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Anogenital distance is associated with serum reproductive hormones, but not with semen quality in young men

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STUDY QUESTION: Is anogenital distance associated with semen parameters and serum reproductive hormone levels in males?

SUMMARY ANSWER: Anogenital distance is associated with serum reproductive hormones, but not with semen quality.

WHAT IS KNOWN ALREADY: Epidemiological studies have suggested that anogenital distance (AGD) may be associated with testicular dysfunction in adult men. However, the role of AGD in estimating male reproductive function remains unclear.

STUDY DESIGN, SIZE, DURATION: We examined the associations between AGD and semen parameters and reproductive hormones levels in 656 young college students in a Male Reproductive Health in Chongqing College Students (MARHCSs) cohort study in June of 2014.

PARTICIPANTS/MATERIALS, SETTING, METHODS: In this study, two variants of AGD (AGD_{AP} and AGD_{AS}) were measured in 656 university students. Serum levels of testosterone (T), estradiol (E2), progesterone (P), prolactin (PRL), luteinizing hormone (LH), follicle-stimulating hormone (FSH), sex hormone-binding globulin (SHBG) and inhibin-B; and semen quality outcomes, including semen volume, sperm concentration, total sperm number, sperm progressive motility, total motility and morphology, were assessed. The associations between AGD and semen parameters/reproductive hormones levels were analyzed using multiple regression analysis.

MAIN RESULTS AND THE ROLE OF CHANCE: Both AGD_{AS} and AGD_{AP} were not associated with any semen parameters. In the non-parametric correlation analysis, AGD_{AP} were correlated with sperm progressive motility and reproductive hormones of E2, testosterone, SHBG and the testosterone/LH ratio. However, body mass index (BMI) also significantly correlated with serum testosterone ($r = -0.216$, $P = <0.0001$) and SHBG ($r = -0.229$, $P = <0.001$). In the multiple regression models, AGD_{AP} was negatively associated with the serum E2 level (95% CI, −0.198 to −0.043; $P = 0.002$) and positively associated with the ratio of T/E2 (95% CI, 0.004–0.011; $P = 0.001$) after an adjustment for BMI and other confounders.

LIMITATIONS, REASONS FOR CAUTION: Using only a single semen sample to predict male reproductive function over a longer period is a potential limitation of the present study. The other limitation is the cross-sectional nature of the study design. Longitudinal data from an extended follow-up on a large cohort would be more definitive.

WIDER IMPLICATIONS OF THE FINDINGS: Our results do not support previous studies where AGD is associated with male semen quality. The utility of AGD in predicting reproductive outcomes in adult males should thus be considered prudently.

† N.Z., L.S. and H.Y.contributed equally to this work.

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Key words: anogenital distance / testicular dysgenesis syndrome / semen quality / reproductive hormones / MARHCS study

Introduction

There is a common hypothesis that cryptorchidism, hypospadias, testis cancer and poor semen quality are symptoms of testicular dysgenesis syndrome (TDS) (Skakkebaek *et al.*, 2001; Sharpe and Skakkebaek, 2008). This hypothesis proposes that during the stage of testis formation and organization, certain causes of endocrine disruptions, such as infection, genetics, lifestyles and environmental exposure, may cause abnormal testis development (dysgenesis) and lead to an increased risk of reproductive disorders (Martin *et al.*, 2008; Hu *et al.*, 2009). However, there are immense difficulties involved when testing this hypothesis, because it is hardly likely for adult males to establish the link directly between these disorders to earlier events within the fetal testis when dysgenesis is presumed to occur (Sharpe and Skakkebaek, 2008). Thus, a sensitive indicator is needed to estimate fetal androgen exposure and accordingly also used to provide further insights into the origin of TDS disorders.

Anogenital distance (AGD) is the distance between the center of the anus and the genitals, and it may serve as a retrospective measure for fetal androgen exposure during the masculinization programing window (MPW) (Hsieh *et al.*, 2008; Scott *et al.*, 2008; Welsh *et al.*, 2008). Epidemiology studies have reported that boys with hypospadias and cryptorchidism have shorter AGD (Hsieh *et al.*, 2008, 2012; Jain and Singal, 2013). In a recent birth cohort study, AGD and penile length were significantly lower in boys with hypospadias or cryptorchidism than in healthy boys (Thankamony *et al.*, 2014). Since hypospadias and cryptorchidism are potential manifestations of TDS at birth, AGD has been established as a biomarker that determines both fetal endocrine disruption and TDS in humans (Dean and Sharpe, 2013).

Recent interest has focused on the associations between AGD and reproductive functions in adult males. Over the past decade, some population-based studies have shown the relationships between AGD and fatherhood, sperm concentration and total sperm count in adult men (Eisenberg *et al.*, 2011; Mendiola *et al.*, 2011, 2015; Eisenberg and Lipshultz, 2015). Since declining semen quality, especially decreased sperm concentration, has been considered one of the symptoms associated with TDS. Previous studies have shown that the most important determinant factor for sperm count is the Sertoli cell number, and the final Sertoli cell number is largely dependent on perinatal events (Sharpe *et al.*, 2003). Therefore, during the MPW, a subtle variation in androgen exposure may cause sperm count variation in healthy adult males. AGD is a sensitive developmental end-point for prenatal androgen exposure. If we can identify the reliability role of the relationship between AGD and male reproductive functions, it will help us predict the TDS hypothesis in humans. However, the use of AGD in human studies is still rare, and the results remain controversial.

The Male Reproductive Health in Chongqing College Students (MARHCSs) study was established in 2013 as a perspective cohort study that recruited voluntary male healthy college students from three universities in Chongqing. The primary objectives of the MARHCS study are to investigate male reproductive health in young adults (Yang *et al.*, 2015). We investigated the relationship between AGD and semen quality/serum reproductive hormone levels in this cohort study, seeking to fully explore the predictable value of AGD measurements on adult reproductive function.

Materials and Methods

Ethical approval

The study was approved by the Ethics Committees of the Third Military Medical University, and a signed informed consent was obtained from each participant.

Study population

Subjects were participants in the MARHCS Study (Yang *et al.*, 2015). The study included a physical examination; collection and examinations of blood, urine and semen samples; and a detailed social–physical–behavioral questionnaire. In June of 2013, a baseline was established within the group of voluntary male college students in the University Town of Chongqing, and a total of 796 eligible subjects finished all investigations in the baseline stage (Yang *et al.*, 2015). The first follow-up was carried out in June of 2014. The participants were recruited from eligible participants from the 2013 baseline investigation. A total of 666 subjects attended the follow-up procedure, of which 10 failed to provide a semen sample. Therefore, 656 (82.4%) of the 796 eligible subjects were followed up and finished all the procedures, including the assessment of AGD.

Semen collection and analyses

The methods for semen collection and analyses have been previously described in detail (Yang *et al.*, 2015). Briefly, all participants were asked to stay abstinent for 2–7 days before contributing a semen sample. Semen samples were obtained by masturbation and collected in sterile plastic containers, then immediately incubated in a water-bathed at 37°C. Once the ejaculates liquefied, a routine semen analysis was performed within 60 min. Conventional semen parameters were measured according to World Health Organization (WHO) guidelines (WHO, 2010). Semen appearance was recorded by observation; semen volume was measured by weighing, assuming 1 g of weight was equal to 1 ml of volume; sperm concentration and sperm motility were assessed by computer-aided sperm analysis (SCA CASA System; Microptic S.L., Barcelona, Spain). Sperm morphology was identified

from semen smears. Diff-Quik stained and assessed using the WHO criteria.

To reduce the variation of assessment of sperm characteristics, all analyses of semen quality were performed by one technician. This technician was well trained in semen analysis and participated in the Continuous Quality Control System (an external quality control system established using WHO guidelines) under the supervision of the Chongqing Science and Technology Commission.

Physical examination

Physical examination, performed by an experienced urologist. The presence of varicocele or other andrology abnormalities was recorded, and testicular size was estimated using Prader's orchidometer (FUAN enterprise, Shanghai, China). Body weight and height were measured using a digital scale (OMRON, HBF-370, Shanghai, China).

In this study, two variants of AGD were obtained using measurement methods that have been described elsewhere (Mendiola *et al.*, 2011; Parra *et al.*, 2016). AGD_{AP} was measured form the cephalad insertion of the penis to the center of the anus, and the AGD_{AS} was measured from the posterior base of the scrotum to the center of the anus. The participant was placed in a supine, frog-legged position with his thighs at a 45° angle to the examination table. To measure the AGD precisely, a single urologist conducted all AGD measurements for all participants using a stainless-steel digital caliper. The examiner measured each AGD variant twice. The mean value of the two measurements was then used. Intra-examiner coefficients of variation for AGD_{AS} and AGD_{AP} were <3 and 8%, respectively. Neither the urologist nor the support staff had any knowledge of the participant's semen quality.

Assessment of serum reproductive hormone levels

The blood serum was separated by centrifugation, coded and frozen at −80°C until analysis. The serum levels of six hormones [follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), progesterone (P), testosterone (T) and prolactin (PRL)) were analyzed at the clinical laboratory of Southwest Hospital (Chongqing, China), using the Beckman UniCel DXI 800 Immunoassay System (Beckman Coulter, Inc., Brea, CA, USA) and commercial test kits. The detection range was 1.2–187.1 mIU/ml for FSH, 0.7–85.9 mIU/ml for LH, 0.4–30.4 ng/ml for P, 0.6–75.7 ng/ml for PRL, 20–4233 pg/ml for E2 and 0.1–14.8 ng/ml for T. The intra-assay coefficient of variation was 3.5% for FSH, 3.8% for LH, 6.1% for P, 1.6% for PRL, 12% for E2 and 3.9% for T.

The detection of the serum levels of Inhibin-B and sex hormone-binding globulin (SHBG) were performed manually using commercial kits according to the manufacturer's instructions. Inhibin-B levels were determined by using a double antibody ELISA Kit (DSL-10-84100 ACTIVE® Inhibin B ELISA, USA) with inter- and intra-assay CVs of 7.6 and 4.6%, respectively, with a sensitivity of 10 pg/ml. SHBG was measured by a solid phase enzyme-linked immunosorbent assay Kit (DEMEDITEC SHBG ELISA, Germany). The inter- and intra-assay CVs were 4.8 and 5.9%, with a sensitivity of 0.77 nmol/l. We calculated free antigen index as (total T × 100/SHBG). Hormone ratios were calculated by simple division.

Statistical analysis

The basic characteristics of the study population were described using untransformed data. Continuous variables were represented as mean ± standard deviation (SD). Semen parameters and reproductive hormones were presented as mean ± SD, median and percentiles (5th, 50th and 95th). Bivariate associations between AGD and each of the semen parameters and reproductive hormones were evaluated using a Spearman correlation coefficient analyses.

We analyzed the associations between AGD measurements and the sperm parameters and serum hormone levels using multivariate linear regression analysis. For any parameters with skewed distribution, we performed statistical transformations on these variables to better approximate the normality assumption of the model. Specifically, we applied log transformation to the sperm concentration and total sperm count, a cube-root transformation to sperm progressive motility and total sperm motility. All serum hormone parameters except those for testosterone were log-transformed (base 10) in the regression models. Selection of risk factors for the final model was based on their

Table I Basic characteristics of the study population.

Characteristics	No. of subjects	Values[a]
Age (years)	656	20.1 ± 1.6
Abstinence time (day)	656	4.2 ± 1.4
Percent body fat (%)	656	16.8 ± 5.3
Time to start semen analysis (min)	656	26.0 ± 10.9
Testicular volume (ml)	656	19.4 ± 4.7
Anogenital distance (AGD)		
AGD_{AS} (mm)	656	39.0 ± 10.7
AGD_{AP} (mm)	656	116.1 ± 10.9
Body mass index (BMI), *n* (%)		
<18.5	67	10.2
18.5–23.9	512	78.0
24.0–27.9	66	10.0
≥28	11	1.8
Race, *n* (%)		
Han	594	90.5
Others	62	9.5
Tobacco smoking, *n* (%)		
Never	484	73.8
Ever	18	2.8
Current	154	23.4
Alcohol consumption, *n* (%)		
Never	320	48.8
Ever	10	1.5
Current	326	49.7
Tea intake, *n* (%)		
Never	355	54.1
Ever	155	23.6
Current	146	22.3

[a]Represented as 'mean ± SD' or 'percentage'.

importance in the literature and biological plausibility. A coefficient was thought to be a potential confounder if the regression coefficient changed by >10% when it was included one by one in the multivariate models. Finally, race, body mass index (BMI), abstinence time and sample collection time were included in the models as potential confounders. Multiplicity adjustment by a Bonferroni correction was conducted for the regression analyses by multiplying the crude *P*-value by the number of analyses. The statistical analyses were performed using the Statistical Package for the Social Sciences, Version 16.0 (SPSS, Chicago, IL, USA).

Results

Population characteristics

The basic characteristics are summarized in Table I. A total of 656 eligible subjects finished all investigations in the follow-up stage. Participants were 19–23 years in age (mean age, 20.1), and predominant race was Han (90.5%). About 23.4% of the subjects were current smokers, and almost half (49.7%) were current drinkers. The AGD_{AS} and AGD_{AP} values were normally distributed. The mean value for AGD_{AS} was 39.0 ± 10.7 mm, and for AGD_{AP} was 116.1 ± 10.9 mm. The reproductive hormone levels and semen quality parameters are given in Table II.

AGD and reproductive hormones and semen quality: a univariate analysis

Table III shows the correlations between AGD measurements and reproductive hormones and semen quality. In the non-parametric correlation analysis, AGD_{AP} showed a positive correlation with sperm progressive motility ($r = 0.084$, $P = 0.032$) and with a testosterone/E2 ratio ($r = 0.022$, $P = 0.041$). On the other hand, AGD_{AP} significantly and negatively correlated with the reproductive hormones of E2, testosterone, SHBG and the testosterone/LH ratio ($r = -0.034$, $P = 0.036$; $r = -0.131$, $P = 0.001$; $r = -0.142$, $P = <0.0001$; $r = -0.099$, $P = 0.013$, respectively). However, there were no significant correlations between AGD_{AS} and the semen quality parameters and reproductive hormone levels.

Correlation between AGD and BMI

As we expected, AGD_{AS} and AGD_{AP} were highly correlated ($r = 0.649$, $P < 0.0001$). BMI, height, weight and body fat percentage were significantly correlated with AGD_{AP} ($r = 0.588$, $P = <0.0001$; $r = 0.257$, $P = 0.001$; $r = 0.610$, $P < 0.0001$; $r = 0.573$, $P < 0.0001$, respectively). Only weight and BMI were correlated with AGD_{AS} ($r = 0.112$, $P = 0.001$; $r = 0.086$, $P = 0.027$, respectively). We also found correlations between BMI and serum hormone levels. As shown in Fig. 1, BMI inversely correlated with serum testosterone ($r = -0.216$, $P < 0.0001$) and SHBG ($r = -0.229$, $P < 0.0001$).

Multivariate analysis for semen parameters, reproductive hormone levels and AGD

The final multiple regression models are summarized in Table IV. After adjusting for race, BMI and abstinence time, AGD measurements were not associated with any semen quality parameter. We only observed a

Table II Distributions of semen parameters and reproductive hormone levels.

Variable	Mean ± SD	Median	Percentiles		
			5th	50th	95th
Semen parameters					
Semen volume (ml)	3.82 ± 1.89	3.56	1.74	3.56	6.52
Sperm concentration (10^6/ml)	69.36 ± 61.25	51.80	13.74	51.80	194.32
Total sperm count (10^6)	252.79 ± 221.74	193.45	42.57	193.45	732.5
Progressive motility (PR, %)	55.32 ± 16.27	57.00	25.60	57.00	78.82
Total motility (PR + NP, %)	86.05 ± 12.19	89.40	60.00	89.40	98.9
Normal morphology (%)	11.92 ± 7.49	10.00	2.35	10.00	27.00
Serum reproductive hormones					
Estradiol (pg/ml)	26.29 ± 13.33	25.50	6.85	25.50	49.15
FSH (mIU/ml)	3.93 ± 1.85	3.59	1.69	3.59	7.57
LH (mIU/ml)	4.74 ± 1.79	4.41	2.44	4.41	7.68
Prolactin (ng/ml)	11.83 ± 5.79	10.87	5.42	10.87	21.19
Progesterone (ng/ml)	0.59 ± 0.38	0.52	0.16	0.52	1.30
Testosterone (nmol/l)	13.98 ± 3.63	13.74	8.88	13.74	20.33
Inhibin B (pg ml)	375.78 ± 207.18	348.39	150.61	348.38	667.16
SHBG (nmol/l)	39.70 ± 23.06	34.10	13.71	34.10	87.04
FTI (testosterone/SHBG)	0.48 ± 0.42	0.40	0.16	0.40	0.99
Testosterone/LH	3.32 ± 1.41	3.08	1.54	3.07	5.74
Testosterone/E2	0.77 ± 1.11	0.53	0.25	0.53	1.85

FSH, follicule-stimulating hormone; LH, luteinizing hormone; SHBG, serum sex hormone-binding globulin; FTI, free testosterone index; PR, progressive; NP, non-progressive.

Table III Univariate correlations for men's semen parameters and reproductive hormone levels and AGD.

	AGD_{AS} (mm)		AGD_{AP} (mm)	
	r	*P*-values	*r*	*P*-values
Semen parameters				
Semen volume (ml)	−0.012	0.766	−0.050	0.205
Sperm concentration (10^6/ml)	0.016	0.679	0.035	0.370
Total sperm count (10^6)	−0.001	0.975	0.000	0.998
Progressive motility (PR, %)	0.025	0.501	0.084	**0.032***
Total motility (PR + NP, %)	0.032	0.418	−0.052	0.186
Normal morphology (%)	0.022	0.578	−0.065	0.094
Serum reproductive hormones				
Estradiol (pg/ml)	−0.054	0.176	−0.034	**0.036***
FSH (mIU/ml)	0.007	0.869	−0.024	0.554
LH (mIU/ml)	−0.010	0.803	−0.003	0.931
Prolactin (ng/ml)	−0.002	0.955	0.008	0.834
Progesterone (ng/ml)	−0.029	0.464	0.009	0.815
Testosterone (nmol/l)	−0.025	0.523	−0.131	**0.001****
Inhibin B (pg/ml)	−0.037	0.360	0.050	0.218
SHBG (nmol/l)	0.013	0.740	−0.142	**<0.001****
FTI (testosterone /SHBG)	−0.042	0.309	0.078	0.056
Testosterone/LH	−0.030	0.450	−0.099	**0.013***
Testosterone/E2	0.028	0.489	0.022	**0.041***

FSH, follicle-stimulating hormone; LH, luteinizing hormone; SHBG, serum sex hormone-binding globulin; FTI, free testosterone index.
*$P < 0.05$, **$P < 0.01$.

weak positive association between AGD_{AP} and sperm concentration (95% CI, −0.003 to 0.015; $P = 0.062$).

We also compared the AGD measurements with the semen quality parameter dichotomized with the WHO reference (WHO, 2010) (semen volume <1.5 ml, sperm concentration <15 million/ml, total sperm count <39 million/ml, motile sperms <40% and morphologically normal sperm <4%) using binary logistic regression analyses, adjusting for the same set of confounders. However, still no associations between the AGD measurements and semen parameters were found (data not shown).

In the preliminary non-parametric correlation analysis, testosterone, SHBG and the testosterone/LH ratio showed negative correlations with AGD_{AP}. However, when adjusted for BMI, which is strongly associated with serum hormone levels, the associations were no longer significant ($P > 0.05$). In the multiple regression models, we only found that AGD_{AP} negatively related to the serum E2 level (95% CI, −0.198 to −0.043; $P = 0.002$) and positively related to the ratio of testosterone/E2 (95% CI, 0.004–0.011; $P = 0.001$) when adjusted for BMI and other confounders. Multiplicity adjustment was conducted using the Bonferroni correction. However, the correlation between AGD_{AP} and E2 was not significant after the Bonferroni correction (corrected $P = 0.068$). Only the correlation between AGD_{AP} and the ratio of testosterone/E2 remained significant (corrected $P = 0.034$).

Discussion

To the best of our knowledge, this study is the first attempt to assess AGD measures in Asian and is the largest population-based study to date to explore the relationship between AGD and reproductive parameters in adult men. In order to eliminate the potential impact of age, we conducted this study in our MARHCS cohort. Moreover, we standardized the assessment techniques of AGD, and implemented strict quality control procedures throughout the period of the study, further suggesting that our findings are robust.

By searching PubMed, we found there were five studies carried out in male populations with comparable age with our study (Mendiola *et al.*, 2011, 2015; Eisenberg *et al.*, 2012; Eisenberg and Lipshultz, 2015; Parra *et al.*, 2016). We summarize these studies in Table V. Of these, three were carried out in the USA; two were carried out in Spain, while our study was the only one conducted in Asia. There were three studies that investigated the AGD in general college students with a homogeneous age (median) from 19.4 to 20.0 years. Mendiola *et al.* (2011) reported the median values of AGD variants as 51.7 mm for AGD_{AS} and 126.0 mm for AGD_{AP} in young men at the University of Rochester, New York (median age, 19.4). Parra *et al.* (2016) reported the median value of AGD_{AS} as 48.3 mm and AGD_{AP} as 128.0 mm in university students in Southern Spain (median age, 20.0). The present study found the median values of AGD variants were 38.0 mm for AGD_{AS} and 115.0 mm for AGD_{AP} (median age, 20.0). These data indicate there are ethnic and regional differences among the continents (Asia, America and Europe) for the two variants of AGD.

In rodents, AGD is the distance from the posterior base of the genitalia to the anal opening. However, studies have applied a variety of measurements for AGD in human. Salazar-Martinez *et al.* (2004) refers to the AGD as the distance from the anus to the most posterior midline point of the scrotum. Torres-Sanchez *et al.* (2008) considered the distance from the tip of the coccyx to the center of the anus as the AGD. Because AGD is affected by body weight, some studies used the anogenital index (AGI = AGD/weight) in children (Swan *et al.*, 2005; Huang *et al.*, 2009; Suzuki *et al.*, 2012). However, Eisenberg *et al.* (2013) suggested that AGI may not be necessary when dealing with adult men because it cannot completely remove the effect of weight. AGD_{AS} and AGD_{AP} are the most commonly used measurements in human studies, and AGD_{AS} is considered to be the most reliable and repeatable measurement (Swan *et al.*, 2005; Thankamony *et al.*, 2009; Sathyanarayana *et al.*, 2010) because this variant is unaffected by BMI/obesity and age, especially in adult men. Mendiola *et al.* (2011) suggested that different AGD measurements might indeed reflect androgen exposures at different life stages.

In this study, we found that AGD_{AP} was associated with reproductive hormone levels. But AGD_{AS} and AGD_{AP} were not associated with any semen parameters. Previous studies on AGD and reproductive parameters in males also have shown inconsistent results. Several studies have reported that AGD measurements were associated with semen quality and serum reproductive hormones. However, most of these studies were conducted on patients attending andrology or infertility clinics (Eisenberg *et al.*, 2012; Eisenberg and Lipshultz, 2015; Mendiola

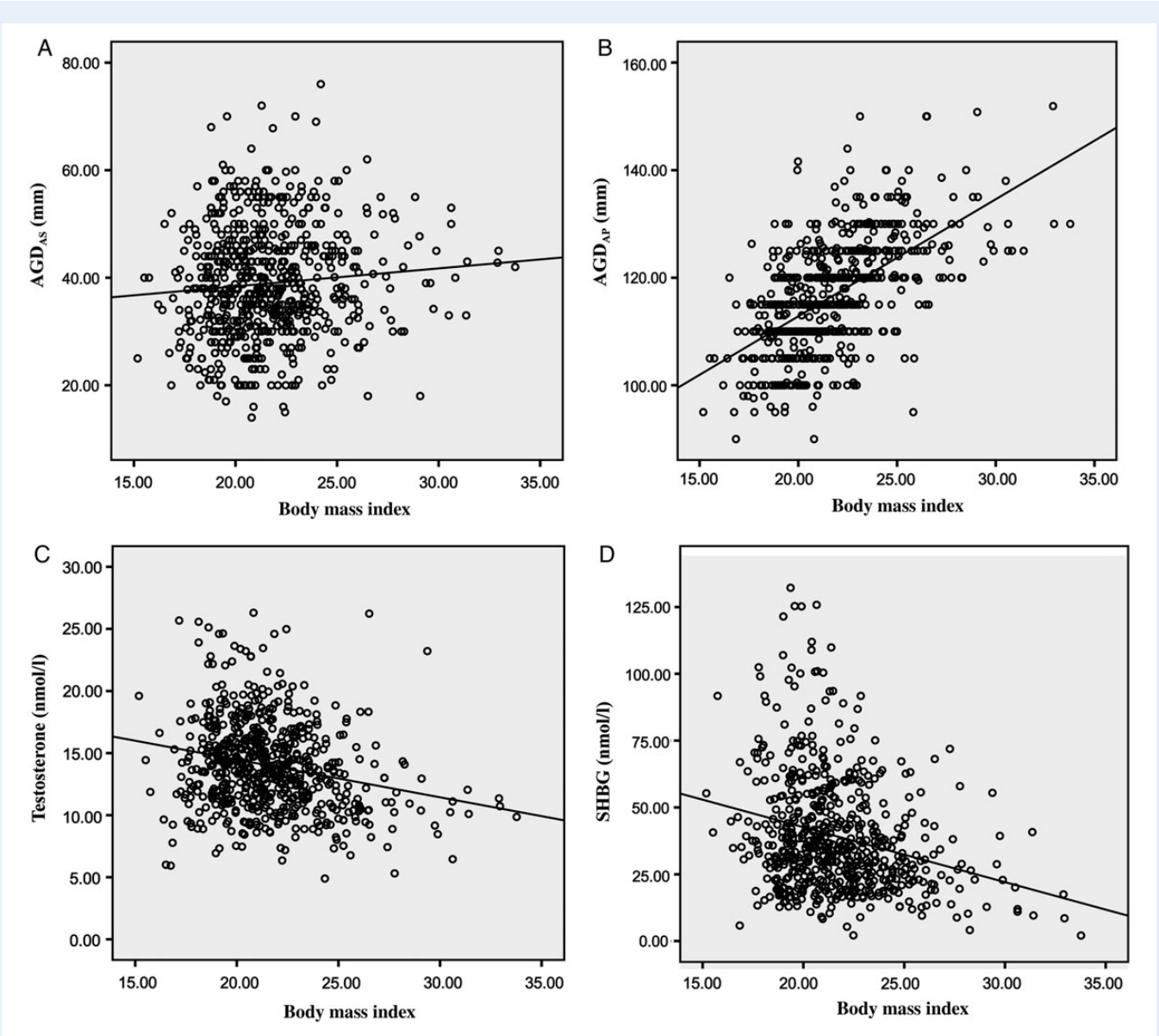

Figure 1 The crude associations between the BMI and AGD and serum hormone levels. (**A**) AGD$_{AS}$ ($r = 0.086$, $P = 0.027$). (**B**) AGD$_{AP}$ ($r = 0.588$, $P = <0.0001$). (**C**) Testosterone ($r = -0.216$, $P = <0.0001$). (**D**) SHBG ($r = -0.229$, $P = <0.0001$).

et al., 2015). Infertile men may have infertility reasons that can cause testis dysfunction, so therefore, their AGD may also have changed as a consequence of infertility. Only two studies have been conducted on unselected men similar to our study, but with conflicting results. Mendiola et al. (2011) reported that AGD$_{AS}$ was associated with sperm concentration, motility, morphology, total sperm count and total motile count in young men at the University of Rochester, New York ($n = 126$). On the contrary, Parra et al. (2016) reported the relationship between AGD and semen quality and serum reproductive hormone levels in university students from Southern Spain ($n = 215$), and found that both AGD$_{AS}$ and AGD$_{AP}$ were not associated with any semen parameters or any of the reproductive hormone levels. The reason for these conflicting results may be due to racial or geographic differences.

We observed significantly negative correlations between AGD$_{AP}$ and reproductive hormones of testosterone and SHBG, which conflicts with the previous studies (Eisenberg et al., 2012; Mira-Escolano et al., 2014), although these associations were not found in the multiple regression models after an adjustment for BMI. On the other hand, the serum levels of testosterone and SHBG were also affected by BMI (see Fig. 1). Many of the previous studies have demonstrated that BMI is negatively associated with T levels and SHBG levels (Seidell et al., 1990; Muller et al., 2003; Nielsen et al., 2007; Travison et al., 2007; Vandenput et al., 2010; Rohrmann et al., 2011; Gates et al., 2013). In the current study, 78.0% of the participated subjects have normal BMI (BMI, 18.5–23.9, $n = 512$). Only 11.8% of subjects were overweight (BMI, >24, $n = 77$) based on the recommended BMI for Chinese adults (Zhou, 2002).

Table IV Multivariable linear regression analysis for men's semen parameters and reproductive hormone levels and AGD.

Variable	AGD_{AS} (mm)		AGD_{AP} (mm)	
	β (95% CI)	*P*-values	β (95% CI)	*P*-values
Semen parameters[a]				
Semen volume (ml)	−0.004 (−0.010 to 0.003)	0.386	0.000 (−0.001 to 0.002)	0.689
Sperm concentration (10^6/ml)	0.002 (−0.006 to 0.010)	0.667	0.007 (−0.003 to 0.015)	0.062
Total sperm count (10^6)	0.002 (−0.008 to 0.015)	0.635	0.010 (−0.009 to 0.023)	0.071
Progressive motility (PR, %)	0.000 (−0.003 to 0.002)	0.299	−0.001 (−0.003 to 0.001)	0.074
Total motility (PR + NP, %)	0.000 (−0.004 to 0.003)	0.849	−0.003 (−0.006 to 0.000)	0.838
Normal morphology (%)	−0.001 (−0.005 to 0.003)	0.914	−0.003 (−0.007 to 0.001)	0.213
Serum reproductive hormones[b]				
Estradiol (pg/ml)	−0.001 (−0.174 to 0.002)	0.579	−0.126 (−0.198 to −0.043)	**0.002****[c]
FSH (mIU/ml)	0.001 (−0.021 to 0.007)	0.832	0.001 (−0.008 to 0.010)	0.458
LH (mIU/ml)	0.000 (−0.012 to 0.015)	0.504	−0.002 (−0.051 to 0.003)	0.232
Prolactin (ng/ml)	0.000 (−0.013 to 0.027)	0.863	−0.001 (−0.049 to 0.015)	0.650
Progesterone (ng/ml)	−0.001 (−0.005 to 0.010)	0.670	0.003 (−0.001 to 0.006)	0.191
Testosterone (nmol/l)	−0.008 (−0.019 to 0.010)	0.559	0.012 (−0.010 to 0.038)	0.339
Inhibin B (pg/ml)	−0.002 (−0.006 to 0.001)	0.175	−0.001 (−0.004 to 0.002)	0.490
SHBG (nmol/l)	0.002 (−0.001 to 0.007)	0.129	0.000 (−0.003 to 0.003)	0.983
FTI (testosterone /SHBG)	−0.005 (−0.008 to 0.000)	0.153	0.000 (−0.003 to 0.004)	0.903
Testosterone/LH	−0.001 (−0.010 to 0.005)	0.396	0.002 (−0.004 to 0.013)	0.094
Testosterone/E2	−0.001 (−0.006 to 0.003)	0.657	0.007 (0.004 to 0.010)	**0.001****[d]

[a]Controlling for race, BMI and abstinence time.
[b]Controlling for race, BMI and sample collection time.
[c]*P*-value for Bonferroni's correction was 0.068.
[d]*P*-value for Bonferroni's correction was 0.034.
**$P < 0.01$.
FSH, follicle-stimulating hormone; LH, luteinizing hormone; SHBG, serum sex hormone-binding globulin; FTI, free testosterone index.

In the multiple regression models, we also examined BMI for the normal range group and the overweight group. Same as in the previous results, AGD_{AP} were associated with the serum E2 level and the testosterone/E2 ratio in the normal range group. No associations were found between AGD measurements and semen parameters or with serum reproductive hormone levels in the overweight group (data not shown). Therefore, we can speculate that the strong influence of BMI may conceal the actual relationships between AGD and the reproductive hormones.

There have been studies that suggest that the finding of a relationship between AGD and the FSH and/or inhibin-B or free testosterone in men would directly support what is already known from the rats experiment (Mendiola *et al.*, 2011; Dean and Sharpe, 2013). Yet until now, only two studies have assessed AGD and reproductive hormones in adult males, and they found no associations between AGD and a FSH or inhibin-B or free testosterone (Eisenberg *et al.*, 2012; Parra *et al.*, 2016).

Our results suggest that the serum level of E2 may adversely impact the AGD_{AP} among man. Recently in a rat study, Mitchell *et al.* (2015) demonstrated that there is a degree of plasticity in AGD in adulthood. They treated adult male rats with estrogen diethylstilbestrol (DES) for 5 weeks and found that DES not only suppressed the circulating testosterone and reduced seminal vesicle weight, but it also induced a significant reduction in AGD. These data support the important observations that AGD is not irrevocably fixed by adulthood, but instead changes to a small but progressive extent throughout adulthood, implying that AGD may serve as a lifelong clinical biomarker of fetal androgen action. Our study is the first to show the relationship between AGD and E2 in humans. This finding still needs to be confirmed by future research.

The limitations of our analysis include the fact that only one semen sample was evaluated for each subject. Since the number of subjects evaluated was relatively high, this sample size would tend to minimize the potential effect of the sample variability of semen quality. Moreover, an earlier study has determined that it makes little difference in epidemiological studies whether an analysis includes men who give one semen sample or two (Stokes-Riner *et al.*, 2007). The other limitation in the current study is the cross-sectional nature of its design. Longitudinal data from an extended follow-up on a large cohort would be more definitive.

In conclusion, we found that AGD is associated with serum reproductive hormones, but not with semen quality. Our data are in discordance with previous studies where AGD is associated with male semen quality. The utility of AGD in predicting reproductive outcomes in adult males still needs to be verified in more diverse populations.

Table V Comparison of AGD parameters among adult male populations published between 2011 and 2016.

Study (publication year)	Year of subjects recruited	Region	Sample size	Population source	Age (years)	AGD_{AS} (mm)	AGD_{AP} (mm)	Parameters analyzed	Results
This study	2014	Chongqing, China	665	General college students	20.0 (20.0–21.0)[a]	38.0 (21.0–51.0)[a]	115.0 (100.0–134.0)[a]	Semen parameters and serum reproductive hormones	AGD_{AP} were associated with reproductive hormone levels. But AGD_{AS} and AGD_{AP} were not associated with any semen parameters
Eisenberg and Lipshultz, 2015	2010–2011	Houston, USA	473	Patients from a urology clinic	43.0 (13.0)[b]	41.9 (13.5) for father; 36.4 (12.9) for childless[c]	NA	Semen parameters	Anogenital distance was significantly longer in men with higher sperm concentration, total sperm count and total motile sperm count
Parra *et al.* (2016)	2010–2011	Murcia, Spain	215	General college students	20.0 (18.0–22.0)[a]	48.3 (11.6)[b]	128.0 (12.0)[b]	Semen parameters and serum reproductive hormone	AGD measures were not associated with any semen parameters or any of the reproductive hormone levels
Mendiola *et al.* (2011)	2009–2010	New York, USA	126	General college students	19.4 (18.8–20.3)[c]	51.7 (43.1–61.1)[c]	126.0 (118–135)[c]	Semen parameters	AGDAS was associated with sperm concentration, motility, morphology, total sperm count, and total motile count
Eisenberg *et al.* (2012)	2010	Houston, USA	116	Patients from a urology clinic	36.1 (8.0)[b]	34.3 (13.3)[b]	NA	Serum reproductive hormones	Anogenital distance was significantly associated with serum testosterone levels
Mendiola *et al.* (2015)	2012–2013	Murcia, Spain	91	Men attending infertility services	36.0 (33.0–38.0)[c]	45.9 (36.2–55.2)[c]	138.0 (90.0–120)[c]	Semen parameters	Significant positive associations between AGDAS and sperm concentration, total sperm count and total sperm motile count were detected

[a]Results are presented as median with 5th and 95th percentile.
[b]Data are shown as mean (SD).
[c]Data are shown as median (25th and 75th percentile).
NA, not available.

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Authors' roles

N.Z. contributed to statistical analyses, interpretation of data and drafted the paper. L.S. and H.Y. assisted in statistical analyses and interpretation of data. The study was conceived and designed by Z.C. and J.C. The data were collected by N.Z., L.S., H.Y., Q.C., X.W., H.Y., L.T., H.C., G.Z., X.L., L.H., P.Z., K.P., T.L., J.L., L.A. and Z.Z. All co-authors interpreted the data and participated in finalizing the manuscript. All co-authors approved the final version of the manuscript.

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Conflict of interest

None declared.

References

Dean A, Sharpe RM. Clinical review: 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**:2230–2238.

Eisenberg ML, Lipshultz LI. Anogenital distance as a measure of human male fertility. *J Assist Reprod Genet* 2015;**32**:479–484.

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.

Eisenberg ML, Jensen TK, Walters RC, Skakkebaek NE, Lipshultz LI. The relationship between anogenital distance and reproductive hormone levels in adult men. *J Urol* 2012;**187**:594–598.

Eisenberg ML, Hsieh TC, Lipshultz LI. The relationship between anogenital distance and age. *Andrology* 2013;**1**:90–93.

Gates MA, Mekary RA, Chiu GR, Ding EL, Wittert GA, Araujo AB. Sex steroid hormone levels and body composition in men. *J Clin Endocrinol Metab* 2013;**98**:2442–2450.

Hsieh MH, Breyer BN, Eisenberg ML, Baskin LS. Associations among hypospadias, cryptorchidism, anogenital distance, and endocrine disruption. *Curr Urol Rep* 2008;**9**:137–142.

Hsieh MH, Eisenberg ML, Hittelman AB, Wilson JM, Tasian GE, Baskin LS. Caucasian male infants and boys with hypospadias exhibit reduced anogenital distance. *Hum Reprod* 2012;**27**:1577–1580.

Hu GX, Lian QQ, Ge RS, Hardy DO, Li XK. Phthalate-induced testicular dysgenesis syndrome: Leydig cell influence. *Trends Endocrinol Metab* 2009;**20**:139–145.

Huang PC, Kuo PL, Chou YY, Lin SJ, Lee CC. Association between prenatal exposure to phthalates and the health of newborns. *Environ Int* 2009;**35**:14–20.

Jain VG, Singal AK. Shorter anogenital distance correlates with undescended testis: a detailed genital anthropometric analysis in human newborns. *Hum Reprod* 2013;**28**:2343–2349.

Martin OV, Shialis T, Lester JN, Scrimshaw MD, Boobis AR, Voulvoulis N. Testicular dysgenesis syndrome and the estrogen hypothesis: a quantitative meta-analysis. *Environ Health Perspect* 2008;**116**:149–157.

Mendiola J, Stahlhut RW, Jorgensen N, Liu F, Swan SH. Shorter anogenital distance predicts poorer semen quality in young men in Rochester, New York. *Environ Health Perspect* 2011;**119**:958–963.

Mendiola J, Melgarejo M, Monino-Garcia M, Cutillas-Tolin A, Noguera-Velasco JA, Torres-Cantero AM. Is anogenital distance associated with semen quality in male partners of subfertile couples? *Andrology* 2015;**3**:672–676.

Mira-Escolano MP, Mendiola J, Minguez-Alarcon L, Melgarejo M, Cutillas-Tolin A, Roca M, López-Espín JJ, Noguera-Velasco JA, Torres-Cantero AM. Longer anogenital distance is associated with higher testosterone levels in women: a cross-sectional study. *BJOG* 2014;**121**:1359–1364.

Mitchell RT, Mungall W, McKinnell C, Sharpe RM, Cruickshanks L, Milne L, Smith LB. Anogenital distance plasticity in adulthood: Implications for its use as a biomarker of fetal androgen action. *Endocrinology* 2015;**156**:24–31.

Muller M, den Tonkelaar I, Thijssen JH, Grobbee DE, van der Schouw YT. Endogenous sex hormones in men aged 40-80 years. *Eur J Endocrinol* 2003;**149**:583–589.

Nielsen TL, Hagen C, Wraae K, Brixen K, Petersen PH, Haug E, Larsen R, Andersen M. Visceral and subcutaneous adipose tissue assessed by magnetic resonance imaging in relation to circulating androgens, sex hormone-binding globulin, and luteinizing hormone in young men. *J Clin Endocrinol Metab* 2007;**92**:2696–2705.

Parra MD, Mendiola J, Jorgensen N, Swan SH, Torres-Cantero AM. Anogenital distance and reproductive parameters in young men. *Andrologia* 2016;**48**:3–10.

Rohrmann S, Shiels MS, Lopez DS, Rifai N, Nelson WG, Kanarek N, Guallar E, Menke A, Joshu CE, Feinleib M *et al.* Body fatness and sex steroid hormone concentrations in us men: results from NHANES III. *Cancer Causes Control* 2011;**22**:1141–1151.

Salazar-Martinez E, Romano-Riquer P, Yanez-Marquez E, Longnecker MP, Hernandez-Avila M. Anogenital distance in human male and female newborns: a descriptive, cross-sectional study. *Environ Health* 2004;**3**:8.

Sathyanarayana S, Beard L, Zhou C, Grady R. Measurement and correlates of ano-genital distance in healthy, newborn infants. *Int J Androl* 2010;**33**:317–323.

Scott HM, Hutchison GR, Jobling MS, McKinnell C, Drake AJ, Sharpe RM. Relationship between androgen action in the "male programming window," fetal sertoli cell number, and adult testis size in the rat. *Endocrinology* 2008;**149**:5280–5287.

Seidell JC, Bjorntorp P, Sjostrom L, Kvist H, Sannerstedt R. Visceral fat accumulation in men is positively associated with insulin, glucose, and c-peptide levels, but negatively with testosterone levels. *Metabolism* 1990;**39**:897–901.

Sharpe RM, Skakkebaek NE. Testicular dysgenesis syndrome: mechanistic insights and potential new downstream effects. *Fertil Steril* 2008;**89**:e33–e38.

Sharpe RM, McKinnell C, Kivlin C, Fisher JS. Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. *Reproduction* 2003;**125**:769–784.

Skakkebaek NE, Rajpert-De Meyts E, Main KM. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Hum Reprod* 2001;**16**:972–978.

Stokes-Riner A, Thurston SW, Brazil C, Guzick D, Liu F, Overstreet JW, Wang C, Sparks A, Redmon JB, Swan SH. One semen sample or two? Insights from a study of fertile men. *J Androl* 2007;**28**: 638–643.

Suzuki Y, Yoshinaga J, Mizumoto Y, Serizawa S, Shiraishi H. Foetal exposure to phthalate esters and anogenital distance in male newborns. *Int J Androl* 2012;**35**:236–244.

Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM, Mao CS, Redmon JB, Ternand CL, Sullivan S *et al.* Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environ Health Perspect* 2005;**113**:1056–1061.

Thankamony A, Ong KK, Dunger DB, Acerini CL, Hughes IA. Anogenital distance from birth to 2 years: a population study. *Environ Health Perspect* 2009;**117**:1786–1790.

Thankamony A, Lek N, Carroll D, Williams M, Dunger DB, Acerini CL, Ong KK, Hughes IA. Anogenital distance and penile length in infants with hypospadias or cryptorchidism: comparison with normative data. *Environ Health Perspect* 2014;**122**:207–211.

Torres-Sanchez L, Zepeda M, Cebrian ME, Belkind-Gerson J, Garcia-Hernandez RM, Belkind-Valdovinos U, López-Carrillo L. Dichlorodiphenyldichloroethylene exposure during the first trimester of pregnancy alters the anal position in male infants. *Ann N Y Acad Sci* 2008;**1140**:155–162.

Travison TG, Araujo AB, Kupelian V, O'Donnell AB, McKinlay JB. The relative contributions of aging, health, and lifestyle factors to serum testosterone decline in men. *J Clin Endocrinol Metab* 2007;**92**:549–555.

Vandenput L, Mellstrom D, Karlsson MK, Orwoll E, Labrie F, Ljunggren O, Ohlsson C. Serum estradiol is associated with lean mass in elderly Swedish men. *Eur J Endocrinol* 2010;**162**:737–745.

Welsh M, Saunders PT, Fisken M, Scott HM, Hutchison GR, Smith LB, Sharpe RM. Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. *J Clin Invest* 2008;**118**:1479–1490.

WHO. *WHO Laboratory Manual for the Examination and Processing of Human Semen*, 5th edn. Geneva, Switzerland: WHO Press, 2010.

Yang H, Chen Q, Zhou N, Sun L, Bao H, Tan L *et al.* Lifestyles associated with human semen quality: results from MARHCS cohort study in Chongqing, China. *Medicine (Baltimore)* 2015;**94**:e1166.

Zhou BF. Predictive values of body mass index and waist circumference for risk factors of certain related diseases in Chinese adults—study on optimal cut-off points of body mass index and waist circumference in Chinese adults. *Biomed Environ Sci* 2002;**15**:83–96.