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Maternal first trimester serum levels of free-beta human chorionic gonadotropin and male genital anomalies

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Running title: Maternal levels of hCG and male genital anomalies

Abstract

Study question: Are maternal first trimester levels of serum free-beta hCG associated with the development of hypospadias or undescended testis (UDT) in boys?

Summary answer: Overall, first trimester maternal levels of serum free-beta hCG are not associated with hypospadias or UDT. However, elevated levels were found in severe phenotypes (proximal hypospadias and bilateral UDT) suggesting an altered pathway of hormonal release in early pregnancy.

What is known already: Human chorionic gonadotropin peaks in first trimester of pregnancy stimulating fetal testosterone production which is key to normal male genital development. Endocrine-disrupting insults early in pregnancy have been associated with increased risk of common genital anomalies in males such as hypospadias and UDT. One plausible etiological pathway is altered release of hCG.

Study design, size, duration: We conducted a record-linkage study of two separate populations of women attending first trimester aneuploidy screening in two Australian states, New South Wales (NSW) and Western Australia (WA), in 2006 - 2009; and 2001 - 2003, respectively.

Participants/materials, setting, methods: Included were women who gave birth to a singleton live-born male infant. There were 12,099 boys from NSW and 10,518 from WA included, of whom 90 and 77 had hypospadias; and 107 and 109 UDT, respectively. Serum levels of free-beta hCG were ascertained from laboratory databases and combined with relevant birth outcomes and congenital anomalies via record linkage of laboratory, birth, congenital anomalies and hospital data. Median and quartile levels of gestational age specific free-beta hCG multiple of the median (MoM) were compared between affected and unaffected boys. Logistic regression was used to evaluate the association between levels of free-beta hCG MoM and hypospadias or UDT, stratified by suspected placental dysfunction and co-existing anomalies. Where relevant, pooled analysis was conducted.

Main results and the role of chance: There was no difference in median hCG levels amongst women with an infant with hypospadias (NSW=0.88 MoM, p=0.83; WA=0.84 MoM, p=0.76) or UDT

(NSW=0.89 MoM, p=0.54; WA=0.95 MoM, p=0.95), compared with women with an unaffected boy (NSW=0.92 MoM; WA=0.88 MoM). Low (<25th centile) or high (>75th centile) hCG levels were not associated with hypospadias or UDT, nor when stratifying by suspected placental dysfunction and co-existing anomalies. However, there was a tendency towards high levels for severe types, although confidence intervals were wide. When combining NSW and WA results, high hCG MoM levels (>75th centile) were associated with increased risk of proximal hypospadias (odds ratio (OR) 4.34; 95%CI: 1.08-17.4) and bilateral UDT (OR 2.86; 95%CI: 1.02-8.03).

Limitations, reasons for caution: There were only small numbers of proximal hypospadias and bilateral UDT in both cohorts and although we conducted pooled analyses, results reported on these should be interpreted with caution. Gestational age by ultrasound may have been inaccurately estimated in small and large for gestational age fetuses affecting hCG MoM calculation in those pregnancies. Despite the reliability of our datasets in identifying adverse pregnancy outcomes, we did not have pathology information to confirm tissue lesions in the placenta and therefore our composite outcome should be considered as a proxy for placental dysfunction.

Wider implications of the findings: This is one of the largest population-based studies examining the association between maternal first trimester serum levels of free-beta hCG and genital anomalies - hypospadias and UDT; and the first to compare specific phenotypes by severity. Overall, our findings does not support the hypothesis that alteration in maternal hCG levels is associated with the development of male genital anomalies, however, high hCG free-beta levels found in severe types suggests different underlying aetiology involving higher production and secretion of hCG. These findings require further exploration and replication.

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Trial registration number: Not applicable

Key words: human chorionic gonadotropin, hCG, first trimester pregnancy, first trimester screening, male genital anomalies, hypospadias, undescended testis, cryptorchidism

Introduction

Hypospadias (where the urethral opening develops on the ventral aspect of the penis, scrotum or perineum) and undescended testis (UDT; the absence of one or both testes from the scrotum) are the most common genital anomalies in males (Paulozzi, 1999)**.** Both anomalies require surgical repair during childhood and have been associated with long-term adverse functional, cosmetic and fertility outcomes (Lee and Coughlin, 2001); and with increased risk of malignancy in adulthood (Schnack, et al., 2010). The development of the male genital tract is highly dependent on testosterone production by Leydig cells and the function of their receptors (Blaschko, et al., 2012). The placental hormone human chorionic gonadotropin (hCG), which peaks in production during early gestation, is secreted into both the maternal and foetal circulations, and has important roles in both pregnancy maintenance and testosterone production in the foetal testis (Molsberry, et al., 1982, Scott, et al., 2009). The peak in production during early gestation is critical for the normal fusion of the urethral fold. Thus, alteration in foetal hCG concentrations in early pregnancy might disrupt foetal testosterone production and result in abnormal gonadal development. Animal studies suggest that androgen blockage with flutamide occurring during the masculinization programming window between 8-14 weeks gestation results in the development of hypospadias and UDT (Welsh, et al., 2008). Human studies have also reported an association between prenatal exposures to endocrine disruptive chemicals and male genital anomalies (Kalfa, et al., 2015, Koskenniemi, et al., 2015). hCG is also presumed to play a key role in optimal placental function with studies suggesting altered release in pregnancies affected by adverse outcomes such as preeclampsia and fetal growth restriction (Krantz, et al., 2004, Norris, et al., 2011). Adverse pregnancy outcomes have also been associated with hypospadias and UDT, implicating a possible role of an altered release of hCG to the developing fetus (Aschim, et al., 2004, Biggs, et al., 2002, Fujimoto, et al., 2008).

Limited studies have assessed the association between maternal levels of hCG in early pregnancy with subsequent development of hypospadias and/or UDT, although results have been inconclusive. This in in part due to small numbers (Bernstein, et al., 1988, Burton, et al., 1987, Chedane, et al., 2014, Kiely, et al., 1995) and heterogeneous clinical settings including different pathophysiological pathways and

phenotype severity such as the presence of other congenital anomalies (Brouwers, et al., 2010) or adverse pregnancy outcomes, indicative of poor placental development or function (Krantz, et al., 2004, Norris, et al., 2011).

The aim of the study was to investigate the association between maternal first trimester levels of serum hCG with hypospadias and UDT, and assess whether hCG levels are associated with specific phenotypes.

Materials and Methods

Ethics approval

Ethics approval for access and linkage of data was obtained from the NSW Population and Health Services Research Ethics Committee and the Department of Health WA Human Research Ethics Committee.

Study population and laboratory information

We conducted a record-linkage study of two separate populations of women attending first trimester screening in two Australian states, New South Wales (NSW) and Western Australia (WA), between July 2006 and December 2009; and between August 2001 and October 2003, respectively. Included were women who gave birth to a singleton live-born male infant. Information on free-beta hCG levels was collected as part of the Down syndrome serum screening test and were ascertained from the Pacific Laboratory Medicine Services (PaLMS) database in NSW and from laboratory databases accredited by the Fetal Medicine Foundation in WA. An Immulite 2000 assay system (Siemens Healthcare Diagnostics, Deerfield, IL, USA) was employed to measure free-beta hCG levels and the intra and inter assay variability was 6-8% and 8-10%, respectively. Laboratory data included free-beta hCG expressed as multiple of the medians (MoM) which accounts for differences in free-beta hCG by

gestational week at testing and maternal weight (Wald, et al., 1992). Gestational week at the time of hCG testing was recorded in laboratory data and assessed by ultrasound using the crown-rump length.

Health data sources

Maternal information, pregnancy and male infant outcomes in NSW were obtained via record linkage of information from the PaLMS laboratory database to the NSW Perinatal Data Collection (PDC) and Admitted Patient Data Collection (APDC). The PDC is a statutory surveillance system of all live births and stillbirths in NSW of at least 20 weeks gestation or 400 g birth weight. It includes information on maternal demographic information, pregnancy conditions, birth factors, and infant outcomes. The APDC is a census of all in-patient hospital admissions from NSW public and private hospitals which collects demographic and clinical information, with records for both mothers and live-born infants. All diagnoses and procedures for each admission are coded according to the 10th revision of the International Classification of Diseases, Australian Modification (ICD10-AM) and the Australian Classification of Health Interventions (ACHI), respectively. Demographic and health information in WA was obtained via record linkage of laboratory data to the Western Australian Midwives Notification System (MNS) and the Western Australia Registry of Developmental Anomalies (WARDA). The MNS is a routinely collected database of all births in WA, including similar information to the PDC from NSW. The WARDA is a population-based notification system of anomalies diagnosed in children up to 6 years of age (Western Australian Register of Developmental Anomalies, 2016). Congenital anomalies are coded according to the British Paediatric Association International Classification of Diseases, 9th revision system (BPA-ICD9). Record linkages were conducted separately for each state by the NSW Centre for Health Record Linkage and by Data Linkage Western Australia independent of the research.

Study outcomes and explanatory variables

Study outcomes were defined as any male infant with a recorded diagnosis of hypospadias or UDT requiring corrective surgery; and identified from relevant infant hospital admissions in NSW and

WARDA records in WA. Boys without a relevant recorded diagnosis were considered as the unaffected comparison group. Hypospadias cases were identified from the APDC using the ICD-10-AM code Q54 and from WARDA using the BPA-ICD9 code 7526. Cases were categorized by severity according to the classification by Duckett,1996 (Duckett, 1996) into four phenotypical types: 1) Anterior: which included balanic (Q54.0) or glanular hypospadias (7526.3); 2) Middle: penile (Q54.1) or subcoronal hypospadias (7526.4), 3) Proximal: penoscrotal (Q54.2; 7526.5) and perineal hypospadias (Q54.3; 7526.8) and 4) Unspecified: other (Q54.8) or unspecified hypospadias (Q54.9, 7526.0, 7526.6 and 7526.9). For those boys with more than one recorded hypospadias diagnosis, the most severe was used. Boys with UDT were identified from the APDC if they had a relevant ICD-10-AM code Q53 and underwent corrective surgery; either an orchidopexy (ACHI codes 37803, 37804, 37806 and 37812) or an orchidectomy (30641). Boys with UDT were identified from WARDA using the BPA-ICD9 code 7525. Only those UDT cases requiring corrective surgery are notified and recorded in the WARDA. All UDT cases were categorized by phenotypical type characterized by severity into unilateral or bilateral UDT according to the relevant diagnosis codes. Boys diagnosed with co-existing anomalies, excluding minor anomalies such as tongue-tie, naevus, skin tags, unstable hip and feet defects, were also differentiated from isolated cases. We excluded 16 boys from NSW and 13 from WA with recorded diagnosis of chromosomal anomalies as these are more likely to have abnormal levels of hCG.

The key explanatory variable for this study was first trimester free-beta hCG serum levels expressed as multiples of the median (MoM) of the total population of women attending first trimester screening in each state. Due to non-normality of free-beta hCG MoM distribution, these were expressed as medians and also categorized by quartile cut points at the 25^{th}, 50^{th} and 75^{th} centiles. Covariates included in the analysis were maternal age, weight (kilograms) ascertained at the time of first trimester screening, parity (nulliparae or multiparae), smoking during pregnancy and adverse pregnancy outcomes considered as potential markers of placental dysfunction (Odibo, et al., 2014, Vinnars, et al., 2014). A

composite variable of markers of placental dysfunction was developed comprising the occurrence of preterm birth (<37 weeks gestation), small for gestational age (SGA) defined as birth weight <10th percentile of the nationwide distribution of birth weight by gestational age and infant sex (Dobbins, et al., 2012) or preeclampsia. Gestational age at birth was reported in the PDC data in completed weeks of gestation and determined by the best clinical estimate, including early ultrasound (97%) and last menstrual period. Preeclampsia was determined either by the relevant box being checked in the PDC or MNS record, or if any maternal APDC record had a diagnosis of preeclampsia (ICD-10-AM: O11 and O14) or eclampsia (O15). As maternal weight was missing in 1,736 (14.3%) and 998 (9.5%) of the NSW and WA records, respectively, multiple imputation was applied to predict missing values using existing values from other variables (Schafer and Olsen, 1998). There were 5 (0.5%) women from WA with missing hCG information and these were excluded from the analysis.

Statistical analysis

Overall median and interquartile range (IQR) for 25th and 75th percentile cut points of free-beta hCG MoM levels by maternal characteristics and infant study outcomes were examined and differences assessed using the Kruskal-Wallis test. The characteristics of boys with hypospadias and UDT were compared with unaffected boys using Chi-squared test. Wilcoxon rank sum test was used to assess the median difference in maternal serum levels of free-beta hCG MoM between boys with hypospadias or UDT and unaffected boys. Differences in free-beta hCG MoM levels by phenotypical types of hypospadias and UDT, isolated versus co-existing congenital anomalies and women with suspected placental dysfunction versus those without were also assessed. Multivariable logistic regression analysis was performed to examine the association between low (<25th centile) and high (>75th centile) serum levels of free-beta hCG MoM with hypospadias and UDT, with the reference being levels between the 25th and 75th centiles. Potential confounder variables were only retained in the models if they were significant at $p<0.05$ and changed the association estimates by 10% or more. Secondary analyses were

also conducted by restricting to severe phenotypes of proximal hypospadias or bilateral UDT, those with co-existing congenital anomalies or women with suspected placental dysfunction. Where relevant, pooled odds ratio and corresponding 95% confidence intervals were calculated to combine results from NSW and WA. P-value <0.05 was considered statistically significant and all analyses were performed using SAS, 9.4 (SAS Institute, Cary, NC, USA).

Results

Population characteristics

A total of 12,099 and 10,518 boys in NSW and WA, respectively, whose mother underwent first trimester screening and with recorded serum free-beta hCG levels were included. The majority of samples were collected between 10 and 13 weeks gestation (NSW, n=10,484, 87%; WA, n=10,465, 99.5%). There were 90 (0.7%) and 77 (0.7%) male infants with a recorded diagnosis of hypospadias; while 107 (0.9%) and 109 (1.0%) had UDT in NSW and WA, respectively. Of those with hypospadias, 42 (46.7%), 18 (20%), 6 (6.7%) and 24 (26.7%) in NSW and 43 (55.8%), 27 (35.1%), 3 (3.9%) and 4 (5.2%) in WA had recorded diagnosis of anterior, middle, proximal and unspecified hypospadias, respectively. There were 97 boys (90.7%) in NSW and 102 (93.6%) in WA with a recorded diagnosis of unilateral; and 10 boys (9.3%) and 7 (6.4%) with bilateral UDT, respectively. **Table I** compares the maternal characteristics and infant outcomes for cases and unaffected boys. There was no overall difference in maternal age and smoking between cases and unaffected boys in either NSW or WA, although, higher maternal weight and multiparity was associated with increased rates of UDT in NSW but not in WA. Boys with hypospadias and UDT were more likely to be born to mothers with suspected placental dysfunction and to have co-existing congenital anomalies in both populations (**Table I**).

Maternal free-beta hCG and genital anomalies

Table II presents maternal free-beta hCG levels by maternal characteristics and by phenotypical type of hypospadias and UDT. Median (IQR) levels of free-beta hCG MoM in NSW and WA decreased with increasing maternal weight; and differed by parity and smoking status, while levels were not affected by maternal age in either population. The association between free-beta hCG levels with hypospadias and UDT is presented in **Table III** for NSW and **Table IV** for WA. There were no differences in median free-beta hCG MoM for mothers with boys with recoded diagnosis of hypospadias or UDT versus unaffected boys. Results did not change restricting analyses to those with co-existing congenital anomalies or suspected placental dysfunction and there was no association between low (<25th centile) or high (>75th centile) maternal serum free-beta hCG MoM levels for these factors and hypospadias or UDT.

When comparing results for women having boys with milder hypospadias types in NSW (n=84; median 0.87 MoM; IQR: 0.67-1.27) and in WA (n=74; median 0.93 MoM; IQR: 0.62-1.30), free-beta hCG MoM levels among women who had boys with proximal hypospadias were higher (NSW: n=6; median 1.38 MoM IQR: 0.90-1.51; p=0.21; WA: n=3; median 1.36 MoM IQR: 0.56-4.55; p=0.34). Similar results were obtained comparing unilateral with bilateral UDT (NSW: n=10; median 1.17 MoM; IQR: 0.71-1.75; p=0.43; WA: n=7; median 1.18, IQR: 0.33-1.79; p=0.77). Compared with milder types of hypospadias and unilateral UDT, high free-beta hCG MoM (>75th centile) was associated with proximal hypospadias (NSW: odds ratio (OR) 3.38; 95%CI: 0.52-21.8; WA: OR 7.25; 95%CI: 0.62-85.2) and bilateral UDT (NSW: OR 2.41; 95%CI: 0.55-10.5; WA: OR 2.31; 95%CI: 0.48-11.0). An analysis pooling results for NSW and WA revealed that mothers giving birth to boys with severe types of hypospadias or bilateral UDT were more likely to have higher levels of free-beta hCG MoM in early pregnancy (proximal hypospadias: OR 4.34; 95%CI: 1.08-17.4 and bilateral UDT: OR 2.86; 95%CI: 1.02-8.03). Although numbers of cases were small, confidence intervals excluded unity.

Discussion

This is one of the largest population-based studies examining the association between maternal first trimester serum levels of free-beta hCG and genital anomalies, hypospadias and UDT. Overall, we found that maternal levels of free-beta hCG were not associated with subsequent development of hypospadias or UDT among male infants. However, levels of free-beta hCG tended to be higher among women who had boys with more the severe phenotypical types of proximal hypospadias or bilateral UDT.

There have been four previous studies examining the association between maternal levels of free-beta hCG and hypospadias or UDT and findings are summarized in **Table V**. Our results are consistent with three studies that reported no difference in first trimester maternal levels of free-beta hCG with hypospadias or UDT, compared to controls (Bernstein, et al., 1988, Burton, et al., 1987, Kiely, et al., 1995). In contrast, a recent study found lower levels of free-beta hCG MoM in women who had a boy with UDT (Chedane, et al., 2014), however, the differences reported were small. First trimester maternal levels of free-beta hCG are currently used for screening to detect Down syndrome fetuses and the reported differences in free-beta hCG levels between Down syndrome and controls are considerably larger (cases: median free-beta hCG MoM 1.70 versus controls: median 1.01; $P < 0.0001$) (Koster, et al., 2011). Differences in hCG MoM levels were not evident in our overall findings for hypospadias and UDT. Our results suggest that the majority of these genital anomalies develop in the setting of normal maternal serum hCG levels. Perhaps these cases are associated with altered expression of the androgen receptor in gonadal fetal tissue (Drabik, et al., 2015) or altered secretion or metabolism of foetal hCG that is not reflected by maternal hCG levels.

To our knowledge this is the first study comparing maternal levels of free-beta hCG in specific phenotypical types of hypospadias or UDT. We did find that for boys with severe types, their mothers had higher levels of free-beta hCG but due to small sample size, our results were imprecise. In a previous study including women with severe placental dysfunction and intrauterine growth restriction (n=30), 62% had boys with proximal hypospadias, and authors reported a high mean hCG of 2.5 MoM (Yinon, et al.,

2010). Although the study did not include a comparative control group, their mean levels are consistent with levels of hCG above the 95th percentile reported in population-based studies (Krantz, et al., 2004). These results suggests that severe hypospadias phenotypes may have different underlying aetiology involving higher production and secretion of hCG, similar to that which occurs in Down syndrome pregnancies (Eldar-Geva, et al., 1995). Additionally, high gonadotropins may be associated with bilateral UDT due to deficient androgenic negative feedback, representing an affected hypothalamic-pituitary axis (Thorup, et al., 2012) or disrupted hCG receptors in the fetal testis (Lei, et al., 2001). Limited androgen stimulation may also be a possible factor for the nearly three quarters of severe idiopathic hypospadias cases occurring in the absence of genetic alterations (Boehmer, et al., 2001). However, we had no information in our data to suggest a reduced fetal testosterone secretion in proximal hypospadias or bilateral UDT, nor of the ability of fetal testosterone to inhibit placental hCG secretion. Indeed, placental aromatase might be expected to inactivate fetal testosterone as it enters trophoblast cells (Pasqualini, 2005). Further research is needed to explore the consistency of these findings and potential diagnostic accuracy of maternal hCG in detecting proximal hypospadias or bilateral UDT.

The main strength of this study was the use of large record-linked population-based cohorts of women and boys across NSW and WA. The health datasets used are accurate and reliable with high agreement with medical records (Lain, et al., 2012). Furthermore, our rates of hypospadias and UDT were similar and consistent with population estimates in NSW and WA (Nassar, et al., 2007, Schneuer, et al., 2015). One limitation of the study was the small numbers of proximal hypospadias and bilateral UDT in both cohorts. Although we conducted pooled analyses, results reported on these should be interpreted with caution. Another limitation was that since we assessed gestational age by ultrasound, the hCG concentrations from mothers with fetuses who were small or large for gestational age may have been inaccurately adjusted in MoM calculations. Despite the reliability of our datasets in identifying adverse pregnancy outcomes, we did not have pathology information to confirm tissue lesions in the placenta and therefore our composite outcome should be considered as a proxy for placental dysfunction. Moreover,

we could not differentiate between congenital and acquired UDT cases due to poor reporting of UDT at birth in our data. Congenital and acquired UDT may also have different etiology, although, the proportion of acquired cases in both study cohorts is likely to be low due to limited follow-up information.

Conclusions

First trimester maternal serum levels of free-beta hCG are not associated with subsequent development of hypospadias or UDT. However, high maternal levels of free-beta hCG were found in more severe types of male genital anomalies and suggest an altered pathway of hormonal metabolism in early pregnancy. These findings require further exploration and replication.

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Author's role

J.A.M. conceived the study. C.B. and N.N. initiated and designed the study. V.T. provided approval to access NSW data, performed and supervised the laboratory analysis. F.J.S. and N.N. analyzed the data. A.J.A.H., L.L., J.A.M., A.B., V.T., C.B. and S.E.J. provided clinical and epidemiological advice. F.J.S. and N.N. wrote the manuscript with input from the other authors. All authors reviewed and provided feed-back on the draft manuscript and approved the final manuscript as submitted.

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Conflicts of interests

None declared

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Table I: Maternal characteristics and outcomes of cases of hypospadias and undescended testis (UDT) compared with unaffected boys in New South Wales (NSW) and Western Australia (WA)

	NSW			WA		
Maternal Characteristics	**Hypospadias N=90**	**UDT N=107**	**Unaffected N=11,904**	**Hypospadias N=77**	**UDT N=109**	**Unaffected N=10,333**
Age (years)						
<25	7 (7.8)	7 (6.5)	691 (5.8)	9 (11.7)	12 (11.0)	1,043 (10.1)
25 - 35	48 (53.4)	59 (55.1)	7,014 (58.9)	49 (63.6)	70 (64.2)	6,845 (66.2)
35+	35 (38.9)	41 (38.3)	4,198 (35.3)	19 (24.7)	27 (24.8)	2,445 (23.7)
Weight (kg)						
<55	12 (15.2)	9 (9.6)*	1,826 (17.9)	9 (13.0)	10 (10.5)	1,274 (13.6)
55 - 75	46 (58.2)	59 (62.8)*	6,316 (62.0)	44 (63.8)	71 (74.7)	6,038 (64.5)
75+	21 (26.6)	26 (27.7)*	2,050 (20.1)	16 (23.2)	14 (14.7)	2,045 (21.9)
Parity						
Nulliparae	48 (53.3)	38 (35.5)*	5,489 (46.1)	22 (28.6)	28 (25.7)	3315 (32.1)
Multipara	42 (46.7)	69 (64.5)*	6,415 (53.9)	55 (71.4)	81 (74.3)	7018 (67.9)
Smoking during pregnancy	8 (8.9)	9 (8.4)	686 (5.8)	7 (9.1)	12 (11.0)	1,132 (11.0)
Suspected placental dysfunction[a]	34 (37.7)*	30 (28.0)*	1,681 (14.1)	22 (28.6)*	23 (21.1)	1,531 (14.8)
Congenital anomalies						
Isolated	44 (48.9)	83 (77.6)	NA	29 (37.7)	98 (90.0)	NA
Coexisting anomalies	46 (51.1)*	24 (22.4)*	533 (4.5)	48 (62.3)*	11 (10.0)*	309 (3.0)

* $p<0.05$; [a] Includes preterm birth (<37 weeks gestation), small for gestational age (<10th centile) or preeclampsia; -NA: not applicable

Table II: Serum hCG multiple of median levels by maternal characteristics and phenotypical types in women who had first trimester Down syndrome screening in New South Wales (NSW) and Western Australia (WA)

	NSW				WA			
Characteristics	**n (%)**	**25th**	**50th**	**75th**	**n (%)**	**25th**	**50th**	**75th**
All women (N)	12,099	0.63	0.92	1.38	10,518	0.59	0.88	1.30
Maternal characteristics								
Maternal age (years)			p=0.42				p=0.82	
<25	705 (5.8)	0.60	0.92	1.37	1,064 (10.1)	0.61	0.89	1.34
25 - 35	7,120 (58.8)	0.63	0.92	1.37	6,968 (66.2)	0.60	0.87	1.29
35+	4,273 (35.3)	0.64	0.93	1.39	2,491 (23.7)	0.59	0.88	1.31
Maternal weight (kg)			p<0.001				p<0.001	
<55	1,847 (15.3)	0.67	0.97	1.49	1,294 (13.6)	0.64	0.94	1.38
55 - 75	6,420 (53.1)	0.63	0.92	1.36	6,155 (64.6)	0.60	0.88	1.28
75+	2,096 (17.3)	0.59	0.89	1.35	2,076 (21.8)	0.57	0.85	1.27
Parity			p<0.001				p<0.01	
Nulliparae	5,575 (46.1)	0.64	0.95	1.41	3,366 (32.0)	0.61	0.91	1.31
Multipara	6,524 (53.9)	0.62	0.90	1.35	7,157 (68.0)	0.59	0.86	1.29
Smoking			p=0.04				p<0.01	
Yes	703 (5.8)	0.57	0.88	1.37	1,151 (10.9)	0.54	0.85	1.30
No	11,396 (94.2)	0.63	0.92	1.38	9,372 (89.1)	0.60	0.88	1.30
Phenotypical type								
Hypospadias			p=0.34*				p=0.21*	
Anterior	42 (46.7)	0.66	0.89	1.19	43 (55.8)	0.68	0.90	1.28
Middle	18 (20.0)	0.71	0.91	1.44	27 (35.1)	0.63	0.73	1.29
Proximal	6 (6.7)	0.90	1.38	1.51	3 (3.9)	0.56	1.36	4.55
Unspecified	24 (26.7)	0.64	0.81	1.19	4 (5.2)	0.35	0.56	0.84
UDT			p=0.43				p=0.77	
Unilateral	97 (90.7)	0.65	0.85	1.31	102 (93.6)	0.62	0.93	1.30
Bilateral	10 (9.3)	0.71	1.17	1.75	7 (6.4)	0.33	1.18	1.79

* Comparison between proximal hypospadias and other types

Table III: Univariate association between serum first trimester free-beta hCG multiple of median (MoM) levels with hypospadias and undescended testis (UDT) in New South Wales (NSW)

Serum hCG MoM	Hypospadias N=90 n (%)	Odds ratio[#] (95% CI)	UDT N=107 n (%)	Odds ratio[#] (95% CI)	Unaffected N=11,904 n (%)
All cases - Median (IQR)	0.88 (0.66-1.40)	p=0.83	0.89 (0.65-1.35)	p=0.54	0.92 (0.63-1.38)
<25th centile (<0.63)	20 (22.2)	0.87 (0.52, 1.48)	24 (22.4)	0.86 (0.54, 1.39)	2,934 (24.7)
25-75 centile (0.63-1.38)	47 (52.2)	1.0 (Ref)	57 (53.3)	1.0 (Ref)	6,020 (50.6)
>75th centile (>1.38)	23 (25.6)	1.00 (0.61, 1.65)	26 (24.3)	0.93 (0.58, 1.48)	2,949 (24.8)
Suspected placental dysfunction[*]					
Yes - Median (IQR)	0.97 (0.58-1.41)	p=0.94	0.95 (0.70-1.40)	p=0.99	0.93 (0.63-1.38)
<25th centile (<0.63)	10 (29.4)	1.60 (0.70-3.66)	7 (23.3)	0.91 (0.43-1.92)	415 (24.7)
25-75 centile (0.63-1.38)	14 (41.2)	1.0 (Ref)	15 (50.0)	1.0 (Ref)	852 (50.7)
>75th centile (>1.38)	10 (29.4)	1.57 (0.68-3.60)	8 (26.7)	1.09 (0.54-2.22)	414 (24.6)
No - Median (IQR)	0.85 (0.69-1.26)	p=0.74	0.84 (0.65-1.33)	p=0.41	0.92 (0.63-1.38)
<25th centile (<0.63)	10 (17.9)	0.62 (0.30-1.26)	17 (22.1)	0.84 (0.45-1.56)	2,515 (24.6)
25-75 centile (0.63-1.38)	33 (58.9)	1.0 (Ref)	42 (54.5)	1.0 (Ref)	5,183 (50.6)
>75th centile (>1.38)	13 (23.2)	0.81 (0.43-1.55)	18 (23.4)	0.85 (0.45-1.58)	2,535 (24.8)
Co-existing congenital anomalies					
Yes - Median (IQR)	0.92 (0.69-1.40)	p=0.24	0.94 (0.70-1.51)	p=0.50	0.85 (0.57-1.31)
<25th centile (<0.63)	10 (21.7)	0.62 (0.29, 1.34)	5 (20.8)	0.62 (0.22, 1.80)	164 (30.8)
25-75 centile (0.63-1.38)	24 (52.2)	1.0 (Ref)	12 (50.0)	1.0 (Ref)	245 (46.0)
>75th centile (>1.38)	12 (26.1)	0.99 (0.45, 2.04)	7 (29.2)	1.15 (0.44, 3.00)	124 (23.3)
No - Median (IQR)	0.80 (0.60 - 1.08)	p=0.46	0.85 (0.65-1.33)	p=0.38	0.93 (0.63-1.38)
<25th centile (<0.63)	10 (22.7)	0.91 (0.43-1.91)	19 (22.9)	0.85 (0.50-1.45)	2,770 (24.4)
25-75 centile (0.63-1.38)	23 (52.3)	1.0 (Ref)	45 (54.2)	1.0 (Ref)	5,775 (50.8)
>75th centile (>1.38)	11 (25.0)	0.96 (0.47-1.97)	19 (22.9)	0.87 (0.51-1.49)	2,825 (24.9)

[#] No relevant confounder variables were retained in the models

[*] Including preterm birth (<37 weeks gestation), small for gestational age (<10th centile) or preeclampsia; CI: confidence interval; IQR: interquartile range

Table IV: Univariate association between serum first trimester free-beta hCG multiple of median (MoM) levels with hypospadias and undescended testis (UDT) in Western Australia (WA)

Serum hCG MoM	Hypospadias N=77 n (%)	Odds ratio[#] (95% CI)	UDT N=109 n (%)	Odds ratio[#] (95% CI)	Unaffected N=10,338 n (%)
All cases - Median (IQR)	0.84 (0.63-1.28)	p=0.76	0.95 (0.62-1.32)	p=0.38	0.88 (0.59-1.30)
<25th centile (<0.59)	17 (22.1)	0.84 (0.48-1.47)	24 (22.0)	0.87 (0.54-1.40)	2,538 (24.6)
25-75 centile (0.59-1.30)	42 (54.6)	1.0 (Ref)	57 (52.3)	1.0 (Ref)	5,232 (50.6)
>75th centile (>1.30)	18 (23.4)	0.88 (0.50-1.52)	28 (25.7)	1.00 (0.63-1.58)	2,563 (24.8)
Suspected placental dysfunction[*]					
Yes - Median (IQR)	0.71 (0.47- 1.28)	p=0.57	0.82 (0.54-1.61)	p=0.57	0.86 (0.55-1.25)
<25th centile (<0.59)	8 (36.4)	1.46 (0.56-3.80)	6 (26.1)	0.98 (0.35-2.71)	446 (29.1)
25-75 centile (0.59-1.30)	9 (40.9)	1.0 (Ref)	10 (43.5)	1.0 (Ref)	730 (47.7)
>75th centile (>1.30)	5 (22.7)	1.14 (0.38-3.41)	7 (30.4)	1.44 (0.54-3.81)	355 (23.2)
No - Median (IQR)	0.86 (0.67-1.29)	p=0.88	0.97 (0.66-1.30)	p=0.46	0.88 (0.60-1.30)
<25th centile (<0.59)	9 (16.4)	0.59 (0.28-1.23)	18 (20.9)	0.83 (0.48-1.43)	2,092 (23.8)
25-75 centile (0.59-1.30)	33 (60.0)	1.0 (Ref)	47 (54.7)	1.0 (Ref)	4,502 (51.2)
>75th centile (>1.30)	13 (23.6)	0.80 (0.42-1.53)	21 (24.4)	0.91 (0.54-1.53)	2,208 (25.1)
Co-existing congenital anomalies					
Yes - Median (IQR)	0.86 (0.66-1.43)	p=0.84	0.83 (0.52-1.60)	p=0.77	0.91 (0.60-1.37)
<25th centile (<0.59)	9 (18.8)	0.83 (0.36-1.89)	3 (27.3)	2.03 (0.40-10.29)	74 (24.0)
25-75 centile (0.59-1.30)	23 (47.9)	1.0 (Ref)	4 (36.4)	1.0 (Ref)	150 (48.5)
>75th centile (>1.30)	16 (33.3)	1.28 (0.64-2.58)	4 (36.4)	2.35 (0.51-10.76)	85 (27.5)
No - Median (IQR)	0.81 (0.56 - 1.01)	p=0.24	0.95 (0.62-1.30)	p=0.38	0.88 (0.59-1.29)
<25th centile (<0.59)	8 (27.6)	0.87 (0.38-1.99)	21 (21.4)	0.82 (0.49-1.36)	2,464 (24.7)
25-75 centile (0.59-1.30)	19 (65.5)	1.0 (Ref)	53 (54.1)	1.0 (Ref)	5,082 (50.7)
>75th centile (>1.30)	2 (6.9)	0.22 (0.05-0.93)	24 (24.5)	0.93 (0.57-1.51)	2,478 (24.7)

[#] No relevant confounder variables were retained in the models

[*] Including preterm birth (<37 weeks gestation), small for gestational age (<10th centile) o preeclampsia; CI: confidence interval; IQR: interquartile range

Table V: Summary of previous studies reporting the association between maternal first trimester serum levels of hCG with hypospadias or undescended testis (UDT)

Study	Hypospadias N	free-beta hCG	UDT N	free-beta hCG	Controls N	free-beta hCG	p-value for difference
Burton, et al., 1987	-	-	25	35.8[1] ku/litre	32	35.5[1] ku/litre	Not reported
Bernstein, et al., 1988	-	-	24	85.1 (1.9)[1] IU/ml	24	74.2 (1.9)[1] IU/ml	0.28
Kiely, et al., 1995	26	Not reported	31	Not reported	96	Not reported	0.09[2]
Yinon, et al., 2010	30	2.5 (1.5)[1] MoM	-	-	No controls	-	-
Chedane, et al., 2014	-	-	51	0.8[1] MoM	306	1.0[1] MoM	<0.01
Present study							
New South Wales	90	0.88 (0.66-1.40)[3] MoM	107	0.89 (0.65-1.35)[3] MoM	11,904	0.92 (0.63-1.38)[3] MoM	0.83 (Hypospadias); 0.54 (UDT)
Western Australia	77	0.84 (0.63-1.28)[3] MoM	109	0.95 (0.62-1.32)[3] MoM	10,338	0.88 (0.59-1.30)[3] MoM	0.76 (Hypospadias); 0.38 (UDT)

[1] Mean (SD: standard deviation [where available]); [2] Multiple comparisons; [3] Median (interquartile range); - not measured; MoM: multiple of the median