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ORIGINAL ARTICLE *Andrology*

Anogenital distance is associated with semen quality but not reproductive hormones in 1106 young men from the general population

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STUDY QUESTION: Is anogenital distance (AGD) associated with semen quality and reproductive hormones in men from the general population?

SUMMARY ANSWER: Short AGD measured from the anus to the base of scrotum (AGD_{AS}) was associated with reduced sperm counts and morphology but not with sperm motility or reproductive hormones.

WHAT IS KNOWN ALREADY: AGD is longer in males than in females. In rodents, AGD is a well-established and sensitive marker of disruption during the masculinization programming window *in utero* and it has been suggested to be so in humans as well. Therefore, the average AGD would be expected to be shorter in men with poor semen quality, which some studies have confirmed while others have not.

STUDY DESIGN, SIZE, DURATION: This cross-sectional population-based study was of 1106 men included between 2012 and 2016.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Men from the general Danish population (median age 19 years), unselected with regard to fertility status and semen quality, delivered a semen sample, had a blood sample drawn, which was analyzed for concentrations of reproductive hormones, and answered a comprehensive questionnaire. They also had a physical examination performed including determination of AGD measured as the distance between anus and scrotum (AGD_{AS}) and penis (AGD_{AP}). Odds ratios (OR) and 95% CI were estimated for a man having abnormal semen parameters according to the World Health Organization's reference values or a low/high concentration of reproductive hormones (defined as the lowest or highest 10%) depending on AGD. AGD was categorized in four strata: ≤10th percentile, 10th–30th percentile, 30th–50th percentile and >50th percentile.

MAIN RESULTS AND THE ROLE OF CHANCE: Men with the 10% shortest AGD_{AS} had a more than doubled risk (OR: 2.19, 95% CI: 1.40–3.42) of being in the subfertile range for either sperm concentration (<15 million/mL) or sperm morphology (<4%) compared to men with AGD_{AS} above the median (reference). Men in the 10th–30th percentile also had an increased OR of 1.48 (95% CI: 1.06–2.08) but not men in the 30th–50th percentile (OR: 1.14, 95% CI: 0.81–1.62). AGD_{AP} was only weakly related to semen quality. AGD was not associated with testicular volume or any of the reproductive hormones.

LIMITATIONS, REASONS FOR CAUTION: Limitations include the potential non-differential misclassification of reproductive outcomes based on a single semen and blood sample and some between-examiner differences in AGD measurements which introduces noise and may result in an underestimation of observed associations.

WIDER IMPLICATIONS OF THE FINDINGS: Our study of men from the general population confirmed associations between AGD and semen quality, supporting the hypothesis that AGD in humans could be a marker of fetal testicular development. This suggests that the low semen quality in Danish men may partly be explained by prenatal factors.

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Introduction

The potential of male adult semen quality may be established *in utero*, more specifically in the 'masculinization programming window', which occurs between gestational weeks 8 and 14 in humans (Welsh *et al.*, 2014). Exposures, including maternal lifestyle and exposure to environmental chemicals, affecting androgen action during this sensitive window are suspected to have a negative impact on the adult reproductive function by disrupting normal differentiation and development of the male reproductive system at this time (Dean and Sharpe, 2013; Welsh *et al.*, 2014; Thankamony *et al.*, 2016). Consequences of such disruptions, compromising the development and function of the testicular Leydig and Sertoli cells, have been proposed to constitute a testicular dysgenesis syndrome (TDS) in humans (Skakkebaek *et al.*, 2001). The interrelated signs of TDS, cryptorchidism, hypospadias, testicular cancer, decreased testosterone production, impaired spermatogenesis and, more recently, short anogential distance (AGD) (Skakkebaek *et al.*, 2016) are associated with reduced fecundity (Skakkebaek *et al.*, 2001, 2016). However, in humans, studying the effects of exposures *in utero* on adult male reproductive function, including semen quality, is challenging due to the long interval between fetal exposure and adult reproductive function, which can only be assessed 20 years later.

AGD, the distance from anus to the genitals, is longer in males than in females and is, in rodents, a well-established and sensitive marker of disruption during the masculinization programming window (Salazar-Martinez *et al.*, 2004; Foster, 2006; Dean and Sharpe, 2013; Liu *et al.*, 2014; Welsh *et al.*, 2014). Maternal exposure to chemicals with anti-androgenic properties such as phthalates have been shown to be associated with shorter AGD in the male human offspring (Swan *et al.*, 2005; Bustamante-Montes *et al.*, 2013; Barrett *et al.*, 2015; Bornehag *et al.*, 2015), suggesting that AGD is a relevant endpoint also for humans (Arbuckle *et al.*, 2008; Liu *et al.*, 2014). Thus, AGD has been an increasingly used tool in epidemiological research. AGD (body size standardized) is stable over the lifespan in rodents and assumed to be stable throughout life in humans as well (McIntyre *et al.*, 2001). Furthermore, short male AGD has been recognized as part of TDS (Skakkebaek *et al.*, 2016). Therefore, average AGD would be expected to be shorter in men with poor semen quality. Cross-sectional studies have shown associations between shorter AGD and reproductive parameters in adulthood, such as lower testosterone levels, poor semen quality and infertility (Mendiola *et al.*, 2011; Eisenberg *et al.*, 2011, 2012a; Zhou *et al.*, 2016; Foresta *et al.*, 2018). However, most studies have been conducted among infertile men and few among unselected men have been performed and the results have been conflicting; in young US men, short AGD was associated with poorer semen quality, whereas no association was observed among men from Spain or China (Mendiola *et al.*, 2011; Parra *et al.*, 2016; Zhou *et al.*, 2016).

To address this question, we examined the association between AGD and semen quality and reproductive hormones in a large cross-sectional study including more than 1000 young men, unselected with respect to reproductive function. The study was carried out with standardized techniques ensuring comparability to the other studies conducted in similar populations of Western men (Mendiola *et al.*, 2011; Parra *et al.*, 2016).

Materials and Methods

Study population

Because of the military draft in Denmark, all 18-year-old men, except those suffering from severe chronic illness, are required to undergo a physical examination to determine their fitness for military service. We approached the draftees during this examination and invited them to participate in a study of semen quality, regardless of their fitness for military service. Men who agreed to participate in this study were given an appointment for examination at the Department of Growth and Reproduction at Rigshospitalet (Copenhagen, Denmark) and were compensated for their time (500 DKK ~ 67 Euro). All participants completed a questionnaire prior to the day of study participation, at which they delivered a semen sample, had a blood sample drawn, and had a physical examination, including measurements of AGD, which was included in the program from 2012. A detailed description of the study setup has been published previously (Jørgensen *et al.*, 2002, 2012; Priskorn *et al.*, 2018a). The overall participation rate was 24% and did not change when AGD measurements were introduced.

A total of 1395 men were examined from 2012 to 2016, but there were several exclusions. Due to potential variation of AGD by ethnicity (Eisenberg *et al.*, 2011; Mendiola *et al.*, 2011), we excluded 246 men (16.6%) because they or their mothers were not born in Denmark. Further, 27 men with missing AGD measurements and eight men with

AGD measurements more than 3 SD from the mean, as well as eight men with current or recent use of anabolic steroids (self-reported or suspected based on hormone profile) were excluded, leaving 1106 men for further analysis.

Ethical approval

The study was conducted in accordance with the Helsinki Declaration and ethical approval was obtained from the local ethical committee (journal no. H-KF-289428). All participants gave written informed consent.

Physical examination, including measurement of AGD

For measurement of AGD, the man was placed in lithotomy position with his thighs at a 45° angle to the examination table. Using a caliper, the distance was measured from the center of the anus to the posterior base of the scrotum (anoscrotal distance, AGD_{AS}), and to the cephalad insertion of the penis (anopenile distance, AGD_{AP}), as described in Mendiola *et al.* (2011). Each AGD variant was measured three times with numbers on the caliper facing away from the examiner and the average was calculated. The examinations were equally distributed over the study period (from mid-2012 to 2016), and four examiners did 80% of the examinations (ranging from 190 to 261 examinations each), with some overlap between examiners over the study period. In addition, testis size was measured using ultrasonography, and varicocele (grades 1–3) was assessed by palpation. Waist and hip circumference were measured as well as weight and height, from which waist-to-hip ratio and BMI was calculated. The examiner had no knowledge of the man's semen quality or concentrations of reproductive hormones at the time of the examination.

Semen analysis

All men provided a semen sample by masturbation in a room close to the semen laboratory. Men had been asked to abstain from ejaculation for at least 48 h before sample delivery. However, they were not excluded if this was not the case, and reported abstinence time is included in the analysis. The semen sample was kept at 37°C until analysis as described in Jørgensen *et al.* (2002), which is in accordance with the most recent guideline from the World Health Organization (WHO) (World Health Organization, 2010). In short, semen volume was assessed by weighing, sperm concentration was determined in diluted samples using a Bürker–Türk haemocytometer and the total sperm count was calculated (semen volume × sperm concentration). For sperm motility, two drops of well-mixed semen were placed on a glass slide, examined under microscope and the spermatozoa were classified as progressively motile, locally motile or immotile. Fixed and Papanicalaou stained morphology slides were prepared and evaluated according to 'strict criteria' (Menkveld *et al.*, 1990) and the total morphologically normal count was calculated (percentage morphologically normal × total sperm count). For all assessments, measurements were done in duplicates and the average was used.

Reproductive hormone analyses

Serum levels of FSH, LH and estradiol (the latter only for years 2012–2013) were determined using a time-resolved immunofluorometric assay (Delfia, Wallac, Turku, Finland). From 2014, estradiol levels were assessed with a radioimmune assay (Pantex, USA). In 2012–2013 testosterone levels were assessed using time-resolved fluoroimmunoassay (DELFIA, Wallac, Turku, Finland) and from 2014, with ELISA (Access2, Beckman Coultier Ltd, High Wycombe, UK). Inhibin B was throughout determined by a specific two-sided enzyme-immunometric assay (Inhibin B gen II, Beckman Coulter Ltd, High Wycombe, UK). All hormones were analyzed in the same laboratory. The hormones were analyzed yearly in batches including reanalysis of a number of controls from the previous year to ensure comparability over time. Free testosterone was calculated (cFT) based on the measured serum concentrations of total testosterone and sex hormone-binding globulin and assuming a fixed albumin value according to Vermeulen *et al.* (1999) and the ratios of inhibin B/FSH and cFT/LH were calculated.

Questionnaire

All participants completed a questionnaire prior to the examination including information on general and reproductive health, lifestyle and their mother's pregnancy. For the latter part, the men were asked to consult their mother. At the examination, responses were reviewed with the participant to clarify missing or ambiguous information. (For further information see Jensen *et al.*, 2004, 2007, 2014.)

Statistical analyses

First, descriptive statistics on clinical assessment, self-reported variables, semen quality and reproductive hormones were calculated, both for the total population and across AGD_{AS} strata. The distributions were compared by chi-square test for categorical variables and Kruskal Wallis test for continuous variables, except for semen parameters. Pearson's correlation coefficient was calculated to assess the association between AGD_{AS} and AGD_{AP}. AGD and semen parameters were plotted together with a locally weighted scatter-plot smoother (LOESS) curve for the association.

Potential predictors for AGD, including year of examination, examiner and measures of body size (weight, length, BMI, waist-to-hip ratio), were investigated in linear regression analyses. For each body size measure, individual *z*-scores were calculated as the difference between the man's value and the mean value in the population divided by the population standard deviation, and *z*-scores were examined in multivariable models. Associations between the prenatal factors, cryptorchidism, *in-utero* exposure to maternal smoking and low birthweight, and AGD were examined in multiple linear regression analyses adjusted for height, BMI, examiner, year of examination and examiner*year interaction.

The association between AGD and semen quality, reproductive hormones and testicular size were initially analyzed in multivariable linear regression analyses including the same covariates as described below. Several analyses were conducted, including AGD categorized and continuous, the latter investigating both linear and quadratic models. Semen parameters and hormones were transformed (by cubic root, square root or natural logarithm) to fulfill model assumptions of constant variance and normally distributed residuals.

Unadjusted and adjusted associations between AGD and main outcomes (semen parameters and reproductive hormones) were then investigated in logistic regression analyses estimating odds ratios (OR) and 95% CI for a man having one or more abnormal semen parameters or a low/high concentration of different reproductive hormones depending on AGD. Abnormal semen parameters were defined using WHO reference limits for subfertility: semen volume <1.5 mL, sperm concentration <15 million/mL, morphology <4%, progressive motility (A + B) < 32%, and motility (A + B + C) < 40% (World Health Organization, 2010), and abnormal hormones were defined as the lowest 10% of observations (for inhibin B, inhibin B/FSH, cFT, cFT/LH and estradiol) or the highest 10% (for FSH and LH).

Based on the literature, LOESS curves and models including the squared AGD term, we hypothesized that associations with sperm parameters would be strongest for very short AGD (≤10th percentile), and that little change would be seen for long AGD (above the median) (Mendiola *et al.*, 2014; Skakkebaek *et al.*, 2016). Therefore, AGD was categorized in four strata based on percentiles as no obvious cutoffs are available: ≤10th percentile, 10th–30th percentile, 30th–50th percentile and >50th percentile.

Table I Basic characteristics, reproductive hormones and anogenital distance of participants, cross-sectionally investigated 2012–2016, for the total population presented as mean (standard deviation) and median (5th–95th percentiles) and by AGD_{AS} as median (5th–95th percentiles).

	N with data	All included men				AGD_{AS} strata								*P*-value for difference[c]
						≤10th percentile		10th–30th percentile		30th–50th percentile		>50th percentile		
		Mean	SD	Median	(5–95 pctl)	Median	(5–95 pctl)	Median	(5–95 pctl)	Median	(5–95 pctl)	Median	(5–95 pctl)	
Physical appearance, etc.														
Age (years)	1106	19.3	(1.3)	18.9	(18.4–21.8)	19.0	(18.4–21.7)	19.0	(18.4–21.8)	18.9	(18.4–22.3)	18.9	(18.4–21.6)	0.6
Height (m)	1104	1.83	(0.07)	1.83	(1.72–1.94)	1.83	(1.71–1.96)	1.83	(1.71–1.93)	1.83	(1.72–1.93)	1.82	(1.72–1.94)	0.06
Weight (kg)	1095	74.8	(10.6)	73.7	(58.8–94.0)	72.6	(56.8–95.2)	74.0	(58.9–93.0)	72.4	(57.8–91.6)	74.3	(59.4–95.1)	0.03
BMI (kg/m^2)	1093	22.4	(2.8)	22.1	(18.2–27.5)	21.8	(17.9–26.2)	22.1	(18.2–26.9)	21.5	(17.9–27.1)	22.4	(18.5–28.3)	<0.001
Waist-to-hip ratio	1096	0.82	(0.05)	0.82	(0.77–0.90)	0.82	(0.75–0.92)	0.82	(0.77–0.90)	0.82	(0.77–0.90)	0.82	(0.76–0.90)	0.6
Testis size by ultrasound, mean (mL)	1096	13.5	(3.6)	13.2	(8.3–20.0)	12.8	(7.6–20.9)	13.5	(8.1–20.4)	13.6	(7.8–19.8)	13.2	(8.5–19.8)	0.2
Ejaculation abstinence (h)	1098	72	(62)	60	(36–132)	61	(35–133)	61	(36–155)	60	(37–121)	60	(36–134)	0.3
Hormones														
FSH (IU/L)	1101	3.1	(2.0)	2.7	(1.0–6.7)	2.6	(1.0–7.5)	2.7	(1.0–6.8)	2.5	(0.9–6.7)	2.7	(1.1–6.6)	0.5
Inhibin B (pg/mL)	1099	181	(63)	178	(91–286)	179	(72–327)	181	(82–289)	177	(87–293)	176	(93–279)	0.5
Inhibin B/FSH ratio	1099	93	(157)	68	(16–248)	68	(12–331)	68	(15–265)	71	(16–262)	67	(18–204)	0.4
LH (IU/L)	1100	3.7	(1.6)	3.4	(1.6–6.6)	3.4	(1.5–6.2)	3.5	(1.6–7.0)	3.2	(1.5–5.9)	3.4	(1.7–6.7)	0.08
cFT (pmol/L)	1092	453	(156)	433	(269–696)	412	(260–626)	430	(254–667)	420	(270–736)	444	(277–714)	0.05
cFT/LH ratio	1091	143	(72)	130	(61–270)	126	(60–257)	121	(64–271)	142	(56–306)	131	(62–260)	0.03
Estradiol (pmol/L)	1101	81	(28)	80	(39–131)	76	(37–120)	80	(32–126)	77	(39–137)	81	(42–134)	0.1
Anogental distance														
AGD_{AS} (mm)	1106	60.5	(12.9)	60.0	(40.0–82.1)	40.0	(28.6–43.1)	49.4	(44.2–52.7)	56.2	(53.0–59.4)	68.0	(60.3–88.3)	–
AGD_{AP} (mm)	1105	130.5	(11.6)	129.2	(112.3–151.3)	123.2	(110.0–142.6)	126.9	(111.2–143.0)	127.4	(111.7–148.3)	133.0	(116.9–153.3)	<0.001
Lifestyle and health				Percentages										
Self-rated health good/very good (%)	1103			87		84		90		88		86		0.3
Self-rated physical fitness good/very good (%)	1102			58		56		58		60		58		0.9
Taken medication (%)[a]	1100			13.6		14.6		16.1		11.8		13.2		0.6
Fever within the past 3 months (%)	1099			8.8		13.6		5.4		7.1		10		0.01
Treated for cryptorchidism (%)	1093			1.2		1.0		0.9		1.4		1.2		1.0
Varicocele (%)[b]	1096			9.1		8.7		9.9		10.5		8.4		0.8

Continued

Table I *Continued*

	N with data	All included men			AGD_{AS} strata						P-value for difference[c]
					≤10th percentile		10th–30th percentile		30th–50th percentile	>50th percentile	
		Mean	SD	Median (5–95 pctl)	Median	(5–95 pctl)	Median	(5–95 pctl)	Median (5–95 pctl)	Median (5–95 pctl)	
Ever had chlamydia or gonorrhea (%)	1103			5.5	2.9		5.1		7.1	5.6	0.5
Drank alcohol recent week (%)	1104			66	62		67		69	66	0.6
Cigarette smokers (>1 cigarette/day) (%)	1087			31	31		32		35	30	0.6
Mother smoked during pregnancy (%)	1012			15.7	18.3		12.1		13.1	17.6	0.2
Birthweight <2500 g (%)	855			6.0	5.0		6.6		5.6	6.0	1.0

AGD_{AP}, anogenital distance from anus to penis; AGD_{AS}, anogenital distance from anus to scrotum; cFT, calculated free testosterone; 5–95 pctl, 5th–95th percentile.
[a]Taken any medication for at least a week during the 3 months prior to participation in the study.
[b]Grade 2 or 3 varicocele detected at the physical examination.
[c]P-value for comparison of results between the four AGD groups. Kruskal–Wallis test has been used for continuous variables and chi-square test for categorical variables.

The width of the group representing the 10% of men with shortest AGD was broader than the following 10% groups and thus a larger proportion of men were included in these groups, covering the range of AGD with a higher density of men to limit the number of groups. In addition, trend was assessed across strata of AGD by regressing semen parameters on mean AGD level in logistic regression analyses. The impact of the choice of cut-offs on the results was investigated in sensitivity analyses repeating the analyses using several different categorizations, including one with 10 categories of equal size. Based on the initial analyses identifying predictors of AGD, adjusted models all included height, BMI, examiner (four main examiners and 'others'), examination year and examiner*year interaction. Based on the literature, models investigating semen parameters also included period of abstinence (using linear splines with knots at 48 and 96 h (Jørgensen *et al.*, 2001)), and motility analyses additionally included time between sample delivery and analysis. Analyses of reproductive hormones furthermore included smoking (yes/no) and time of blood sampling.

All *P*-values were two-sided, and a *P*-value of <0.05 was considered statistically significant. Statistical analyses were performed using PASW GradPack V.22.0 (SPSS Inc.).

Results

Basic description of study population

The basic description of the total study population and stratified by AGD_{AS} is shown in Table I. The mean age was 19.3 years and mean BMI 22.4 kg/m^2, 31% were regular smokers, and 66% had consumed alcohol within the week prior to study participation. Overall, the men's self-rated health was good (87% had good or very good health) and they reported a low frequency of reproductive disorders (1.2% reported to have been treated for cryptorchidism and 5.5% to have been diagnosed with chlamydia or gonorrhea). The mean AGD_{AS} and AGD_{AP} were 60.5 and 130.5 mm, respectively (Table I); AGD_{AP} was on average 2.2 times longer than AGD_{AS} and the two AGD measurements were correlated ($r = 0.40$, $P < 0.001$).

Predictors of AGD

Examination year and examiner

Overall, AGD measurements increased slightly over time (for AGD_{AS} from 59.4 mm in the first half of the study to 61.5 mm in the last ($P = 0.009$). Differences were also seen between examiners and an examiner*examination year term was significant in multivariable models.

Body size

We found a 1.9 mm increase in AGD_{AS} per BMI *z*-score ($P < 0.001$) and a 1.5 mm increase per weight *z*-score ($P < 0.001$). AGD_{AP} was more sensitive to the men's body size than was AGD_{AS}. In addition to BMI and weight, AGD_{AP} was associated with height and waist-to-hip ratio (all $P < 0.001$), and the absolute and relative increase in AGD_{AP} per *z*-score for different size measures was larger than for AGD_{AS} (Table II). BMI and height were uncorrelated and when both of these variables were included, they each remained significant predictors of AGD_{AP}, and therefore both were included in final models to ensure comparability of models, although height was unrelated to AGD_{AS}.

Prenatal factors

Neither cryptorchidism, in-utero exposure to maternal smoking or low birthweight was associated with AGD in adjusted linear regression analyses.

AGD and semen quality

Overall, AGD_{AS} was positively associated with sperm concentration, total sperm count, percentage morphologically normal spermatozoa and total morphologically normal count but not semen volume or sperm motility. In multiple linear regression analyses, all measures of sperm count and morphology showed a significant linear association with the continuous untransformed AGD_{AS} variable. When a squared AGD_{AS} term was included in the regression models, this term was, in most cases, also significant or of borderline significance suggesting a non-linear association; a conclusion supported by the stratified analysis. Overall, associations between AGD and semen parameters decreased as AGD increased, as seen in the LOESS curve in Fig. 1. Results from multiple logistic regression analyses showed that men with an AGD_{AS} ≤10th percentile were more likely to have semen parameters below the WHO reference levels for normal semen quality. Compared to the reference group (men with an AGD above the median), men with AGD_{AS} ≤10th percentile had an OR of 1.91 (95% CI: 1.14–3.19) of being below the reference limit for sperm concentration, 1.81 (95% CI: 1.06–3.10) for total sperm count, 1.89 (95% CI: 1.18–3.03) for percentage morphologically normal spermatozoa and 2.45 (95% CI: 1.48–4.06) for total normal spermatozoa, while men in the 10th–30th percentile and 30th–50th percentile were not statistically significantly different from the reference. However, significant trends across the categories were observed (Table III and Fig. 2). The OR of being below the reference limit for either sperm concentration or morphology was 2.19 (95% CI: 1.40–3.42) for men ≤10th percentile, 1.48 for men in the 10th–30th percentile (95% CI: 1.06–2.08) and not significantly different from the reference for men in the 30th–50th percentile (OR: 1.14, 95% CI: 0.81–1.62) (Table III). In general, results from linear and logistic regression and from unadjusted and adjusted analyses yielded similar conclusions. As the proportion of men reporting to have had a fever was higher among the men with short AGD, analyses were repeated including this covariate, which did not alter the results. Sensitivity analyses of alternative categorizations yielded similar results as the results presented (Supplementary Tables SI and SII).

While there was a significant trend across AGD_{AP} categories for sperm concentration, and estimates were, in most cases, similar in direction to those seen for AGD_{AS}, AGD_{AP} was not significantly associated with most semen parameters (Supplementary Table III, Supplementary Figs S1 and S2).

AGD, testicular size and reproductive hormones

Despite a smaller testicular volume for men whose AGD_{AS} was ≤10th percentile compared to other men (above the 10th percentile) (Table I), testicular size was not statistically significantly associated with AGD_{AS} in any analyses, while testis size increased slightly with increasing AGD_{AP} (0.2 mL increase per cm increase in AGD_{AP}, $P = 0.04$) in unadjusted but not adjusted analyses. AGD was not consistently associated with any of the reproductive hormones in adjusted logistic or linear regression analyses (Table IV).

Discussion

We report a significant positive association between AGD_{AS} (AGD measured as the distance between anus and scrotum) and semen quality in our cross-sectional study of more than 1000 men from the general population. Men with the shortest AGD_{AS} (≤10th percentile) had a more than doubled risk of being in the subfertile range for either sperm concentration or sperm morphology compared to men with AGD_{AS} above the median. On the other hand, AGD_{AP} (measured as the distance between anus and penis) was largely unrelated to semen quality, and we saw no clear association between AGD and testicular size or levels of testosterone or other reproductive hormones measured.

Two previous studies were conducted in populations comparable to ours, including primarily Caucasian men of similar age; young US men (the Rochester Young Men's Study, $N = 103$) and young Spanish men (the Murcia Young Men's Study, $N = 215$). In all three studies, semen analysis methods were the same, and all reproductive hormone levels were analyzed in the same laboratory in Copenhagen. The same measurement technique for AGD was used as Mendiola, who conducted the majority of measurements in US and Spanish men, was the one who trained the examiners in the present study. Still, especially AGD_{AS} was on average longer in our study (AGD_{AS}: 60.5 mm and AGD_{AP}: 130.5 mm) compared to that in Caucasian men in USA (51.2 and 128.0 mm) and Spain (48.3 and 128.0 mm), which cannot be explained by the observed differences in BMI or other basic characteristics (see Supplementary Table SIV for basic description of the three populations). An association between AGD and semen quality was observed in our study and the study of young US men, which were different regarding sperm counts (average semen quality was higher in the young US men), and not in young Spanish men who had a semen quality similar to that in our study. In young Chinese men of similar age (Zhou *et al.*, 2016) no association between AGD and semen quality was found, whereas AGD was found to be associated with semen parameters in a recent Italian study (Foresta *et al.*, 2018). In both Spain and China, a recent decline in semen quality has been suggested based on studies among university students and semen donors, with increased agricultural industrialization and environmental pollution as possible explanations (Mendiola *et al.*, 2013; Huang *et al.*, 2017). Thus, the lack of association between AGD and semen quality in the Spanish and Chinese populations might be due to higher environmental exposures during adult life which could have affected the young men's semen quality but not their AGD, which was determined *in utero* (Parra *et al.*, 2016). This hypothesis is supported by the finding that AGD_{AS} was positively associated with semen quality and fatherhood status in slightly older US and Spanish men attending infertility services, (Eisenberg *et al.*, 2011; Mendiola *et al.*, 2015). In contrast, the observed associations in the present study corroborate that the reported low semen quality in Danish men may partly be explained by prenatal factors (Priskorn *et al.*, 2018a). The magnitude of association was moderate supporting that only some cases of poor semen quality are due to TDS and that adult exposures and lifestyle can impair spermatogenesis as well (Jørgensen *et al.*, 2010b)

Despite the known association between semen quality and reproductive hormones in men from the general population (Jørgensen *et al.*, 2010a, 2016), the observed association between AGD and semen quality was not reflected in the levels of reproductive

Table II Association between anogenital distance and body size (z-scored height, weight, BMI, and waist-to-hip ratio) in linear regression analyses of participants, cross-sectionally investigated 2012–2016.

	AGD_{AS} (mm)		AGD_{AP} (mm)	
	Estimate	***P*-value**	**Estimate**	***P*-value**
Height (z-score)	−0.6	0.118	1.7	<0.001
Weight (z-score)	1.5	<0.001	6.6	<0.001
BMI (z-score)	1.9	<0.001	6.4	<0.001
Waist-to-hip ratio (z-score)	0.1	0.800	2.0	<0.001

AGD_{AP}, anogenital distance from anus to penis; AGD_{AS}, anogenital distance from anus to scrotum; BMI, body mass index.

hormones. No association between AGD_{AS} and either hormones or semen quality in the young Spanish and Chinese men was reported (Parra *et al.*, 2016; Zhou *et al.*, 2016). However, in primarily infertile men, a positive association between AGD_{AS} and testosterone levels were found (Eisenberg *et al.*, 2012a). Our population was overall healthy, and the associations with semen quality were moderate, which could mask a potential association between AGD and hormones that might be present in infertile men, where the distributions of reproductive hormone levels would be expected to be adversely shifted (Andersson *et al.*, 2004). Children born with hypospadias or cryptorchidism have, on average, shorter AGD (Hsieh *et al.*, 2012; Jain and Singal, 2013; Thankamony *et al.*, 2014). In our data, none of the

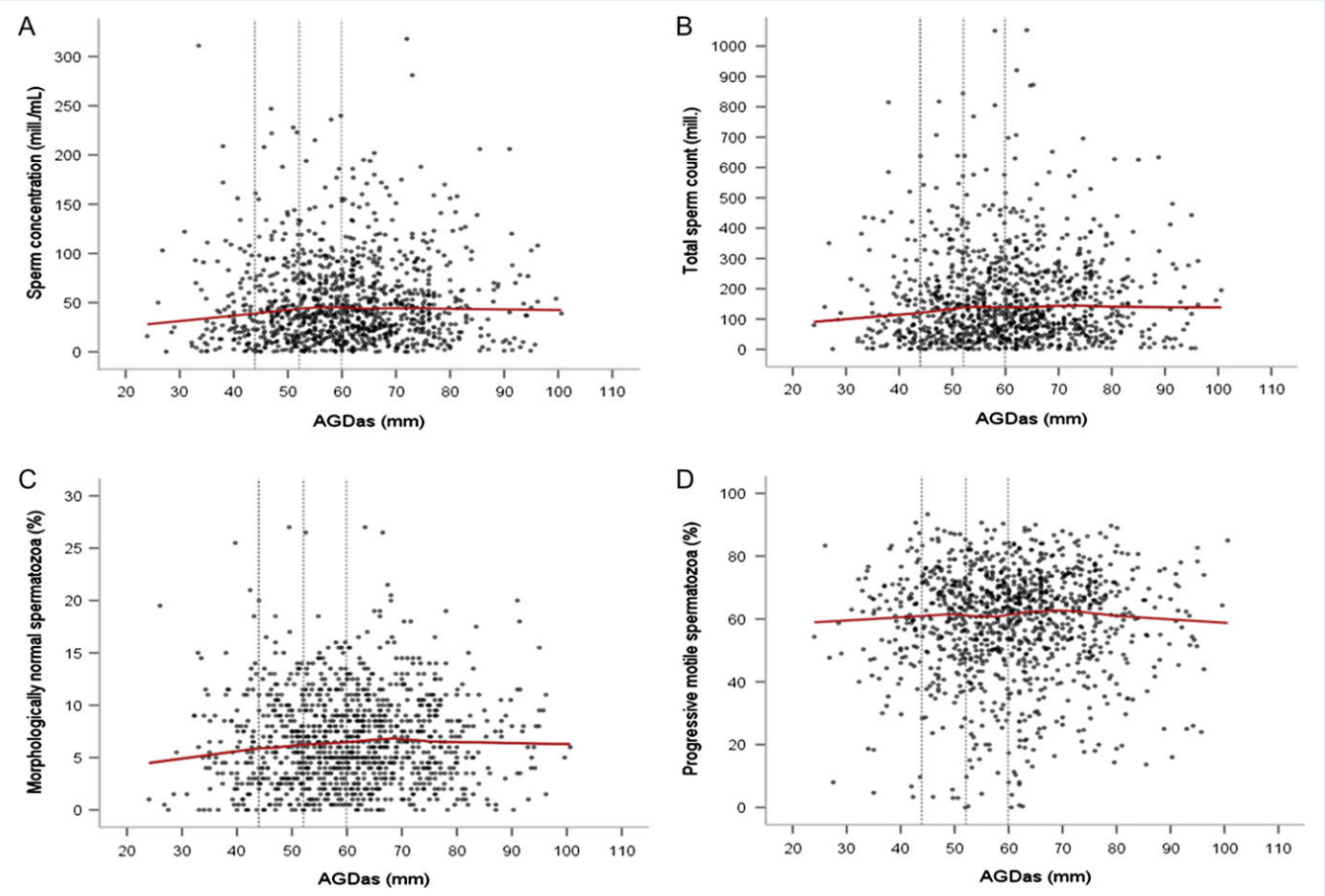

Figure 1 Plot of AGD_{AS} and semen parameters for participants, cross-sectionally investigated 2012–2016. (**A**) Sperm concentration, (**B**) total sperm count, (**C**) morphologically normal spermatozoa and (**D**) progressive motile spermatozoa. Abbreviations: AGD_{AS}, anogenital distance from anus to scrotum. Red line = LOESS (locally weighted scatter-plot smoother) curve; dotted lines=10th, 30th and 50th percentile (cutoffs for AGD strata). For visual purposes, the *y*-axis for sperm concentration and total sperm count is cut at +3 SD, but observations above +3 SD still contribute to the calculation of the LOESS curve.

Table III Associations between AGD_{AS} and semen parameters of participants, cross-sectionally investigated 2012–2016, presented as raw values (median with 5th–95th percentiles) and results from logistic regression analyses (odds ratio with 95% confidence interval for having abnormal semen parameters according to WHO).

	Raw values	Events	Unadjusted model		Adjusted model[a]	
	Median (5–95 pctl)	%	OR[b]	95% CI	OR[b]	95% CI
Semen volume (event: <1.5 mL)						
AGD_{AS}, ≤10th percentile	3.4 (1.1–5.7) mL	7%	1.084	(0.469–2.506)	1.173	(0.491–2.806)
AGD_{AS}, 10th–30th percentile	3.3 (1.2–6.2) mL	8%	1.419	(0.795–2.532)	1.414	(0.772–2.590)
AGD_{AS}, 30th–50th percentile	3.2 (1.4–5.8) mL	5%	0.813	(0.406–1.629)	0.774	(0.369–1.624)
AGD_{AS}, >50th percentile	3.2 (1.3–5.9) mL	6%	Ref.		Ref.	
P-trend			0.5		0.5	
Total population (*N* = 1104)	3.2 (1.3–5.9) mL	7%				
Sperm concentration (event: <15 million/mL)						
AGD_{AS}, ≤10th percentile	30 (1–132) mill./mL	26%	1.769*	(1.082–2.891)	1.905*	(1.137–3.190)
AGD_{AS}, 10th–30th percentile	40 (2–145) mill./mL	20%	1.266	(0.851–1.885)	1.268	(0.834–1.927)
AGD_{AS}, 30th–50th percentile	41 (3–147) mill./mL	18%	1.081	(0.714–1.636)	1.057	(0.688–1.624)
AGD_{AS}, >50th percentile	43 (4–150) mill./mL	17%	Ref.		Ref.	
P-trend			0.03		0.03	
Total population (*N* = 1101)	41 (3–147) mill./mL	19%				
Total sperm count (event: <39 million)						
AGD_{AS}, ≤10th percentile	95 (3–435) mill.	23%	1.603	(0.963–2.668)	1.811*	(1.057–3.103)
AGD_{AS}, 10th–30th percentile	136 (7–513) mill.	19%	1.237	(0.822–1.859)	1.279	(0.829–1.975)
AGD_{AS}, 30th–50th percentile	127 (14–460) mill.	16%	0.972	(0.822–1.859)	0.979	(0.622–1.541)
AGD_{AS}, >50th percentile	128 (11–452) mill.	17%	Ref.		Ref.	
P-trend			0.09		0.049	
Total population (*N* = 1096)	125 (8–456) mill.	17%				
Normal morphology (event: <4%)						
AGD_{AS}, ≤10th percentile	5.3 (0.0–15.0) %	37%	1.869*	(1.197–2.917)	1.893*	(1.184–3.028)
AGD_{AS}, 10th–30th percentile	6.0 (0.5–14.0) %	33%	1.519*	(1.077–2.144)	1.434	(0.998–2.060)
AGD_{AS}, 30th–50th percentile	6.0 (0.5–14.5) %	27%	1.188	(0.828–1.704)	1.203	(0.827–1.750)
AGD_{AS}, >50th percentile	6.5 (1.0–14.5) %	24%	Ref.		Ref.	
P-trend			0.001		0.004	
Total population (*N* = 1089)	6.0 (0.5–14.5) %	28%				
Total normal spermatozoa (event: <1.56 million)						
AGD_{AS}, ≤10th percentile	4.6 (0.0–56.5) mill.	30%	2.174*	(1.350–3.501)	2.451*	(1.480–4.061)
AGD_{AS}, 10th–30th percentile	7.2 (0.0–46.9) mill.	22%	1.431	(0.968–2.114)	1.463	(0.969–2.209)
AGD_{AS}, 30th–50th percentile	8.0 (0.2–34.4) mill.	17%	1.013	(0.662–1.551)	1.013	(0.653–1.571)
AGD_{AS}, >50th percentile	8.4 (0.1–47.4) mill.	17%	Ref.		Ref.	
P-trend			0.002		0.001	
Total population (*N* = 1086)	7.6 (0.1–45.3) mill.	19%				
Progressive motile spermatozoa (event: <32%)						
AGD_{AS}, ≤10th percentile	60 (11–83) %	9%	0.910	(0.434–1.905)	0.882	(0.407–1.909)
AGD_{AS}, 10th–30th percentile	63 (26–84) %	7%	0.665	(0.361–1.224)	0.691	(0.364–1.309)
AGD_{AS}, 30th–50th percentile	61 (23–81) %	9%	0.984	(0.574–1.688)	0.995	(0.567–1.745)
AGD_{AS}, >50th percentile	63 (22–83) %	10%	Ref.		Ref.	
P-trend			0.4		0.4	
Total population (*N* = 1097)	62 (23–83) %	9%				
Motile spermatozoa (event: <40%)						
AGD_{AS}, ≤10th percentile	66 (16–89) %	8%	0.878	(0.403–1.912)	0.834	(0.369–1.884)
AGD_{AS}, 10th–30th percentile	69 (34–89) %	6%	0.613	(0.320–1.175)	0.621	(0.312–1.237)

Continued

Table III *Continued*

	Raw values	Events	Unadjusted model		Adjusted model[a]	
	Median (5–95 pctl)	%	OR[b]	95% CI	OR[b]	95% CI
AGD_{AS}, 30th–50th percentile	67 (35–87) %	9%	0.958	(0.546–1.682)	0.982	(0.545–1.771)
AGD_{AS}, >50th percentile	69 (32–88) %	9%	Ref.		Ref.	
P-trend			0.3		0.3	
Total population (*N* = 1097)	68 (32–88) %	8%				
Sperm concentration or morphology						
AGD_{AS}, ≤10th percentile		49%	2.062*	(1.346–3.159)	2.188*	(1.398–3.424)
AGD_{AS}, 10th–30th percentile		41%	1.486*	(1.074–2.055)	1.480*	(1.055–2.078)
AGD_{AS}, 30th–50th percentile		35%	1.134	(0.812–1.585)	1.142	(0.806–1.616)
AGD_{AS}, >50th percentile		32%	Ref.		Ref.	
P-trend			0.07		<0.001	
Total population (*N* = 1089)		36%				
Concentration, morphology or motility						
AGD_{AS}, ≤10th percentile		49%	1.853*	(1.211–2.836)	1.939*	(1.237–3.040)
AGD_{AS}, 10th–30th percentile		42%	1.388*	(1.006–1.915)	1.376	(0.981–1.939)
AGD_{AS}, 30th–50th percentile		36%	1.085	(0.780–1.510)	1.102	(0.779–1.558)
AGD_{AS}, >50th percentile		34%	Ref.		Ref.	
P-trend			0.003		0.003	
Total population (*N* = 1089)		37%				

AGD_{AS}, anogenital distance from anus to scrotum; OR, odds ratio; 5–95 pctl, 5th–95th percentile; 95% CI, 95% confidence interval.
[a]Adjusted for height, BMI, examiner, examination year, examiner*year and period of abstinence, and in all analyses including motility, time between sample delivery and analysis.
[b]Odds ratio of being below the WHO reference level for low semen quality, referred to as 'event' in the above table.
*$P < 0.05$.

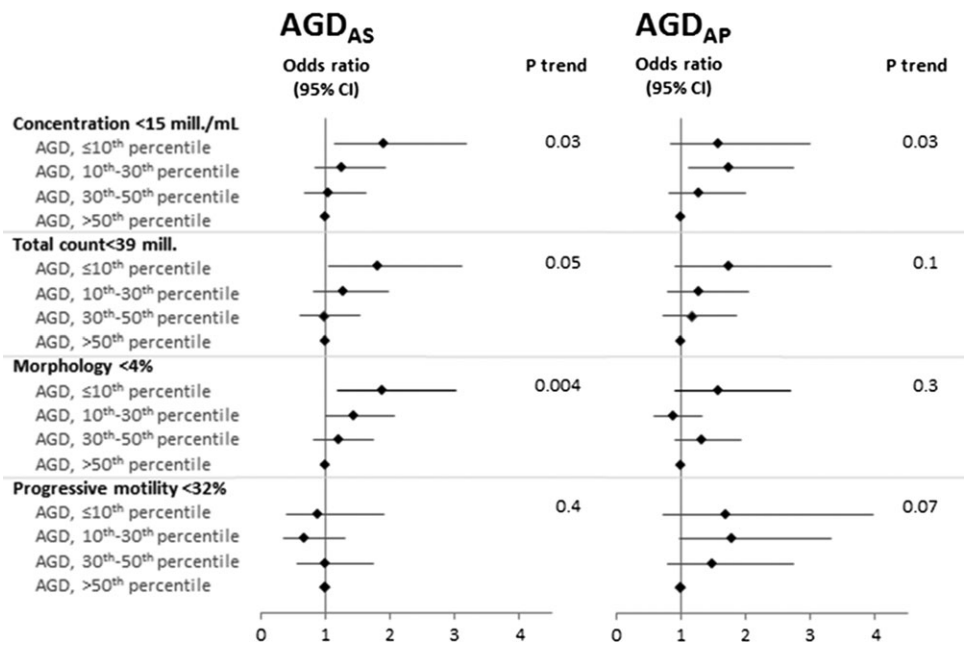

Figure 2 Odds ratios and 95% confidence intervals for having semen parameters below the WHO reference levels depending on anogenital distance. Abbreviations: AGD_{AP}, anogenital distance from anus to penis; AGD_{AS}, anogenital distance from anus to scrotum. Odds ratio of having: sperm concentration below 15 million/mL; total sperm count below 39 million; percentage morphologically normal spermatozoa below 4%; or percentage progressive motile spermatozoa below 32%. Analyses are adjusted for height, BMI, examiner, examination year, examiner*year and period of abstinence and, for motility, time between sample delivery and analysis.

Table IV Associations between anogenital distance and reproductive hormones of participants, cross-sectionally investigated 2012–2016, presented as results from logistic regression analyses (odds ratio with 95% confidence interval for having low/high hormone levels).

	AGD_{AS}			AGD_{AP}		
	Events	Adjusted model[a]		Events	Adjusted model[a]	
	%	OR[b]	(95% CI)	%	OR[b]	(95% CI)
FSH (event: >90th percentile)						
AGD, ≤10th percentile	15%	1.492	(0.779–2.855)	13%	1.994	(0.938–4.239)
AGD, 10th–30th percentile	7%	0.691	(0.377–1.264)	8%	0.790	(0.413–1.514)
AGD, 30th–50th percentile	10%	1.072	(0.625–1.839)	9%	1.010	(0.568–1.795)
AGD, >50th percentile	10%	Ref.		11%	Ref.	
P-trend		0.8			0.4	
Total population (*N* = 1101)	10%			10%		
Inhibin B (event: ≤10th percentile)						
AGD, ≤10th percentile	12%	1.122	(0.567–2.220)	7%	0.874	(0.363–2.106)
AGD, 10th–30th percentile	9%	0.750	(0.424–1.326)	9%	1.139	(0.625–2.078)
AGD, 30th–50th percentile	11%	1.105	(0.652–1.870)	12%	1.558	(0.908–2.672)
AGD, >50th percentile	10%	Ref.		11%	Ref.	
P-trend		0.8			0.7	
Total population (*N* = 1099)	10%			10%		
Inhibin B/FSH (event: ≤10th percentile)						
AGD, ≤10th percentile	14%	1.593	(0.827–3.067)	13%	2.243*	(1.046–4.809)
AGD, 10th–30th percentile	8%	0.688	(0.368–1.286)	8%	0.941	(0.494–1.793)
AGD, 30th–50th percentile	12%	1.388	(0.820–2.347)	9%	1.070	(0.593–1.929)
AGD, >50th percentile	9%	0.600		11%	Ref.	
P-trend		0.7			0.2	
Total population (*N* = 1099)	10%			10%		
LH (event: >90th percentile)						
AGD, ≤10th percentile	9%	0.811	(0.381–1.725)	9%	1.114	(0.489–2.540)
AGD, 10th–30th percentile	15%	1.552	(0.958–2.514)	12%	1.520	(0.862–2.678)
AGD, 30th–50th percentile	7%	0.668	(0.359–1.242)	9%	1.105	(0.619–1.974)
AGD, >50th percentile	10%	Ref.		10%	Ref.	
P-trend		0.7			0.4	
Total population (*N* = 1093)	10%			10%		
cFT (event: ≤10th percentile)						
AGD, ≤10th percentile	15%	1.534	(0.804–2.925)	11%	0.671	(0.300–1.500)
AGD, 10th–30th percentile	12%	1.136	(0.667–1.936)	8%	0.635	(0.339–1.191)
AGD, 30th–50th percentile	9%	1.084	(0.612–1.919)	10%	0.818	(0.462–1.448)
AGD, >50th percentile	9%	Ref.		11%	Ref.	
P-trend		0.3			0.2	
Total population (*N* = 1092)	10%			10%		
cFT/LH (event: ≤10th percentile)						
AGD, ≤10th percentile	12%	0.905	(0.434–1.887)	11%	1.115	(0.510–2.439)
AGD, 10th–30th percentile	11%	1.179	(0.699–1.990)	10%	1.099	(0.610–1.980)
AGD, 30th–50th percentile	9%	1.209	(0.702–2.082)	8%	0.853	(0.470–1.546)
AGD, >50th percentile	10%	Ref.		10%	Ref.	
P-trend		0.8			0.8	
Total population (*N* = 1091)	10%			10%		

Continued

Table IV *Continued*

	AGD_{AS}			AGD_{AP}		
	Events	Adjusted model[a]		Events	Adjusted model[a]	
	%	OR[b]	(95% CI)	%	OR[b]	(95% CI)
Estradiol (event: ≤10th percentile)						
AGD, ≤10th percentile	15%	1.699	(0.839–3.442)	6%	0.842	(0.309–2.300)
AGD, 10th–30th percentile	12%	1.209	(0.684–2.139)	12%	1.483	(0.807–2.725)
AGD, 30th–50th percentile	11%	1.112	(0.615–2.009)	9%	0.910	(0.494–1.677)
AGD, >50th percentile	8%	Ref.		11%	Ref.	
P-trend		0.2			0.4	
Total population (*N* = 1101)	10%			10%		

AGD_{AP}, anogenital distance from anus to penis; AGD_{AS}, anogenital distance from anus to scrotum; cFT, calculated free testosterone; OR, odds ratio.
[a]Adjusted for BMI, examiner, examination year, examiner*examination year, smoking, time of blood sampling.
[b]Odds ratio (OR) of being in the lowest 10% (for inhibin B, inhibin B/FSH, cFT, cFT/LH and estradiol) or in the highest 10% (for FSH and LH), referred to as 'event' in the above table.
*$P < 0.05$.

participants reported having been operated for testicular cancer or hypospadias. Only 26 men reported congenital cryptorchidism, which was not associated with either AGD variant, or with semen quality.

While this study suggests that men with short AGD are at increased risk of being in the subfertile range, semen quality varied considerably across the range of AGD, and many men with short AGD had normal semen quality. This suggest that AGD and the maximum potential for semen quality, which is highly dependent on Sertoli cell number (Scott *et al.*, 2007; Sharpe, 2010), are not determined within the same developmental window. While AGD is determined in the masculinization programming window in both rodents and humans (Martino-Andrade *et al.*, 2016), rodent studies have shown that although *in utero* exposures to anti-androgenic compounds reduces fetal Sertoli cell proliferation, a continued exposure was needed to suppress a compensatory increase in Sertoli cell numbers perinatally (Auharek *et al.*, 2010). Consequently, withdrawal of an adverse exposure after the end of the masculinization programming window could result in a better semen quality than would be predicted by AGD alone. Thus, the clinical interpretation of a short AGD may not be straightforward. However, fertility problems in the absence of a short AGD might reflect a problem that is not of prenatal origin, and thus, potentially reversible (Sharpe, 2010). Based on 46 men, Eisenberg *et al.* (2012b) reported that men with longer AGD were more likely to experience improved semen quality after varicocelectomy, confirming a potential clinical relevance. Thus, further studies on selected populations could help identify subgroups of infertile men whose AGD measurement may have clinical applicability.

A major strength of the present study is the large study population. We believe that the men participating in the study represent the general population of young men regarding reproductive function as the men, due to their age, have essentially no knowledge of their reproductive potential. Furthermore, a previous study conducted when the original study was initiated showed that levels of reproductive hormones were similar in participants and non-participants (Andersen *et al.*, 2000). Limitations include the potential misclassification of reproductive outcomes based on a single semen sample and blood sample and some between-examiner differences in AGD measurements. We hypothesized that AGD, as well as the potential for adult reproductive function, is determined *in utero*. However, rodent studies are inconsistent on the permanence of body size adjusted AGD, and AGD has been shown to be slightly responsive to changes in androgen action prenatally (Mitchell *et al.*, 2015; Kita *et al.*, 2017), which may limit the use of AGD as a readout of androgen action *in utero*. No studies have followed a population of human males with repeated AGD measurements throughout life, and therefore it is possible that AGD in humans may be modifiable and inferences about fetal development based on AGD measures in adults may be premature. However studies of repeated measurements during childhood suggest that AGD is a relatively stable phenotypic signature (Papadopoulou *et al.*, 2013; Priskorn *et al.*, 2018b).

In conclusion, our study confirmed associations between AGD and semen quality in men from the general population. By contrast, we did not observe associations between AGD and reproductive hormone levels or testicular size. Our findings support the hypothesis that AGD in humans is a marker of fetal testicular development, whereas the clinical value of measuring AGD is questionable.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

Substantial contribution to conception and design: L.P., J.M., T.K.J., S.H.S. and N.J. Data acquisition: L.P., A.K.B., L.N., M.K. and N.J. Data analysis: L.P., S.H.S. and N.J. Data interpretation: all authors. Drafting the article: L.P., S.H.S. and N.J. Revising the article critically for important intellectual content: all authors. Final approval of the article: all authors.

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Conflict of interest

The authors have nothing to declare.

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