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ORIGINAL ARTICLE

The relationship between anogenital distance and the androgen receptor CAG repeat length

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Anogenital distance (AGD) is used to define degree of virilization of genital development, with shorter length being associated with feminization and male infertility. The first exon of the androgen receptor (AR) consists of a polymorphic sequence of cytosine–adenine–guanine (CAG) repeats, with longer CAG repeat lengths being associated with decreased receptor function. We sought to determine if there is an association between AGD and AR CAG repeat length. A cross-sectional, prospective cohort of men evaluated at a urology clinic at a single institution was recruited. AGD (the distance from the posterior scrotum to the anal verge) and penile length (PL) were measured. Sanger DNA sequence analysis was used to define CAG repeat length. AGD and CAG repeat lengths in 195 men were determined. On unadjusted analysis, there was no linear relationship between CAG repeat length and PL ($P=0.17$) or AGD ($P=0.31$). However, on sub-population analyses, those men with longer CAG repeat lengths (>26) had significantly shorter AGDs compared to men with shorter CAG repeat lengths. For example, the mean AGD was 41.9 *vs.* 32.4 mm with a CAG repeat length ⩽26 *vs.* >26 ($P=0.01$). In addition, when stratifying the cohort based on AGD, those with AGD less than the median (i.e. 40 mm) had a longer CAG repeat length compared to men with an AGD >40 mm ($P=0.02$). In summary, no linear relationship was found between AGD and AR CAG repeat length overall.

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INTRODUCTION

A marker for genital development, anogenital distance (AGD) has been examined in both animals and humans.[1–4] In humans, a relationship among AGD, sperm count and testosterone production was described, suggesting a relationship between genital tract development and function.[5–7] Nevertheless, the cause of varied AGDs between men remains uncertain. Rodent studies suggest that *in utero* androgen signaling during the masculinization programing window determines adult AGD with minimal influence from postnatal exposures to androgens.[8] This correlation implies that AGD may reflect fetal determinants in the adult of prenatal androgen and estrogen exposures.

Although *in utero* environmental insults have been hypothesized to compromise normal human genital development,[3,9,10] other intrinsic fetal factors may lead to abnormal testicular function and development. Androgen signaling is critical to the development of the male phenotype and the effects are mediated through the androgen receptor (AR). Thus, mutations in the AR may explain discrepancies in genital formation.

The AR is located on the short arm of the X chromosome and is encoded by eight exons.[11] Exon 1 contains the polymorphic glutamine segment coded by the CAG repeat tract. The normal range for the CAG repeat tract of the AR is approximately 9–34 repeats and is known to vary with race and possibly fertility.[11–13] The CAG repeat length is thought to correlate with androgen sensitivity, where shorter lengths display increased androgen sensitivity and longer lengths are more androgen-resistant, perhaps due to differential affinity of nuclear protein coactivators for the AR.[14,15] Indeed, many groups have examined a relationship between the length of the polyglutamine repeat in the AR and male factor infertility.[16–18] To date, no work has examined the relationship between a man's AGD and his AR CAG repeat length.

MATERIALS AND METHODS

The methods of collection and cohort assembly were previously reported.[5,7] Briefly, after obtaining Institutional Review Board approval from Baylor College of Medicine (USA), patients ($n=195$) were recruited from a urology clinic specializing in reproductive and sexual medicine from August 2010 through October 2011. All men provided written consent for participation. Age, self-reported race, height and weight were recorded.

Genital measurements

The methods of genital measurement were previously described.[5,7] Briefly, in the supine, frog-legged position with the legs abducted allowing the soles of the feet to meet, the distance from the posterior aspect of the scrotum to the anal verge was measured using a digital caliper (model No. 01407A; Neiko, USA). This was defined as the AGD.

From the same position, the stretched penile length (PL) was measured from the base of the dorsal surface of the penis to the tip of the glans.

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Table 1 Demographic, reproductive and anthropomorphic characteristics of the cohort (*N*=195)

Characteristic		*Value*
Age (year), mean±s.d.		45.1±14.4
Race, *n*(%)	Caucasian	151 (77.4)
	Others	44 (22.6)
Height (cm), mean±s.d.		178.5±6.6
Weight (kg), mean±s.d.		92.0±15.0
BMI category, *n*(%)	Normal	28 (14.4)
	Overweight	88 (45.1)
	Obese	79 (40.5)
Father, *n*(%)	No	98 (50.2)
	Yes	97 (49.7)
Reason for office visit, *n*(%)[a]	General urology	21 (10.9)
	Erectile dysfunction	10 (5.2)
	Hypogonadism	64 (33.3)
	Infertility	75 (39.1)
	Vasectomy	1 (0.5)
	Vasectomy reversal	21 (10.9)

Abbreviation: BMI, body mass index.
[a] Totals that add up to less than 195 indicate incomplete data.

AGD was measured by five investigators (four authors (MLE, TCH, MGM and RCW) and one collaborator). Previous data suggested reproducibility of the measurements with the correlation coefficient of 0.91 for both AGD and PL measurements. Moreover, the within-subject standard deviation was 4.1 mm for AGD and 5.4 mm for stretched PL. In addition, there was no evidence for the measurement error being proportional to the magnitude of the measurement.[5] Within-observer variability was not assessed in this measured population. However, investigators using a similar technique on paid volunteers reported relatively small variability (2.1%–2.7% of the mean AGD).[6] Moreover, previous measurements performed in children (MLE participated) also report good reproducibility (intraobserver coefficient of variation is 3.3).[19]

DNA isolation and analysis

After the clinical encounter, all participants had approximately 10 ml of blood drawn. Genomic DNA was isolated from peripheral blood leukocytes using the Qiagen DNeasy Blood and Tissue Extraction kit (Qiagen, Inc., Valencia, CA, USA). The CAG repeat region of the AR was amplified by polymerase chain reaction using sequence-specific primers as previously described, and Sanger DNA sequencing performed by Genewiz, Inc. (South Plainfield, NJ, USA).[17,20] The data were then analyzed using Mutation Surveyor (Softgenetics, Inc., State College, PA, USA) to calculate the number of CAG repeats in both the sense and antisense directions. Specimens were frozen at collection then all analyzed simultaneously at recruitment completion.

Statistical analysis

One-way analysis of variance (ANOVA) was used to compare normally distributed continuous variables. The Wilcoxon rank sum test was used to compare non-parametrically distributed continuous variables. Pearson's correlation coefficients were calculated to determine the association between continuous variables. Logistic regression models were used to determine the relationship between genital measures and ARCAG repeat length after dichotomizing CAG and AGD length based on experience gained from prior analyses conducted on AR CAG length.[11] Covariates that have been shown to affect AGD were selected for inclusion *a priori* including age, race and fatherhood status. Models for PL were adjusted for age, race and body mass index (BMI). Given the non-parametric distribution of genital measures and CAG repeat length, regression models were also run with log-transformed variables with no differences in the overall conclusions. Effect modification of fatherhood was assessed using the likelihood ratio test by entering AGD along with fatherhood as well as the term for their product in the multivariable model. In addition, stratified analyses were also performed to judge effect modification. $P<0.05$ was considered significant, and all P values were two sided. Analyses were performed using Stata 10 (StataCorp LP, College Station, TX, USA).

RESULTS

In all, 195 men had both AGD measured and AR CAG repeat length determined with a mean age (s.d.) of 45.1 (14.4) years. Men were seen for a variety of urological complaints. A majority of the men were Caucasian with equal numbers of fathers and childless men (**Table 1**). The mean number of CAG repeats was 21.7±3.3. The mean AGD (s.d.) was 41.3 (13.4) mm and the mean PL (s.d.) was 113.2 (26.0) mm.

Since the normal AR repeat length varies, we focused on each extreme. Men with longer CAG repeat lengths (>26) had significantly

Table 2 AGDs stratified by CAG repeat length[a]

AR CAG repeat length cutoff (repeat no.)	*⩽ cutoff*		*> cutoff*			*Multivariable analysis[b]*	
	n	*Mean AGD (mm), mean±s.d.*	n	*Mean AGD (mm), mean±s.d.*	P*	*OR (95% CI)*	P
15	6	45.1±12.6	189	41.2±13.4	0.41	1.15 (0.64, 2.05)	0.64
16	10	43.6±10.1	185	41.2±13.6	0.39	1.08 (0.67, 1.72)	0.76
17	18	41.3±9.4	177	41.3±13.8	0.70	0.87 (0.58, 1.31)	0.51
18	28	40.0±10.5	167	41.5±13.8	0.91	0.82 (0.58, 1.15)	0.26
19	42	42.5±13.1	153	40.9±13.5	0.34	1.04 (0.79, 1.36)	0.80
24	159	41.3±12.8	36	41.4±15.8	0.61	0.95 (0.72, 1.26)	0.74
25	176	41.8±13.4	19	36.7±12.9	0.09	1.41 (0.91, 2.17)	0.12
26	183	41.9±13.4	12	32.4±10.9	0.01	2.20 (1.12, 4.32)	0.02
27	188	41.7±13.4	7	29.0±7.2	0.01	4.00 (1.22, 13.11)	0.02
28	192	41.5±13.3	3	28.0±10.4	0.07	4.43 (0.70, 28.09)	0.11

Abbreviations: AGD, anogenital distance; CAG, cytosine–adenine–guanine; CI, confidence interval; OR, odds ratio.
[a] Listed lengths represent the extremes of long and short repeats.
[b] Multivariable OR (95% CI) adjusted for age, race and fatherhood states the odds for having a CAG repeat length less than the cutoff for each 10 mm increase in AGD.
*P value represents Wilcoxon rank sum analyses.

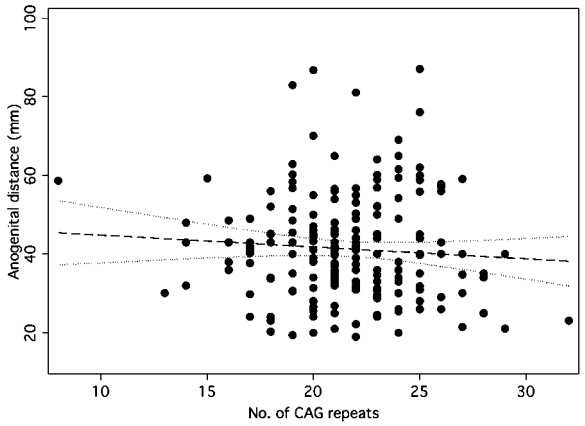

Figure 1 Scatterplot and linear best fit line (dashed line) with 95% confidence interval (dotted line) demonstrating relationship between anogenital distance and CAG repeat length. CAG, cytosine–adenine–guanine.

shorter AGD compared to men with shorter CAG repeat lengths (**Table 2**). For example, the mean AGD was 41.9 *vs.* 32.4 mm with a CAG repeat length $\leqslant$26 *vs.* >26. The relationship remained after multivariable adjustment. For short CAG repeat lengths, the AGD length differences were not significantly different (**Table 2**). There was no difference in AGD length across intermediate CAG repeat lengths. In addition, there was no linear relationship between AGD and the number of CAG repeats (P=0.31; **Figure 1**).

When examining PL, there was no linear relationship between PL and CAG repeat length (P=0.17), nor when examining longer or shorter CAG repeat lengths.

When stratifying the cohort based on AGD, those with AGD shorter than the median (i.e. 40 mm) had a longer CAG repeat length compared to men with an AGD >40 mm (22.3 *vs.* 21.1; P=0.02; **Table 3**; **Figure 2**). After adjusting for age, race and fatherhood status, the relationship remained whereby for each additional AR CAG repeat, the odds of a shorter AGD increased by 11% (95% confidence interval: 1–22%; **Table 3**). A similar trend existed for other AGD lengths around the median AGD but none reached statistical significance (**Table 3**). In contrast, men with a PL longer and shorter than the median (116.5 mm) had similar CAG repeat lengths (21.8 *vs.* 21.5; P=0.48). After adjusting for age, race and BMI, no relationship was observed between PL and CAG repeat length (data not shown).

Of the 12 men with a CAG repeat length >26, only nine (75%) had an AGD less than the median AGD for the group (40 mm). Of the seven men with a CAG repeat length >27, six (86%) had an AGD less than the median. Of the three men with a CAG repeat length >28, all had an AGD less than the median AGD.

Of the 49 men with an AGD in the lowest quartile (AGD <31 mm), only six (12%) had a CAG repeat length >26.

DISCUSSION

While no relationship was found between AGD and AR CAG repeat length overall, the current study suggests that men with the longest CAG repeat lengths had shorter AGDs. Moreover, men with an AGD above the median had a shorter CAG repeat length compared to men with an AGD below the median. However, there was no linear relationship between AGD and CAG repeat length for the overall cohort. In addition, no relationship was found between PL and AR CAG repeat length.

During sexual development, the immature genital precursors migrate ventrally *via* an androgen-mediated pathway.[21] A marker for genital development, the AGD has been examined in both animal and humans.[1–4] Investigators have also used AGD to show that agents which have the potential to disrupt androgen signaling in animal models can lead to abnormal genital lengths and even alter testicular function as measured by testosterone and sperm production.[22–25]

While the final determination of AGD is likely complex, androgen sensitivity likely plays a role for a subset of men. The current study demonstrated that a majority of the men with the longest CAG repeat lengths, and conceivably the most impaired androgen signaling, had significantly shorter AGDs. However, the converse was not true. Of men in the shortest quartile group of AGDs, only 12% had longer CAG repeat lengths. Thus, AR sensitivity is unlikely to be the sole factor in determining AGD, and other factors operating during the fetal period, likely through androgen-mediated pathways, also impact normal genital development. It is possible that the complex network of AGD determinants limited our ability to identify a linear relationship between AGD and AR CAG repeat length.

Several limitations warrant mention. While the AR CAG repeat length was not known during data collection, urological diagnoses such as infertility, which may correlate with CAG repeat length, were known. Thus, it was not always possible to blind observers to the men's diagnoses which theoretically can lead to observer bias. In addition, multiple observers measured genital lengths in men. While we have previously established reproducibility of measurements, variation in assessments is possible and in the current data set within and between observers, variations were not measured to minimize patient discomfort and dropout. While longer AR CAG repeat length was associated with AGD, a shorter AR CAG distance (i.e. <19 repeats) was not. Moreover, the number of men with longer CAG repeat lengths was small. Thus, the identified association with AGD may have resulted from chance alone. This becomes increasingly likely given the number of comparisons tested in **Tables 2** and **3** and the absence of significant P values after Bonferonni correction. In addition, a relationship with

Table 3 CAG repeat lengths stratified by AGD around the median

AGD cutoff (mm)	*⩽ cutoff*		*> cutoff*			*Multivariable analysis*[a]	
	n	*CAG, mean±s.d.*	n	*CAG, mean±s.d.*	P*	*OR (95% CI)*	P
36	81	22.3±3.3	114	21.3±3.2	0.06	1.07 (0.98, 1.18)	0.15
38	88	22.1±3.3	107	21.3±3.2	0.16	1.04 (0.94, 1.14)	0.44
40	102	22.3±3.3	93	21.1±3.1	0.02	1.11 (1.01, 1.22)	0.03
42	112	22.1±3.3	83	21.2±3.2	0.13	1.08 (0.98, 1.19)	0.11
44	124	21.9±3.3	71	21.3±3.1	0.39	1.05 (0.96, 1.16)	0.30

Abbreviations: AGD, anogenital distance; CAG, cytosine–adenine–guanine; CI, confidence interval; OR, odds ratio.
[a] Multivariable OR (95% CI) adjusted for age, race and fatherhood states the odds of having a shorter AGD for each additional CAG repeat.
**P* value represents Wilcoxon rank sum analyses.

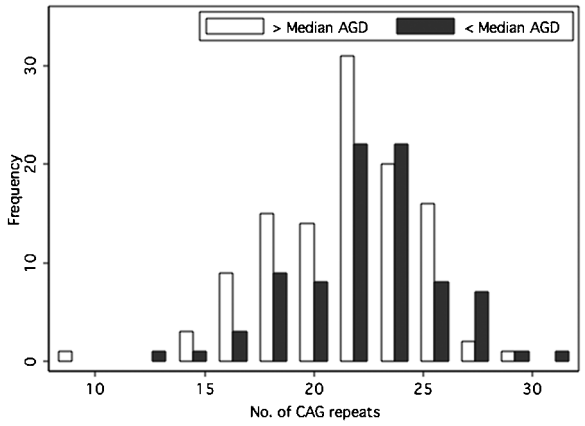

Figure 2 Distribution of CAG repeat lengths in men that had an anogenital length less than (black bars) and greater than (white bars) the median (40 mm). AGD, anogenital distance; CAG, cytosine–adenine–guanine.

AR CAG repeat number and PL was not found despite the fact that penile development is also known to be under androgen influence. It is also possible that the relative obesity in our cohort prevented accurate assessment of phallic length, which could not be overcome despite adjustment for BMI.

While no linear relationship was found between AGD and AR CAG repeat length, this is the first study to suggest a link between AGD and AR CAG repeat length. As such, AGD may provide some insight into a man's androgen sensitivity. Future studies should examine the relationship between androgen signaling and a man's CAG repeat length.

AUTHOR CONTRIBUTIONS

MLE, DJL and LIL conceived the project. MLE, TCH, AWP, MGM and RCW collected data. MLE, AWP and DJL analyzed the data. MLE drafted the manuscript. All authors provided critical revision of the manuscript.

COMPETING FINANCIAL INTERESTS

All authors declare that there are no competing financial interests.

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