importance in both the full and reduced male sample; these were rs4952922, rs10495960, and rs2301267 (all in/near *LHCGR*), rs47340 (*TSPO*), and rs74091680 (*HSD17B6*). In females, genes related to estrogen signaling appear to play an important role. Specifically, variants in *ESR1*, *CYP19A1*, *CYP1B1*, and *HSD17B8* were consistently selected as high importance in the full sample. Analyses in the subset of females with transferrin <0.50 mg/dl supported the importance of estrogen signaling, although the specific SNPs differed. Two SNPs were selected as high importance in both the full and reduced female sample; these were rs4149448 (*SULT2A1*) and rs4659175 (*HSD3B2*).

DISCUSSION

This study provides the first detailed analysis of environmental and genetic contributors to variation in salivary testosterone during the minipuberty, a developmental period that plays a critical role in genital development and sexual differentiation of the brain. As such, it should be considered exploratory and requires replication. Twin modeling indicated that individual variability in testosterone levels in the minipuberty is primarily explained by environmental factors. In terms of specific environmental contributors, we observed a consistent and robust association between 5 min APGAR scores and salivary testosterone levels in males. In terms of specific genetic contributors, univariate tests did not reveal any variants significantly associated with salivary testosterone after adjusting for FDR. However, we note that this approach is underpowered for identifying SNPs with small effect sizes. The top hit in males was rs10923844, an SNP of unknown function located downstream of *HSD3B1* and *HSD3B2*. The top hits in females were two SNPs located upstream of *ESR1* (rs3407085 and rs2295190). RF analysis, which reflects joint and conditional effects of multiple variables, including those with small individual effect sizes, suggests that genes involved in regulation of reproductive function and cholesterol production, transport, and removal are involved in individual variation in salivary testosterone in males, while genes involved in estrogen signaling are important in females.

MAJOR DEMOGRAPHIC AND MEDICAL HISTORY VARIABLES AND SALIVARY TESTOSTERONE

Higher APGAR scores taken 5 min post-birth were associated with lower salivary testosterone levels in males during the minipuberty. APGAR scores were originally designed to quickly evaluate a newborn's physical condition and to identify any immediate need for extra medical or emergency care. The APGAR score includes five components: heart rate, respiratory effort, muscle tone, reflex irritability, and color, each of which is given a score of 0, 1, or 2 with 2 being the best score (42). A number of factors may influence an APGAR score, including hypoxia, exposure to drugs, trauma, congenital anomalies, infections, and hypovolemia (43). While primarily conceived as a measure of short peripartum stress, some researchers have suggested that it also indexes a suboptimal fetal environment (44). Studies in rodent models provide compelling evidence that the stress response induced by physical or emotional challenges in fetal life affects later reproductive function (45–47). Regarding the HPG axis, maternal stress

Table 7 | Random forest results males.

SNP	beta.hat	lr	mChr	mPos	pval	pval.bh	Index gene	Function class
FULL SAMPLE								
rs10923844	6.26	13.42	chr1	120000000	0.0002	0.07	*HSD3B1*	Downstream
rs2301267	5.66	12.38	chr2	48984391	0.0004	0.07	*LHCGR*	Upstream (*LHCGR*) intronic (*STON1-GTF2A1L*)
rs11897846	2.94	3.29	chr2	48956512	0.07	0.66	*LHCGR*	Intronic
rs10495960	3.97	3.26	chr2	48960032	0.07	0.66	*LHCGR*	Intronic (*LHCGR*) missense (*GTF2A1L*)
rs4952922	4	3.29	chr2	48961396	0.07	0.66	*LHCGR*	Intronic
rs988328	−6.6	8.42	chr6	152000000	0.004	0.35	*ESR1*	Intronic
rs4728533	1.82	0.75	chr7	75586536	0.38	0.90	*POR*	Intronic
rs10504255	3.63	3.64	chr8	59398461	0.06	0.66	*CYP7A1*	Downstream
rs1004467	4.04	2.85	chr10	105000000	0.09	0.66	*CYP17A1*	Intronic
rs12419334	4.62	7.34	chr11	71139472	0.007	0.38	*DHCR7*	Downstream
rs12797951	4.17	6.26	chr11	71143266	0.01	0.57	*DHCR7*	Downstream
rs74091680	7.75	7.47	chr12	57154822	0.006	0.38	*HSD17B6*	Intronic
rs47340	5.66	12.8	chr22	43562829	0.0003	0.07	*TSPO*	Downstream (*TSPO*) 3 prime UTR (*TTLL12*)
rs139036121	−0.21	0.03	chrX	8912628	0.87	0.96	*Xp22 near GWAS hit*	Intergenic b/t *FAM9A* and *FAM9B*
rs5934508	−0.1	0.01	chrX	8918776	0.92	0.97	*Xp22 near GWAS hit*	Intergenic b/t *FAM9A* and *FAM9B*
SUBSET WITH TRANSFERRIN <0.50 ng/dl								
rs56058466	9.15	6.42	chr2	38328870	0.01	0.21	*CYP1B1*	Upstream
rs4952922	11.36	17.97	chr2	48961396	0.00002	0.006	*LHCGR*	Intronic
rs10495960	11.30	17.81	chr2	48960032	0.00002	0.0006	*LHCGR*	Intronic (*LHCGR*) missense (*GTF2A1L*)
rs2301267	7.37	13.36	chr2	48984391	0.0003	0.03	*LHCGR*	Upstream
rs4245818	8.94	10.78	chr2	48985607	0.001	0.06	*LHCGR*	Upstream (*LHCGR)* Intronic (*STON1-GTF2A1L*)
rs11682325	9.86	6.56	chr2	48899807	0.01	0.21	*LHCGR*	Downstream (*LHCGR* Intronic (*STON1-GTF2A1L*)
rs2031367	8.74	7.83	chr6	87807180	0.01	0.17	*CGA*	Upstream
rs6937568	12.46	5.22	chr6	152153964	0.02	0.28	*ESR1*	Intronic
rs62442039	13.38	12.90	chr6	152158090	0.0003	0.03	*ESR1*	Intronic
rs10954724	−4.19	3.16	chr7	75597545	0.08	0.46	*POR*	Intronic
rs17148944	10.69	5.96	chr7	75601867	0.01	0.23	*POR*	Intronic
rs800667	2.91	0.41	chr7	99447241	0.52	0.81	*CYP3A43*	Synonymous
rs881671	−21.87	12.79	chr8	59417107	0.0003	0.03	*CYP7A1*	Upstream
rs8190495	5.80	7.09	chr9	99061884	0.01	0.18	*HSD17B3*	Intronic
rs8190478	6.23	9.07	chr9	99064883	0.003	0.12	*HSD17B3*	Upstream
rs74091680	7.59	2.03	chr12	57154822	0.15	0.55	*HSD17B6*	Intronic
rs2277339	2.67	0.47	chr12	57146069	0.49	0.82	*HSD17B6*	Upstream (*HSD17B6*) missense (*PRIM1*)
rs47340	6.61	6.83	chr22	43562829	0.01	0.20	*TSPO*	Downstream (*TSPO*) 3 prime UTR (*TTLL12*)

during pregnancy disrupts the prenatal surge of testosterone that normally occurs in the developing male rat (48, 49) and is associated with reduced testosterone levels in adult rats (50). No studies have examined stress response during labor and delivery and its impact on reproductive function. Physical stressors associated with lower APGAR scores may explain the association between 5 min APGAR scores and salivary testosterone in males observed in our study, although the direction of effect is opposite to that reported for prenatal stress in rodents. We note that while 5-min Apgar score is a valid predictor of neonatal mortality,

Table 8 | Random forest results females.

SNP	beta.hat	lr	mChr	mPos	pval	pval.bh	Index gene	Function class
FULL SAMPLE								
rs4659175	−3.44	2.08	chr1	119956473	0.15	0.80	*HSD3B2*	Upstream
rs4952222	1.58	0.13	chr2	31799863	0.72	0.97	*SRD5A2*	Intronic
rs56058466	6.90	4.73	chr2	38328870	0.03	0.52	*CYP1B*	Upstream
rs1547387	−8.21	5.75	chr6	33169895	0.02	0.39	*HSD17B8*	Upstream (*HSD17B8*) synonymous (*SLC39A7)*
rs11155820	1.43	0.29	chr6	152204210	0.59	0.96	*ESR1*	Intronic
rs62443560	−13.68	6.59	chr6	152190476	0.01	0.35	*ESR1*	Intronic
rs2982683	2.95	1.37	chr6	152298435	0.24	0.91	*ESR1*	Intronic
rs17081685	1.46	0.14	chr6	152116655	0.71	0.97	*ESR1*	Intronic
rs3798758	−5.50	1.37	chr6	152421854	0.24	0.91	*ESR1*	3 prime UTR
rs2899472	−0.46	0.04	chr15	51516055	0.85	0.99	*CYP19A1*	Intronic
rs9939740	−2.96	1.54	chr16	82121981	0.21	0.88	*HSD17B2*	Intronic
rs6259	−4.61	1.56	chr17	7536527	0.21	0.88	*SHBG*	Missense
rs4149448	5.52	2.68	chr19	48386357	0.10	0.69	*SULT2A1*	Intronic
rs138929	−4.78	3.44	chr22	43562439	0.06	0.58	*TSPO*	Downstream
rs57484470	5.01	0.87	chr22	43545077	0.35	0.92	*TSPO*	Upstream
rs6971	−4.70	2.89	chr22	43558926	0.09	0.68	*TSPO*	Missense
rs7058445	−2.03	0.67	chrX	7172508	0.41	0.94	*STS*	Intronic
SUBSET WITH TRANSFERRIN < 0.50 ng/dl								
rs4659175	−3.76	2.32	chr1	119956473	0.13	1	*HSD3B2*	Upstream
rs10923844	−4.58	2.84	chr1	120059500	0.09	0.90	*HSD3B1*	Downstream
rs232535	−0.60	0.04	chr2	38332303	0.85	1	*CYP1B1*	Upstream
rs162557	−2.85	1.17	chr2	38305451	0.28	1	*CYP1B1*	Upstream
rs28585480	−7.35	7.02	chr4	74290918	0.01	0.86	*ALB*	Downstream
rs2747653	6.47	−1.19	chr6	152446057	1.00	1	*ESR1*	Downstream (*ESR1*) intronic (*SYNE1*)
rs10224569	10.19	0.33	chr7	99248304	0.57	1	*CYP3A5*	Intronic
rs12537277	−0.61	0.04	chr7	75588704	0.84	1	*POR*	Intronic
rs10135310	9.95	−0.28	chr14	64574140	1.00	1	*ESR2*	Downstream (*ESR2*) intronic (*SYNE2*)
rs2781377	10.02	−1.42	chr14	64560092	1.00	1	*ESR2*	Downstream (*ESR2*) stop gained (*SYNE2*)
rs41334947	10.02	−1.42	chr14	64560091	1.00	1	*ESR2*	Downstream (*ESR2*) missense(*SYNE2*)
rs57018718	10.02	−1.42	chr14	64594019	1.00	1	*ESR2*	Downstream (*ESR2*) intronic (*SYNE2*)
rs2414095	−6.29	0.57	chr15	51524292	0.45	1	*CYP19A1*	Intronic
rs12591359	2.23	0.68	chr15	51539368	0.41	1	*CYP19A1*	Intronic
rs4149448	4.08	2.03	chr19	48386357	0.15	1	*SULT2A1*	Intronic
rs2910400	3.56	1.41	chr19	48394042	0.24	1	*SULT2A1*	Upstream

its predictive value for other outcomes continues to be debated (43). Never-the-less, this scoring system remains the only widely used and accepted tool for assessing the vitality of newborn infants across the world. We also note that the majority of children in our sample had 5-min Apgar scores between 7 and 10, which is considered normal, with only a few falling in the intermediate range (scores between 4 and 6). Additional research is necessary to replicate these findings, determine if they extend to individuals with lower APGAR scores, and understand underlying mechanisms. None of the major demographic or maternal history variables examined in this study impacted salivary testosterone levels in females.

HERITABILITY OF SALIVARY TESTOSTERONE

Our results suggest that individual variability in salivary testosterone levels in the minipuberty is predominantly explained by environmental factors. Our findings are similar to those reported by Caramaschi et al. (28) in older infants, but contrast with studies in adolescents and adults, which show high heritability, especially in males (17–23). Caramaschi et al. (28) suggested that the pubertal rise in male testosterone levels has a strong genetic component and that testosterone levels are less influenced by genetic factors when male and female levels are similar and very low as is the case in later infancy. Salivary testosterone levels in our study were significantly higher than those reported by Caramaschi et al. (28): 40.39 vs. 9.60 pg/ml in boys and 39.70 vs. 8.93 pg/ml in girls, but are lower than those reported for adults: 163.81 and 60.86 pg/ml for males and females, respectively (51). Salivary testosterone did not differ significantly between males and females in our sample, but estimated serum levels exhibited a sex difference, which is comparable to published reports on serum testosterone levels in this age range (39, 40). Therefore, our results suggest that environmental factors play a stronger role than genetic factors during the minipuberty despite moderately high testosterone levels and sex differences in serum testosterone. Identifying the environmental factors involved is an important area for future research.

GENETIC FACTORS INFLUENCING SALIVARY TESTOSTERONE

Although our heritability analysis indicated that environmental risk factors play a larger role in determining individual variation in salivary testosterone during the minipuberty than genetic factors, deciphering the genetic component is still important in terms of understanding underlying biochemical pathways. We were also mindful of the fact that heritability by itself does not provide information about the genetic architecture of traits. In principle, a trait with a low heritability can have a single locus that causes variation and a trait with a high heritability can have hundreds of contributing loci (52). The presence of gene–environment correlations and interactions also introduces systematic biases in heritability estimates made under the independence assumption in twin studies, which can result in an underestimation of the genetic component (53) For all these reasons, we performed univariate genetic association analyses and RF analyses of SNPs within ±5 kb of genes involved in testosterone synthesis, transport, signaling, and metabolism for their relationship with salivary testosterone.

No SNPs were significantly associated with salivary testosterone levels after adjusting for FDR in the full male sample or the full female sample. Simulations indicated that theoretically we were well-powered to detect significant SNPs with Beta values >6 within the range of minor-allele frequencies we examined. Thus, our study suggests that common variants with large effect sizes do not play a role in individual differences in salivary testosterone in infants, at least within the genes examined. The top hit in males was rs10923844, a variant of unknown function located downstream of *HSD3B1* and *HSD3B2*, two genes that play a critical role in steroidogenesis. This variant was also identified as an important variable in the RF analysis. Both isoforms of 3-beta-HSD convert pregnenolone to progesterone, 17α-hydroxypregnenolone to 17α-hydroxyprogesterone, dehydroepiandrosterone (DHEA) to androstenedione, and androstenediol to testosterone. Type 1 is primarily expressed in the placenta and non-steroidogenic tissues, while type 2 is primarily expressed in the adrenals and gonads (54). The top hits in females were two SNPs located upstream of *ESR1* (rs3407085 and rs2295190), the gene coding for estrogen receptor alpha, one of two main types of estrogen receptor.

It is increasingly recognized that individual variation in complex phenotypes results, in part, from the joint and conditional effects of many common SNPs whose individual effect sizes are relatively small, making them difficult to identify via standard univariate analyses (55, 56). RF analysis represents a promising approach to this problem and is well-suited to high-dimension, low-sample-size data typical of genetic association studies. RF analysis suggested that regulators of reproductive function and genes related to cholesterol production, transport, and removal play an important role in salivary testosterone levels in males. Five SNPs were selected as high importance in both the full and reduced male sample and thus are particularly promising candidates for follow-up. rs4952922, rs10495960, and rs2301267 are all located in/near *LHCGR*, which codes for the LH/choriogonadotropin receptor. rs47340 is located downstream of *TSPO*, which codes for a protein that interacts with StAR (steroidogenic acute regulatory protein) to transport cholesterol into mitochondria to permit steroid synthesis [data on gene functions from www.genecards.org (57), SNP locations from dbSNP (58) and UCSC genome browser (59)]. rs74091680 is an intronic variant in *HSD17B6*, which codes for an enzyme involved in androgen catabolism. Specifically, the oxidoreductase activity can convert 3 alpha-adiol to dihydrotestosterone, while the epimerase activity can convert androsterone to epi-androsterone. Interestingly, two variants near the Xp22 loci identified in GWAS studies of serum testosterone in adult males (24, 25) were selected in the primary RF analysis. This suggests that some genetic factors influencing testosterone levels are active throughout the lifespan, although this finding was not recapitulated in the subsample analysis.

In females, RF analysis suggested that genes related to estrogen signaling play an important role. Specifically, variants in/near *ESR1*, *CYP19A1*, *CYP1B1*, and *HSD17B8* were consistently selected as high importance in the full sample. *ESR1* codes for estrogen receptor alpha, one of two main types of estrogen receptor. *CYP19A1* codes for aromatase, the enzyme responsible for the conversion of androgens into estrogens. *CYP1B1* codes for an enzyme that metabolizes multiple compounds, including 17β-estradiol. *HSD17B8* inactivates estrogens and androgens, with high activity toward estrogen and low activity toward testosterone. It can also synthesize estradiol from estrone. Analyses in the subset of females with transferrin <0.50 mg/dl supported the importance of estrogen signaling, although the specific SNPs implicated differed. Two SNPs were selected as high importance in both the full and reduced female sample; these were rs4149448 (*SULT2A1*) and rs4659175 (*HSD3B2*). *SULT2A1* codes for a protein, which catalyzes the sulfation of steroids and bile acids in the liver and adrenal glands, and may have a role in the inherited adrenal androgen excess in women with polycystic ovary syndrome (60). *HSD3B2* codes for an enzyme with a critical role in the biosynthesis of all classes of steroid hormones.

LIMITATIONS

The primary limitation of the current study is the use of salivary testosterone levels rather than plasma testosterone levels. While plasma and serum are the traditional matrices for the determination of endocrine parameters including testosterone, saliva offers a non-invasive and stress-free alternative, which enjoys widespread acceptance and has been used for over 40 years (61). In men, salivary testosterone is strongly correlated with the free fraction of testosterone in serum ($r = 0.64$–0.97) (51, 62–64). In women, reported correlations are more moderate ($r = 0.37$–0.85) (51, 63, 65). There is no *a priori* reason to presume that correlations between serum and saliva are different in the neonate, although this has not been directly tested. Collection of saliva maximized parental acceptability and minimized child distress. It is unlikely that we would have achieved a reasonable sample size if we opted to use blood. Substitution of saliva assay results for serum values is known to underestimate testosterone–behavior associations, primarily in females, and this problem may also be applicable to the environmental and genetic associations, which were the focus of this study. Null results, especially in females, should be treated with caution.

We also observed higher levels of transferrin in saliva than expected and moderate correlations between transferrin and testosterone in both sexes (stronger in females), which raises the possibility of blood contamination. For comparison, Granger et al. (41), in a study of children between 6 and 13 years of age, reported a mean ± SD for transferrin of 0.37 ± 0.46 mg/dl and a correlation with testosterone of 0.058. Unfortunately, although the transferrin assay (66) has been available for over a decade, it has not been routinely used in studies of infants making it difficult to determine if the levels that we observed are typical for this age or not. Reassuringly, the mean ± SD for salivary testosterone in our study was highly similar to that reported by other studies in our age range (11) and estimated serum levels are comparable to published reports on serum testosterone in this age range (39, 40). We also addressed this issue statistically. In all our primary analyses, we included transferrin as a covariate. In addition, for the environmental association analysis, univariate genetic analyses, and RF analysis, we performed sensitivity analyses in infants with transferrin levels <0.50 mg/dl (cut-off based on the recommendation of Granger et al. (41). In general, relationships identified in the full sample were also present in the subsample with transferrin levels <0.50 mg/dl. Finally, we note that collecting samples on multiple days would have allowed a more precise evaluation of individual differences in testosterone levels, but was deemed impractical. The sample size for twin modeling was also somewhat underpowered.

CONCLUSION

Despite these limitations, the current study provides novel information about the environmental and genetic contributors to testosterone levels in the early post-natal period. In contrast to the strong genetic contributions observed during puberty, transient activation of the HPG axis in the early post-natal period appears to be heavily influenced by environmental factors. We identified 5 min APGAR score as a significant predictor of salivary testosterone levels in males. Further research is needed to elucidate the biological mechanisms underlying this relationship. Our study also suggests that genetic variants in regulators of reproductive function and cholesterol play an important role in salivary testosterone levels in males, while genes related to estrogen signaling play an important role in females. All results require replication, preferably with serially collected serum samples. Ideally, such a study would also include measures of prenatal testosterone in amniotic fluid or through cordocentesis, providing insight into the genetic architecture of the prenatal testosterone surge as well as the neonatal surge.

AUTHOR CONTRIBUTIONS

Kai Xia, Yang Yu, Mihye Ahn, Hongtu Zhu, and Fei Zou made substantial contributions to the analysis and interpretation of data for this project. John H. Gilmore made substantial contributions to the acquisition of data for this project. Rebecca C. Knickmeyer made substantial contributions to the conception and design of the work; as well as the acquisition, analysis, and interpretation of data. Kai Xia, Yang Yu, Mihye Ahn, and Rebecca C. Knickmeyer drafted the manuscript. All coauthors revised the manuscript for important intellectual content, and approved the final version to be published. RK agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fendo.2014.00187/abstract

REFERENCES

1. Forest MG, Cathiard AM. Pattern of plasma testosterone and ^{4}-androstenedione in normal newborns: evidence for testicular activity at birth. *J Clin Endocrinol Metab* (1975) **41**:977–80. doi:10.1210/jcem-41-5-977
2. Winter JS, Faiman C, Hobson WC, Prasad AV, Reyes FI. Pituitary-gonadal relations in infancy. I. Patterns of serum gonadotropin concentrations from birth to four years of age in man and chimpanzee. *J Clin Endocrinol Metab* (1975) **40**(4):545–51. doi:10.1210/jcem-40-4-545
3. Forest MG, de Peretti E, Bertrand J. Testicular and adrenal androgens and their binding to plasma proteins in the perinatal period: developmental patterns of plasma testosterone, 4-androstenedione, dehydroepiandrosterone and its sulfate in premature and small for date infants as compared with that of full-term infants. *J Steroid Biochem* (1980) **12**:25–36.
4. Kulle AE, Riepe FG, Melchior D, Hiort O, Holterhus PM. A novel ultrapressure liquid chromatography tandem mass spectrometry method for the simultaneous determination of androstenedione, testosterone, and dihydrotestosterone in pediatric blood samples: age- and sex-specific reference data. *J Clin Endocrinol Metab* (2010) **95**(5):2399–409. doi:10.1210/jc.2009-1670
5. Kuiri-Hanninen T, Seuri R, Tyrvainen E, Turpeinen U, Hamalainen E, Stenman UH, et al. Increased activity of the hypothalamic-pituitary-testicular axis in

infancy results in increased androgen action in premature boys. *J Clin Endocrinol Metab* (2011) **96**(1):98–105. doi:10.1210/jc.2010-1359
6. Mann DR, Gould KG, Collins DC. Blockade of neonatal activation of the pituitary-testicular axis: effect on peripubertal luteinizing hormone and testosterone secretion and on testicular development in male monkeys. *J Clin Endocrinol Metab* (1989) **68**:600–7. doi:10.1210/jcem-68-3-600
7. Brown GR, Dixson AF. Investigation of the role of postnatal testosterone in the expression of sex differences in behavior in infnats rhesus macaques (*Macaca mulatta*). *Horm Behav* (1999) **35**:186–94. doi:10.1006/hbeh.1999.1512
8. Main KM, Schmidt IM, Skakkebaek NE. A possible role for reproductive hormones in newborn boys: progressive hypogonadism without the postnatal testosterone peak. *J Clin Endocrinol Metab* (2000) **85**(12):4905–7. doi:10.1210/jcem.85.12.7058
9. Sharpe RM, Fraser HM, Brougham MF, McKinnell C, Morris KD, Kelnar CJ, et al. Role of the neonatal period of pituitary-testicular activity in germ cell proliferation and differentiation in the primate testis. *Hum Reprod* (2003) **18**(10):2110–7. doi:10.1093/humrep/deg413
10. Zivkovic D, Fratric I. Disturbances of sperm maturation and minipuberty: is there a connection? *Biomed Res Int* (2014) **2014**:912746. doi:10.1155/2014/912746
11. Alexander GM, Wilcox T, Farmer ME. Hormone-behavior associations in early infancy. *Horm Behav* (2009) **56**(5):498–502. doi:10.1016/j.yhbeh.2009.08.003
12. Alexander GM, Saenz J. Postnatal testosterone levels and temperament in early infancy. *Arch Sex Behav* (2011) **40**(6):1287–92. doi:10.1007/s10508-010-9701-5
13. Lamminmaki A, Hines M, Kuiri-Hanninen T, Kilpelainen L, Dunkel L, Sankilampi U. Testosterone measured in infancy predicts subsequent sex-typed behavior in boys and in girls. *Horm Behav* (2012) **61**(4):611–6. doi:10.1016/j.yhbeh.2012.02.013
14. Saenz J, Alexander GM. Digit ratios (2D:4D), postnatal testosterone and eye contact in toddlers. *Biol Psychol* (2013) **94**(1):106–8. doi:10.1016/j.biopsycho.2013.05.010
15. Saenz J, Alexander GM. Postnatal testosterone levels and disorder relevant behavior in the second year of life. *Biol Psychol* (2013) **94**(1):152–9. doi:10.1016/j.biopsycho.2013.05.011
16. Alexander GM. Postnatal testosterone concentrations and male social development. *Front Endocrinol* (2014) **5**:15. doi:10.3389/fendo.2014.00015
17. Harris JA, Vernon PA, Boomsma DI. The heritability of testosterone: a study of Dutch adolescent twins and their parents. *Behav Genet* (1998) **28**:165–71. doi:10.1023/A:1021466929053
18. Sluyter F, Keijser JM, Boomsma DI, van Doornen LJ, van den Oord EJCG, Snieder H. Genetics of testosterone and the aggression-hostility-anger (AHA) syndrome: a study of middle aged twins. *Twin Res* (2000) **3**:266–76. doi:10.1375/twin.3.4.266
19. Ring HZ, Lessov CN, Reed T, Marcus R, Holloway L, Swan GE, et al. Heritability of plasma sex hormones and hormone binding globulin in adult male twins. *J Clin Endocrinol Metab* (2005) **90**(6):3653–8. doi:10.1210/jc.2004-1025
20. Hoekstra RA, Bartels M, Boomsma DI. Heritability of testosterone levels in 12-year-old twins and its relation to pubertal development. *Twin Res Hum Genet* (2006) **9**(4):558–65. doi:10.1375/twin.9.4.558
21. Kuijper EA, Lambalk CB, Boomsma DI, van der Sluis S, Blankenstein MA, de Geus EJ, et al. Heritability of reproductive hormones in adult male twins. *Hum Reprod* (2007) **22**(8):2153–9. doi:10.1093/humrep/dem145
22. Koenis MM, Brouwer RM, van Baal GC, van Soelen IL, Peper JS, van Leeuwen M, et al. Longitudinal study of hormonal and physical development in young twins. *J Clin Endocrinol Metab* (2013) **98**(3):E518–27. doi:10.1210/jc.2012-3361
23. Harden KP, Kretsch N, Tackett JL, Tucker-Drob EM. Genetic and environmental influences on testosterone in adolescents: evidence for sex differences. *Dev Psychobiol* (2014) **56**(6):1278–89. doi:10.1002/dev.21207
24. Ohlsson C, Wallaschofski H, Lunetta KL, Stolk L, Perry JR, Koster A, et al. Genetic determinants of serum testosterone concentrations in men. *PLoS Genet* (2011) **7**(10):e1002313. doi:10.1371/journal.pgen.1002313
25. Jin G, Sun J, Kim ST, Feng J, Wang Z, Tao S, et al. Genome-wide association study identifies a new locus JMJD1C at 10q21 that may influence serum androgen levels in men. *Hum Mol Genet* (2012) **21**(23):5222–8. doi:10.1093/hmg/dds361
26. Chen Z, Tao S, Gao Y, Zhang J, Hu Y, Mo L, et al. Genome-wide association study of sex hormones, gonadotropins and sex hormone-binding protein in Chinese men. *J Med Genet* (2013) **50**(12):794–801. doi:10.1136/jmedgenet-2013-101705
27. Prescott J, Thompson DJ, Kraft P, Chanock SJ, Audley T, Brown J, et al. Genome-wide association study of circulating estradiol, testosterone, and sex hormone-binding globulin in postmenopausal women. *PLoS One* (2012) **7**(6):e37815. doi:10.1371/journal.pone.0037815
28. Caramaschi D, Booij L, Petitclerc A, Boivin M, Tremblay RE. Genetic and environmental contributions to saliva testosterone levels in male and female infant twins. *Psychoneuroendocrinology* (2012) **37**(12):1954–9. doi:10.1016/j.psyneuen.2012.04.008
29. Forest MG, Sizonenko PC, Cathiard AM, Bertrand J. Hypophyso-gonadal function in humans during the first year of life: I. Evidence for testicular activity in early infancy. *J Clin Invest* (1974) **53**:819–28. doi:10.1172/JCI107621
30. Konforte D, Shea JL, Kyriakopoulou L, Colantonio D, Cohen AH, Shaw J, et al. Complex biological pattern of fertility hormones in children and adolescents: a study of healthy children from the CALIPER cohort and establishment of pediatric reference intervals. *Clin Chem* (2013) **59**(8):1215–27. doi:10.1373/clinchem.2013.204123
31. Bidlingmaier F, Dorr HG, Eisenmenger W, Kuhnle U, Knorr D. Contribution of the adrenal gland to the production of androstenedione and testosterone during the first two years of life. *J Clin Endocrinol Metab* (1986) **62**(2):331–5. doi:10.1210/jcem-62-2-331
32. Prince FP. The triphasic nature of Leydig cell development in humans, and comments on nomenclature. *J Endocrinol* (2001) **168**(2):213–6. doi:10.1677/joe.0.1680213
33. Knickmeyer RC, Woolson S, Hamer RM, Konneker T, Gilmore JH. 2D:4D ratios in the first 2 years of life: stability and relation to testosterone exposure and sensitivity. *Horm Behav* (2011) **60**(3):256–63. doi:10.1016/j.yhbeh.2011.05.009
34. Patterson N, Price AL, Reich D. Population structure and eigenanalysis. *PLoS Genet* (2006) **2**(12):e190. doi:10.1371/journal.pgen.0020190
35. Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, Reich D. Principal components analysis corrects for stratification in genome-wide association studies. *Nat Genet* (2006) **38**(8):904–9. doi:10.1038/ng1847
36. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* (2007) **81**(3):559–75. doi:10.1086/519795
37. Ahn M, Zhang HH, Lu W. Moment-based method for random effects selection in linear mixed models. *Stat Sin* (2012) **22**(4):1539–62. doi:10.5705/ss.2011.054
38. Liaw A, Wiener M. Classification and regression by randomforest. *R News* (2002) **2**(3):18–22. Available from: http://cran.r-project.org/doc/Rnews/Rnews_2002-3.pdf
39. Garagorri JM, Rodriguez G, Lario-Elboj AJ, Olivares JL, Lario-Munoz A, Orden I. Reference levels for 17-hydroxyprogesterone, 11-desoxycortisol, cortisol, testosterone, dehydroepiandrosterone sulfate and androstenedione in infants from birth to six months of age. *Eur J Pediatr* (2007) **167**(6):647–53. doi:10.1007/s00431-007-0565-1
40. Ji C, Huang XW, Yang RW, Wang X, Zhao ZY. Gonadotropins and sex hormones in healthy Chinese infants. *Indian Pediatr* (2008) **45**(6):489–92. Available from: http://indianpediatrics.net/june2008/june-489-492.htm
41. Granger DA, Cicchetti D, Rogosch FA, Hibel LC, Teisl M, Flores E. Blood contamination in children's saliva: prevalence, stability, and impact on the measurement of salivary cortisol, testosterone, and dehydroepiandrosterone. *Psychoneuroendocrinology* (2007) **32**(6):724–33. doi:10.1016/j.psyneuen.2007.05.003
42. Apgar V. A proposal for a new method of evaluation of the newborn infant. *Curr Res Anesth Analg* (1953) **32**(4):260–7. doi:10.1213/00000539-195301000-00041
43. AAP ACOG. The Apgar score. *Pediatrics* (2006) **117**(4):1444–7. doi:10.1542/peds.2006-0325
44. Li J, Cnattingus S, Gissler M, Vestergaard M, Obel C, Ahrensberg J, et al. The 5-minute Apgar score as a predictor of childhood cancer: a population-based cohort study in five million children. *BMJ Open* (2012) **2**(4):e001095. doi:10.1136/bmjopen-2012-001095
45. Velazquez-Moctezuma J, Dominguez Salazar E, Cruz Rueda ML. The effect of prenatal stress on adult sexual behavior in rats depends on the nature of the stressor. *Physiol Behav* (1993) **53**(3):443–8. doi:10.1016/0031-9384(93)90137-5
46. Wang CT, Huang RL, Tai MY, Tsai YF, Peng MT. Dopamine release in the nucleus accumbens during sexual behavior in prenatally stressed adult male rats. *Neurosci Lett* (1995) **200**(1):29–32. doi:10.1016/0304-3940(95)12071-B
47. Rhees RW, Al-Saleh HN, Kinghorn EW, Fleming DE, Lephart ED. Relationship between sexual behavior and sexually dimorphic structures in the anterior hypothalamus in control and prenatally stressed male rats. *Brain Res Bull* (1999) **50**(3):193–9. doi:10.1016/S0361-9230(99)00191-4

48. Ward IL, Ward OB, Hayden T, Weisz J, Orth JM. Naltrexone normalizes the suppression but not the surge of delta 5-3 beta-hydroxysteroid dehydrogenase activity in Leydig cells of stressed rat fetuses. *Endocrinology* (1990) **127**(1):88–92. doi:10.1210/endo-127-1-88
49. Ward IL, Ward OB, Affuso JD, Long WD III, French JA, Hendricks SE. Fetal testosterone surge: specific modulations induced in male rats by maternal stress and/or alcohol consumption. *Horm Behav* (2003) **43**(5):531–9. doi:10.1016/S0018-506X(03)00061-8
50. Pallares ME, Adrover E, Baier CJ, Bourguignon NS, Monteleone MC, Brocco MA, et al. Prenatal maternal restraint stress exposure alters the reproductive hormone profile and testis development of the rat male offspring. *Stress* (2013) **16**(4):429–40. doi:10.3109/10253890.2012.761195
51. Shirtcliff EA, Granger DA, Likos A. Gender differences in the validity of testosterone measured in saliva by immunoassay. *Horm Behav* (2002) **42**(1):62–9. doi:10.1006/hbeh.2002.1798
52. Visscher PM, Hill WG, Wray NR. Heritability in the genomics era – concepts and misconceptions. *Nat Rev Genet* (2008) **9**(4):255–66. doi:10.1038/nrg2322
53. Johnson W, Penke L, Spinath FM. Heritability in the era of molecular genetics: some thoughts for understanding genetic influences on behavioral traits. *Eur J Pers* (2011) **25**(4):254–66. doi:10.1002/per.834
54. Miller WL, Auchus RJ. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocr Rev* (2011) **32**(1):81–151. doi:10.1210/er.2010-0013
55. Yang JA, Benyamin B, McEvoy BP, Gordon S, Henders AK, Nyholt DR, et al. Common SNPs explain a large proportion of the heritability for human height. *Nat Genet* (2010) **42**(7):565–U131. doi:10.1038/ng.608
56. Visscher PM, Goddard ME, Derks EM, Wray NR. Evidence-based psychiatric genetics, AKA the false dichotomy between common and rare variant hypotheses. *Mol Psychiatry* (2012) **17**(5):474–85. doi:10.1038/mp.2011.65
57. Safran M, Chalifa-Caspi V, Shmueli O, Olender T, Lapidot M, Rosen N, et al. Human gene-centric databases at the weizmann institute of science: genecards, UDB, CroW 21 and HORDE. *Nucleic Acids Res* (2003) **31**(1):142–6. doi:10.1093/nar/gkg050
58. Sherry ST, Ward MH, Kholodov M, Baker J, Phan L, Smigielski EM, et al. dbSNP: the NCBI database of genetic variation. *Nucleic Acids Res* (2001) **29**(1):308–11. doi:10.1093/nar/29.1.308
59. Karolchik DM, Barber GP, Clawson H, Cline MS, Diekhans M, Dreszer TR, et al. The UCSC Genome Browser database: 2014 update. *Nucelic Acids Res* (2014) **42**:D764–70. doi:10.1093/nar/gkt1168
60. Goodarzi MO, Antoine HJ, Azziz R. Genes for enzymes regulating dehydroepiandrosterone sulfonation are associated with levels of dehydroepiandrosterone sulfate in polycystic ovary syndrome. *J Clin Endocrinol Metab* (2007) **92**(7):2659–64. doi:10.1210/jc.2006-2600
61. Groschl M. Current status of salivary hormone analysis. *Clin Chem* (2008) **54**(11):1759–69. doi:10.1373/clinchem.2008.108910
62. Baxendale PM, Reed MJ, James VHT. Testosterone in saliva of normal men and its relationship with unbound and total testosterone levels in plasma. *J Endocrinol* (1980) **4**:46–7.
63. Vittek J, L'Hommedieu DG, Gordon GG, Rappaport SC, Southren AL. Direct radioimmunoassay (RIA) of salivary testosterone: correlation with free and total serum testosterone. *Life Sci* (1985) **37**(8):711–6. doi:10.1016/0024-3205(85)90540-5
64. Navarro MA, Juan L, Bonnin MR, Villabona C. Salivary testosterone: relationship to total and free testosterone in serum. *Clin Chem* (1986) **32**(1 Pt 1):231–2.
65. Magrini G, Chiodoni G, Rey F, Felber JP. Further evidence for the usefulness of the salivary testosterone radioimmunoassay in the assessment of androgenicity in man in basal and stimulated conditions. *Horm Res* (1986) **23**(2):65–73. doi:10.1159/000180291
66. Schwartz EB, Granger DA. Transferrin enzyme immunoassay for quantitative monitoring of blood contamination in saliva. *Clin Chem* (2004) **50**(3):654–6. doi:10.1373/clinchem.2003.028266

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