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Title: First-trimester determination of fetal gender by ultrasound: measurement of the ano-genital distance

Short title: Anogenital distance

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Abstract:

*Introduction*: Early ultrasound fetal sex determination is of obvious interest, particularly in the context of X-linked diseases. In the human, the anogenital distance, i.e., the distance between the caudal end and the base of the genital tubercule is sexually dimorphic. This difference is apparent from 11 weeks of gestation.

The aim of this prospective study was to evaluate the accuracy of anogenital distance measurement during the first trimester ultrasound in the early determination of fetal gender.

*Material and methods*: Fetal gender was assessed by ultrasound in 310 singleton pregnancies at 11-14 weeks of gestation. The optimal cut-off was determined by the minimal p-value technic and validated using bootstrap simulation.

*Results*: 310 women were included. A cut-off of 4.8 mm was determined to predict male (≥ 4.8 mm) or female (< 4.8 mm) fetuses. Sex was correctly determined for 87 % of the males and 89 % of the females. The inter-observer variability was excellent.

*Conclusion:* This study presents a new sonographic sign for early fetal sex determination that has not been previously explored. It appears to be an accurate tool but it requires further validation in larger series.

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Introduction

Early fetal sex determination during pregnancy is of great interest for both physicians and future parents for pregnancies at risk of gender-linked genetic disease. To date, fetal sex is determined either by genetic testing or ultrasonography [1].

Genetic tests are highly accurate in determining fetal sex. Chorionic villus sampling under sonography guidance was the first approved technique but is an invasive procedure associated with a risk of pregnancy loss. Analysis of cell-free fetal DNA in maternal blood is a non-invasive technique but expensive and less available worldwide than ultrasonography [2].

The first non-invasive technique to assess gender is based on second trimester fetal ultrasonography with simple morphologic criteria: i) presence of a penis and scrotum for a male and ii) labia majora and minora for a female. In the absence of sexual anomalies, this simple and worldwide technique has an accuracy of up to 100 % as from 20 weeks of gestation (WG).

In the late nineties, a new method based on first trimester fetal ultrasonography was developed to determine fetal sex earlier. This involved measuring the angle of the genital tubercle to a horizontal line through the lumbosacral skin surface in a mid-sagittal plane with the fetus in a natural position [3,4]. This method gave a 100% sensitivity in fetal sex determination after 13 WG but a lower sensitivity between 11 and 12 WG [3,5-8].

Sexual morphogenesis is a dynamic hormono-dependent phenomenon occurring from the sixth WG. Testosterone secretion by testicular cells is responsible for sexual differentiation in the male. Consequently, the anogenital distance (AGD), i.e., the distance between the caudal extremity of the fetus and the base of the genital tubercule, is testosterone dependent and hence sex dependent. The AGD is greater in males than in females [9,10]. In

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the animal model, it is a marker of fetal exposure to androgens during the masculinization programming window [11,12]. In the human, AGD in male newborns is approximately double that of female newborns. This difference remains significant until 24-30 months of life then decreases up to adulthood [9,10,13]. So far, no study has evaluated the contribution of the ultrasound assessment of AGD between 11 and 13 WG + 6 days (corresponding to the time of the first routine ultrasonography during pregnancy) to determine fetal sex.

Therefore, the aim of the present prospective study was to evaluate whether AGD measurement by ultrasonography between 11 and 13 WG + 6 days could accurately determine fetal sex.

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Materials and Methods

We conducted a prospective study to evaluate fetal gender by ultrasonography between January and December 2014. AGD was performed by one operator (JSA) in 310 consecutive singleton pregnancies between 11 and 13 WG + 6 days (Crown–rump length (CRL), 45–84 mm) during the routine first trimester ultrasonography.

All patients gave their informed consent to participate in the study. The study protocol was accepted by the Ethics Committee of the *Collège National des Gynécologues et Obstétriciens Français*.

A Voluson E8 Expert HD Live (General Electric Company), equipped with a 4–7-MHz convex transducer was used for all scans. The AGD was evaluated in the mid-sagittal plane with the fetus lying in a natural position (neither hyperflexed nor hyperextended), which is the image used for the CRL measurement. A caudal caliper was positioned as for a CRL measurement and a genital caliper placed at the inferior base of the genital tubercule (Figure 1).

In the whole population of women, the optimal cut-off for AGD was retrospectively determined by a minimal p-value approach. This involved dichotomizing the AGD into dummy variables with a cut-off every 0.1 mm. Chi-square tests comparing the rate of male and female newborns for every dummy variable were then calculated. The cut-off with the minimal p value was chosen as the optimal cut-off.

The predictive accuracy of the threshold was assessed by its discrimination. The area under the receiver operating characteristic curve (AUC) measured the threshold's ability to discriminate the sex between patients. An AUC of 0.5 indicates that the model provides no predictive discrimination, while a value of 1.0 indicates perfect discrimination between cases. Measures of predictive accuracy were validated using bootstrap simulation. The threshold was

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fit to 300 samples of equivalent size drawn at random with replacement from the original study population. The measures of predictive performance obtained for each statistic in the bootstrap samples were used to estimate the bias in the model statistics attributable to overfitting.

Inter-observer agreement was evaluated comparing the measurements of two operators (JSA and JC) on another 50-woman sample. Each operator measured the AGD, blinded to the other operator's measurement and the difference between the two measurements was analysed.

Statistical analysis was based on Student's t-test for parametric variables, and the Chi-square test or Fisher's exact test, as appropriate, for categorical variables. Inter-reader agreement was evaluated using intraclass correlation coefficients. Values of $p < 0.05$ were considered to denote significant differences.

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Results:

**Determination of the best cut-off**

The best cut-off was calculated in a population of 310 consecutive women. Gender assignment was possible in all 310 fetuses. Sex at birth was available for all newborns except 10. For 10 of the 310 fetuses (3.2%) to which gender was sonographically assigned, information on phenotypic sex at birth was unavailable (lost to follow-up). The mean gestational age at the time of assessment was 12 WG + 3 days (range 11 WG–13 WG + 6 days) and the mean CRL was 63.7 mm (range 46.8–84 mm).

The AGD of the male fetuses was greater than for female fetuses (mean value 6 mm ($IC_{95\%}$ 5.8-6.2) versus 4.2 mm ($IC_{95\%}$ 4-4.3), $p<0.0001$). The distribution of the fetuses' AGD in male and female is represented in the Figure 2.

A 4.8 mm cut-off was associated with the best p value (minimal p-value approach), (Figure 3).

**Optimal Threshold Accuracy**

We constructed a ROC curve in order to confirm the accuracy of the 4.8 mm cut-off (Figure 4). The score of 4.8 millimeters corresponded to the optimal threshold in terms of clinical utility. Using this cut-off, the sex was correctly determined by ultrasound in 87 % of the males (sensitivity) and in 89% of the females (specificity). The chance of being a male when the AGD was more than or equal to 4.8 mm was 91 % (positive predictive value) and the chance of being a female when the AGD was less than 4.8 mm was 85 % (negative predictive value). Likelyhood ratio (LHR) was 8, area under curve (AUC) 0.93 and $p < 0.0001$ (Table 1).

We divided our population into 3 subpopulations following gestational age: (≤12

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GW), (> 12 GW - ≤13 GW) and (> 13 GW). In the population of women whom gestational age was ≤ 12 WG (n=64), the sex was correctly determined by ultrasound in 66 % of the males (sensitivity) and in 100 % of the females (specificity). The chance of being a male when the AGD was more than or equal to 4.8 mm was 100 % (positive predictive value) and the chance of being a female when the AGD was less than 4.8 mm was 67 % (negative predictive value).

In the population of women whom gestational age was > 12 GW and ≤13 GW (n=192), the sex was correctly determined by ultrasound in 91 % of the males (sensitivity) and in 91 % of the females (specificity). The chance of being a male when the AGD was more than or equal to 4.8 mm was 92.3 % (positive predictive value) and the chance of being a female when the AGD was less than 4.8 mm was 89.8 % (negative predictive value).

In the population of women whom gestational age was > 13 GW (n=44), the sex was correctly determined by ultrasound in 100 % of the males (sensitivity) and in 64 % of the females (specificity). The chance of being a male when the AGD was more than or equal to 4.8 mm was 70 % (positive predictive value) and the chance of being a female when the AGD was less than 4.8 mm was 100 % (negative predictive value).

These results are reported in table 1, together with LHRs, AUC and p.

**Optimal Threshold validation (bootstrap correction)**

The predictive threshold had an AUC of 0.88 after the 300 repetitions of bootstrap sample corrections. The maximal difference in predicted and observed probabilities of sex was 0.003.

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**Reproducibility of AGD measurements:**

Two operators (JSA and JC) separately measured AGD in a 50-woman sample, blinded to each other's measurements. In this sample of women, the difference between the measurements of the two operators was 0.2 ± 0.18 mm. The degree of consistency among AGD measurements was excellent with an inter-observer correlation coefficient at 0.97 ($IC_{95\%}$ =0.95-0.98).

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**Discussion**:

The results of this prospective study suggest that AGD measurement could constitute a new and accurate tool to determine fetal sex as early as the first trimester ultrasonography. The AGD of the male fetuses was greater than for female fetuses (6 versus 4.2 mm, $p<0.0001$). With a cut-off of 4.8 mm a sample of 300 women (mean gestational age 12 WG + 3 days), the sex was correctly determined by ultrasound in 87 % of the males and 89 % of the females.

Emerson et al. first described the « sagittal sign » as from 14 WG as a non-invasive way of determining fetal sex in 1989 [4]. Efrat et al. then studied the relevance of the angle of the genital tubercle to a horizontal line through the lumbosacral skin surface, in a mid-sagittal plane, with the fetus in a natural position [3]. The fetus was identified as male when the angle of the genital tubercle was >30° and as female when <10°. Colmant et al. [1] went on to publish a review of 13 studies to evaluate the predictive value of ultrasound early fetal sex determination based on the « sagittal sign ».[14,3,5,15-19,8,20,7,6,21] They found that ultrasound fetal sex determination gave a sensitivity and specificity of 100% from 13 WG but that sex determination was difficult between 11 and 12 WG. However, in this review sex was not evaluable in 7.5 to 40.6 % of cases depending on the series. This could be explained by the impossibility to estimate fetal sex for fetuses with a sagittal sign comprised between 10 and 30°. In contrast, the AGD was evaluable in all cases in our study.

Very few data are available on fetal AGD differences between males and females. Fowler et al [22] measured the AGD in fetuses between 10 and 20 WG and showed a sex-dependent growth. However, the wide range in WG is not compatible with the use of the routine first trimester ultrasonography which is recommended between 11 and 13 WG + 6

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days mainly to detect Down syndrome and major foetal malformations. In the Fowler et al. study, the authors report that AGD was always over 5 mm for male fetuses between 10 and 14 WG. In the current study, we demonstrated that the mean value of AGD was significantly different between male (6 mm ($IC_{95\%}$ 5.8-6.2)) and female (4.2 mm ($IC_{95\%}$ 4-4.3)) fetuses. These results are in agreement with those of the Fowler study although the cut-off value in our study was 4.8 mm as opposed to 5 mm. This 4.8 mm cut-off demonstrated a high accuracy of AGD in distinguishing male from female fetuses resulting in sex determination in 87 % of the males and 89 % of the females. Moreover, the main issue of a new tool is to display high reproducibility. In the current study, inter-observer variability was performed in 50 patients and we observed an excellent agreement.

Several factors can impact AGD. Indeed, AGD in newborns is a marker of *in utero* exposure to androgens and disturbances during the masculinization-programming window could impact its value. In the rat model, various substances have been administered to pregnant females to evaluate the impact on reproductive organ development in the offspring. [23,24,25]. When the pregnant female rat received flutamide (androgen receptor antagonist) or dibutylphthalate [24,25] the AGD at birth was shorter in male offspring. Exposure to phthalates was also associated with a higher prevalence of cryptorchidism and hypospadias [24]. In the human, AGD has also been measured in boy and girl newborns. In boys, AGD has been found to be shorter in newborns with cryptorchidism [26] and when the mother was exposed to phthalates [27,28]. These results suggest that genital organs should be systematically evaluated at the second trimester ultrasonography in male fetuses with low AGD to detect cryptorchidism and hypospadias. In girls, AGD was found to be greater [27] in newborns when the mother was exposed to phthalates. These results could be particularly relevant, as for male fetuses, to detect early sexual dysmorphism. In addition, in accordance to

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previous studies [29,30] demonstrating the risk of developing endometriosis linked to exposure to phthalates and to xeno-estrogenes, abnormalities in the AGD in female foetuses might be a parameter to take into account in further studies to better understand the physiopathology of this debilating disorder.

Our study has some limitations. First, despite an excellent inter-observer variability in AGD measurements, it was only evaluated in a population of 50 patients and the possibility of bias cannot be excluded. Furthermore, no intra-observer variability was evaluated. However, the small window between 11 and 13 WG + 6 days for performing the first trimester ultrasonography could also be a bias due to the short interval between two ultrasonographies. Second, we did not include a comparison of the outcomes of AGD measurements with other validated techniques (such as sagittal sign or analysis of cell-free fetal DNA in maternal blood) in the study design. A comparative study may hence be necessary. Third, between 11 and 13 WG + 6 days, the AGD changes with fetal development. Consequently, AGD measurements need to be evaluated according to WG. Moreover, to avoid misdiagnosis, AGD measurements should be correlated to the CRL. The use of a CRL-adapted threshold could improve the accuracy of this new tool to predict fetal sex. However, despite the inclusion of 310 pregnancies, the population was too small to perform this assessment. For example, in our study, although the number of women with a gestational age > 13 GW was small (n=44), a 5.5 mm cut off provided both 100% sensitivity and specificity. Cut-offs adapted to the CRL need to be evaluated in larger samples of women.

In conclusion, this prospective study supports the relevance of AGD measurement to determine fetal sex during the routine first trimester ultrasonography performed between 11 and 13 WG + 6 days. Moreover, this non-invasive tool does not incur additional costs. Further

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studies are required to validate the tool including the determination of a CRL-adapted threshold.

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**Table 1: First trimester ultrasound gender assignment with the anogenital distance method.**

| | | | | | | | ROC CURVE caracteristics | |
|---|---|---|---|---|---|---|---|---|
| | N | Sensitivity | Specificity | PPV | NPV | LHR | AUC | p |
| All women | 300 | 87% | 89 % | 91% | 85% | 8 | 0.93 | <0.0001 |
| Women with pregnancy ≤12 GW | 64 | 66% | 100% | 100% | 67% | 17 | 0.89 | <0.0001 |
| Women with pregnancy >12 and ≤13 GW | 192 | 91% | 91% | 92.3% | 89.8% | 10 | 0.96 | <0.0001 |
| Women with pregnancy > 13 GW | 44 | 100% | 64% | 70% | 100% | 2.8 | 1 | <0.0001 |

**Sensitivity = Males at birth that were sonographically assigned males**

**Specificity = Females at birth that were sonographically assigned females**

**PPV = Positive predictive value (Probability to be correctly assigned as male)**

**NPV = Negative predictive value (Probability to be correctly assigned as female)**

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**Figure 1.**

Title: Measurement of the anogenital distance (AGD) during first trimester ulstrasonography: method and examples.

Legend:

A: Diagram of the measurement of the AGD. B: Example of a female assignment (AGD = 4.1 mm). C: Example of a male assignment (AGD = 7.8 mm).

**Figure 2.**

Title: The distribution of the fetuses' AGD in males and females.

Legend: *** = $p < 0.0001$

**Figure 3.**

Title: Optimal cut-offs denoting a correlation between anogenital distance and fetal gender. Best p-value approach.

**Figure 4.**

Title: ROC curve of the anogenital distance measured in fetuses during first trimester ultrasonography to determine fetal gender.

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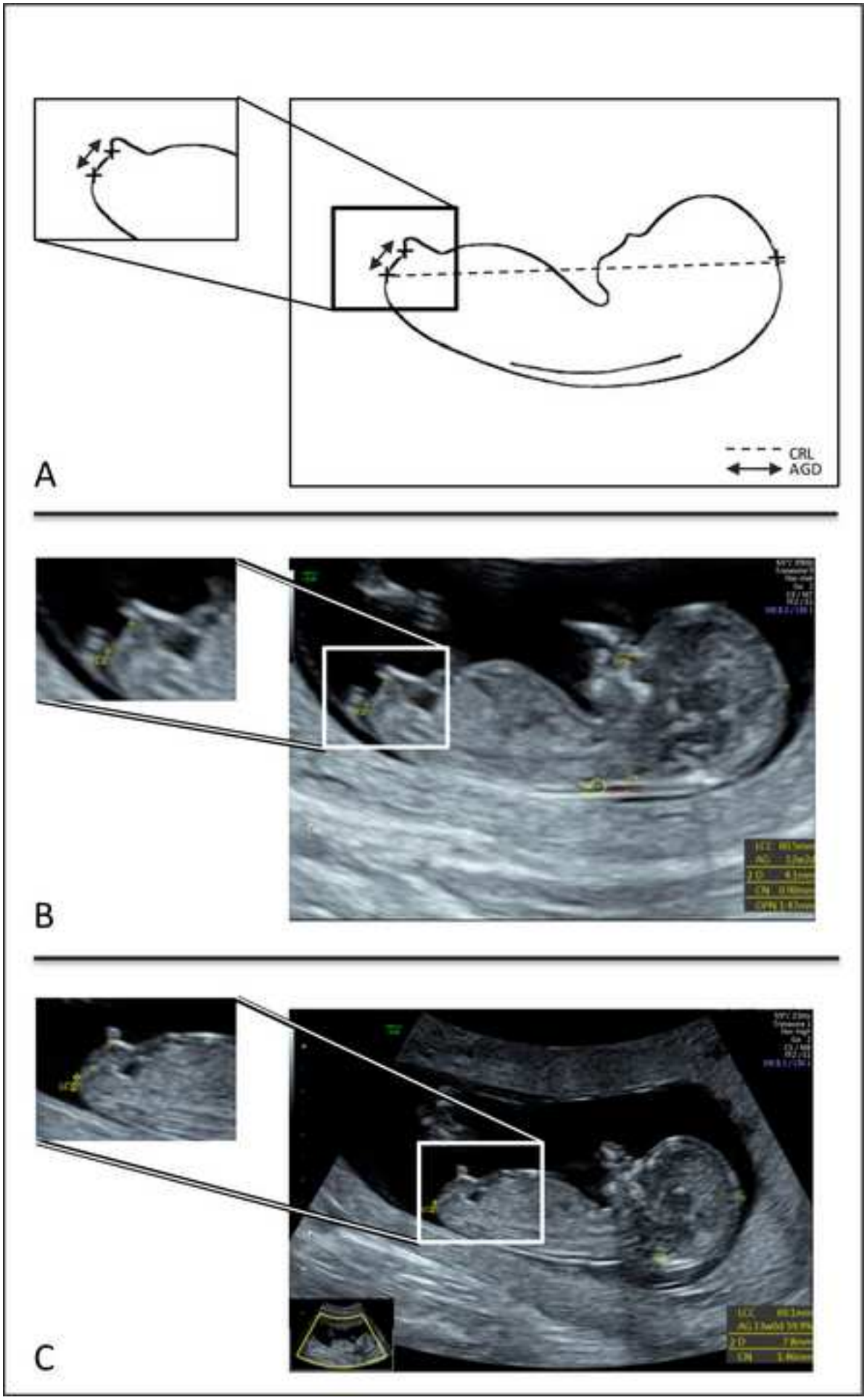


**Figure2**

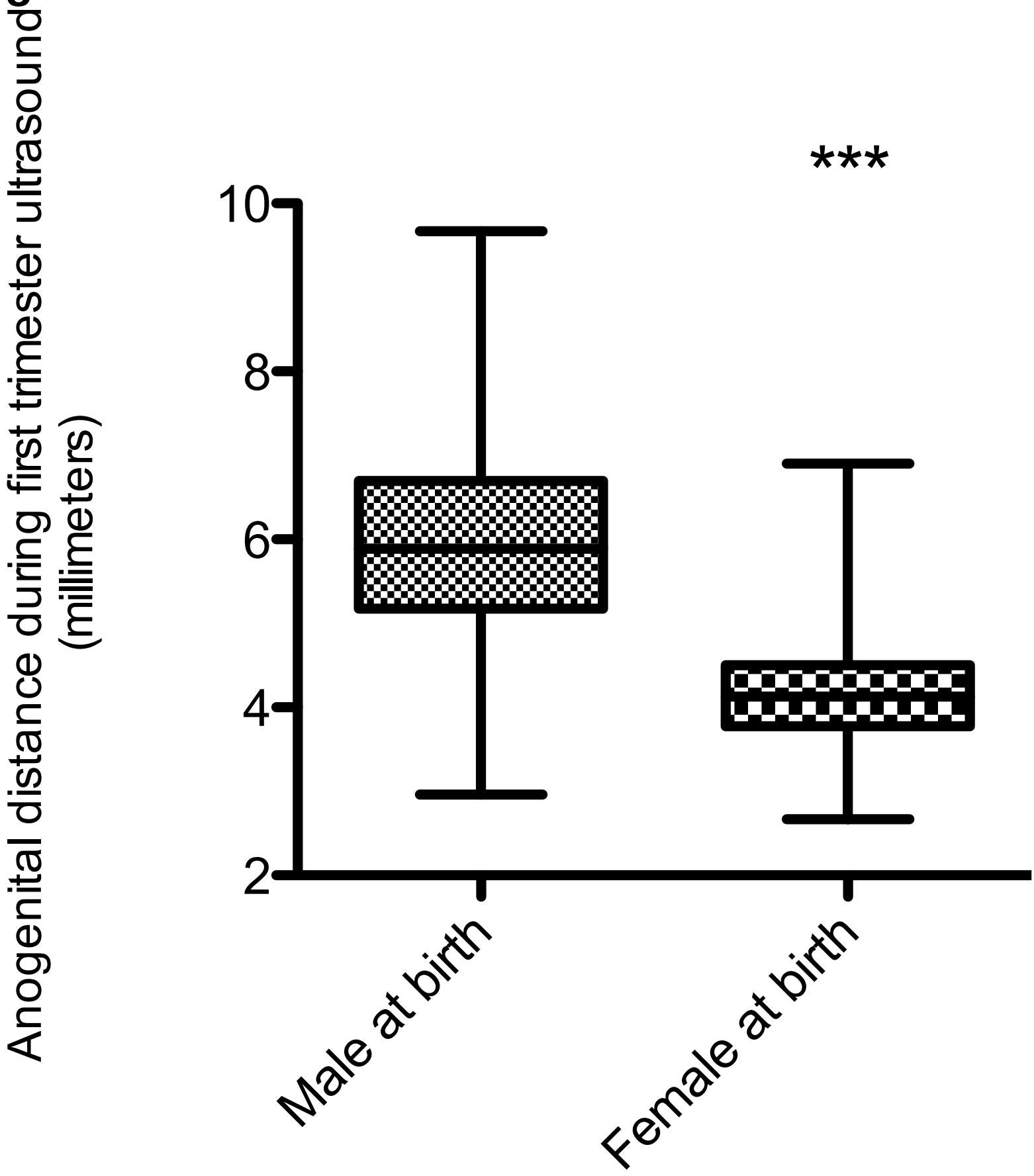

Anogenital distance during first trimester ultrasound
(millimeters)
10
8
6
4
2
***
Male at birth
Female at birth

**Figure3**

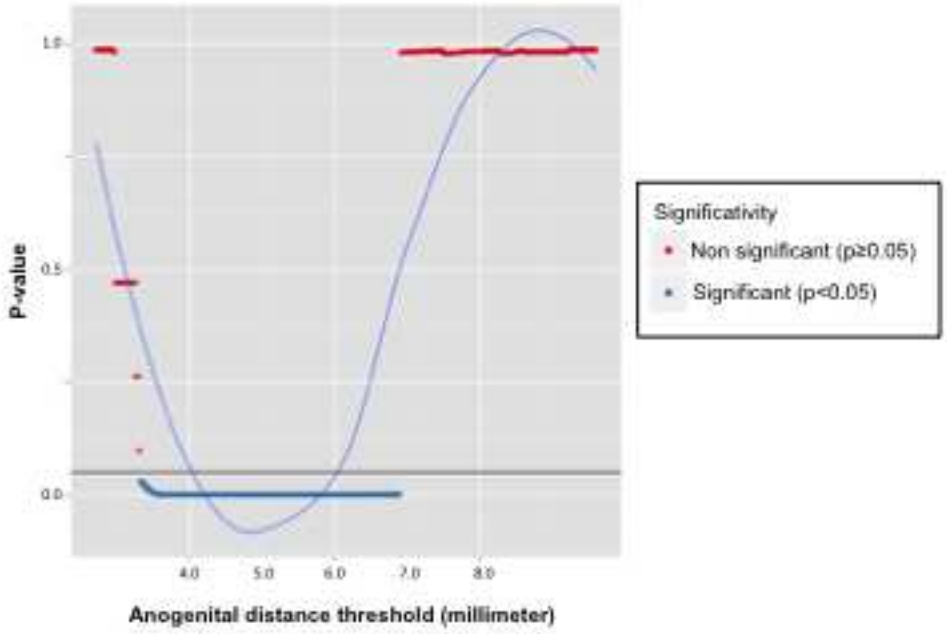

1.0
0.5
0.0
P-value
4.0
5.0
6.0
7.0
8.0
Anogenital distance threshold (millimeter)
Significativity
Non significant (p≥0.05)
Significant (p<0.05)

**Figure4**

