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ORIGINAL ARTICLE

A pilot study of the association between genetic polymorphisms involved in estrogen signaling and infant male genital phenotypes

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Single nucleotide polymorphisms (SNPs) in genes that influence development of the male reproductive tract have been associated with male genitourinary abnormalities. However, no studies have tested the relationship between SNPs and intermediate phenotypes such as anogenital distance (AGD), anoscrotal distance (ASD) and penile width (PW). We tested whether 24 common SNPs in eight genes that influence male genital development were associated with intermediate phenotypes in 106 healthy male infants from the Study for Future Families. We used DNA from buccal smears and linear regression models to assess the relationship between anogenital measurements and SNP genotypes with adjustment for covariates. We found that the rs2077647 G allele, located in the coding region of estrogen receptor alpha (*ESR1*), was associated with a shorter AGD ($P=0.02$; −7.3 mm, 95% confidence interval (CI): −11.6 to −3.1), and the rs10475 T allele, located in the 3′ untranslated region of activating transcription factor 3 (*ATF3*), was associated with a shorter ASD (−4.3 mm, 95% CI: −7.2 to −1.4), although this result was not significant ($P=0.07$) after controlling for multiple comparisons. We observed no association between PW and the SNPs tested. Minor alleles for two SNPs in genes that regulate estrogen signaling during male genital development were associated with AGD and ASD, although the significance of the association was marginal. Our findings suggest that AGD and ASD are influenced by heritable factors in genes known to be associated with frank male genital abnormalities such as hypospadias and cryptorchidism.
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INTRODUCTION

Hypospadias and cryptorchidism are common birth defects affecting approximately 0.5%–4.5% and 2%–8% of infants, respectively, in industrialized countries.[1–4] These conditions are known risk factors for the development of testicular cancer and sperm abnormalities in adult males, suggesting that early programming of genital development can affect male childhood and adult reproductive function.[5] Numerous epidemiological and genetic studies of familial and sporadic hypospadias, cryptorchidism and testicular cancer suggest a multifactorial etiology, reflecting the contribution and interaction of both genetic and environmental factors in the development of these conditions.[6]

Normal male reproductive system development depends on appropriate genetic programming of androgen signaling and functioning during fetal life.[7] The androgen receptor (*AR*) and estrogen receptor genes (alpha (*ESR1*) and beta (*ESR2*)) affect development of all male genitourinary structures including the anus, penis, testes and urinary system.[8,9] Insulin-like factor (*INSL3*) and its receptor *LGR8* trigger testicular descent into the scrotum in the third trimester of pregnancy.[2,10] Several other genes have recently been found to be both differentially expressed in penile tissue of boys with hypospadias and to be estrogen responsive: including activating transcription factor 3 (*ATF3*), connective tissue growth factor (*CTGF*) and cysteine-rich angiogenic inducer (*CYR61*).[11,12]

Variation in repeat sequence number and single nucleotide polymorphisms in genes controlling development of the male reproductive pathway has been associated with male genital abnormalities. For example, polymorphisms in the *ESR1* and *ESR2* genes have been associated with both hypospadias and cryptorchidism,[9,13] suggesting that genetic variation can affect normal male genital development. Similarly, polymorphisms in *INSL3* and *LGR8* have been associated with cryptorchidism.[14,15] In addition, the number of CAG repeats in exon 1 of the *AR* gene may influence *AR* transcription with longer CAG repeat length resulting in reduced *AR* function.[16]

Whether common variation in genes involved in male reproductive development is associated with intermediate phenotypes such as

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anogenital distance (AGD), anoscrotal distance (ASD) and penile width (PW) has not been studied. We define intermediate phenotypes to be in the male reproductive developmental pathway and potentially influenced by the same genetic and environmental factors known to affect male genital development. AGD, ASD and PW are markers used in animal studies as reproductive toxicity endpoints reflecting *in utero* androgen exposure. In animal models, shortened AGD, ASD and smaller PW are associated with decreased androgen exposure and with male reproductive tract birth defects including hypospadias and cryptorchidism.[17–19] In addition, diminished AGD, ASD and PW lengths have been observed in human infants and in the offspring of rats prenatally exposed to some phthalate chemicals, man-made compounds with anti-androgenic properties.[20,21] Similar to animal models, reductions in AGD, ASD and/or PW lengths may be associated with similar birth defects in human males.[22] We tested whether polymorphisms in genes known to be involved in the regulation of male genital development are associated with AGD, ASD and PW in male infants and the extent to which such associations observed were modified by prenatal phthalate exposure.

MATERIALS AND METHODS

Participants

Pregnant women were originally recruited in the Study for Future Families I (SFFI), a multicenter pregnancy cohort study from September 1999 to August 2005. Infants born in SFF I were recruited in a follow-up study, Study For Future Families II (SFFII) which examined prenatal environmental exposures and health outcomes. Methods are described in detail elsewhere.[23,24] Eligibility included: pregnancy that was not medically assisted; woman and her partner was >18 years of age; either partner read and/or spoke Spanish or English; and the father was available to participate.

Buccal smears from infants were collected in 2007–2008 by inserting the swab into the baby's mouth along the cheek and rubbing vigorously along the cheek and under the tongue to collect cells. Study coordinators rolled the applicator on a collection card for several seconds until saturated with saliva. Cards were allowed to dry overnight at room temperature and then transferred to a standard refrigerator for storage.

Women in SFFI were asked to provide a urine sample at their recruitment visit (average 28 weeks pregnancy). Maternal urine samples were assayed for phthalate metabolites by the Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention. The analytical method involved the enzymatic deconjugation of the phthalate metabolites from their glucuronidated form, followed by concentration of the analytes of interest by solid-phase extraction, separation with high-performance liquid chromatography, and detection by isotope-dilution tandem mass spectrometry. Limits of detection are in the low nanogram per milliliter range. Isotopically labeled internal standards and conjugated internal standards were used to increase precision and accuracy of the measurements. Quality control and reagent blank samples were analyzed along with unknown samples to monitor assay performance.[25–27]

There were 454 mothers participating in SFFII who gave birth to 223 male infants. A physical exam including measurement of AGD, ASD and PW was conducted on each infant at an average of 12.6 months of age. AGD was measured from the center of the anus to anterior base of the penis. ASD was measured from the center of the anus to posterior portion of rugated scrotum. PW was measured transversely at the head of the phallus. All measurements were made with precision calipers. Anthropometric measurements (weight, length, head circumference) were also obtained. Examiners underwent a standardized training, and inter-rater variability coefficients for all genital measurements were above 80%. All infants were healthy without any genital birth defects and had testes descended into the scrotum. Within the group of mothers with male infants, seven mothers did not consent to buccal smears. Of the remaining 216 of male infants, 106 had full data on maternal prenatal phthalate concentration, full physical exam data with AGD, ASD and PW measurements, and buccal smears that produced usable DNA. We conducted genetic and statistical analyses on all 106 male infants.

Selection of genes and single nucleotide polymorphisms (SNPs)

We chose candidate genes for which either expression or nucleotide polymorphisms had previously been associated with hypospadias and/or cryptorchidism: *AR*, *ESR1*, *ESR2*, *INSL3*, *LGR8*, *ATF3*, *CYR61* and *CGTF*. For each gene, we identified SNPs for genotyping based on either a prior published association with hypospadias or cryptorchidism or a minor allele frequency $\geqslant$10% and tagging other SNPs (i.e., tagSNPs) with $r^2 \geqslant 0.80$ in individuals of European descent from HapMap data. When multiple tagSNPs were available, we preferentially chose SNPs that were more likely to be functional (e.g., demonstrated to have functional effects, location in a protein-coding exon, 3′ untranslated region, 5′ promoter region, exon/intron boundaries) and SNPs that could be assayed on our genotyping platform.

Genotyping methods

Genomic DNA from study subjects was isolated from FTA cards using the QIAamp DNA Investigator Kit (Qiagen, Valencia, CA, USA), followed by genomic amplification using the Qiagen REPLI-g Mini kit (Qiagen) according to the established manufacturer's protocols.

Genotyping was performed by KBiosciences (Hertfordshire, UK) using a fluorescence-based high-throughput allele-specific PCR technique (http://www.kbioscience.co.uk/KASP_manual.pdf). Genotype calling was performed manually by using the KBiosciences SNPviewer software by KBiosciences.

CAG repeat length was assessed at the Functional Genomics Laboratory of the Center for Ecogenetics and Environmental Health at the University of Washington. The number of CAG repeats in the variable tandem number CAG repeat polymorphism in exon 1 of the *AR* gene was determined by estimating the size of a specific PCR product containing this tri-nucleotide microsatellite. Briefly, fluorescent labeled sense primer FAM-5′-TCCAGAATCTGTTCCAGAGCGTGC-3′ and anti-sense primer 5′-CTCTACGATGGGCTTGGGGAGAAC-3′ were used for PCR with thermocycler conditions of 95 °C for 5 min; 40 cycles of 95 °C for 30 s, 61 °C for 30 s and 72 °C for 30 s. The length of the resultant fluorescent PCR amplicon was determined by using a 377 ABI DNA Sequencer (Applied Biosystems, Foster City, CA, USA) containing fluorescent in-lane size markers according to manufacturer's protocol. The fragment length of the PCR product was called by using the Genotyper software 2.5 (Applied Biosystems). The number of CAG repeats corresponding to the PCR fragment lengths were calculated using several control samples that were previously verified using four-color dye terminator-based DNA sequencing.

Statistical analysis

Multivariable linear regression was used to examine the association between AGD, ASD or PW (in mm) and genotype. A dominant

genetic model was used where subjects who are homozygous or heterozygous for the minor allele are combined for analysis. Infant age, weight and height were assessed at the time of the genital measurements. These measures were associated with infant AGD, ASD and PW, but were highly collinear with one another and thus could not be included simultaneously in the model. Therefore, infant weight percentile (which is a variable that is not collinear with other anthropometric factors) and infant age were used as covariates in analysis; this analytical approach was used in previous analyses of AGD from the SFFII study.[21] Study center is a known confounder from previous AGD analyses within the SFF study (likely due to AGD measurement differences between centers),[24] and its inclusion in the regression models led to appreciable changes in point estimates for the genital measures in our analysis. The final linear regression model included weight percentile, age and study center as covariates. In addition to estimating the main effects of the genotypes, an exploratory multivariable linear regression analysis was conducted to assess potential gene–environment interaction between prenatal phthalate exposure, genetic variants and AGD, ASD and PW by also including terms representing phthalate concentration and the product of phthalate concentration and minor allele carriership. Log transformation of phthalate concentrations was performed to normalize the distribution. Concentrations below the limit of detection were assigned the specific metabolite limit of detection value divided by the square root of two for statistical analysis, as has been performed previously.[28] We examined phthalate metabolites that have individually been associated with AGD [21] and used the sum of log transformed di-ethyl hexyl phthalate (DEHP) phthalate metabolites adjusted for molecular weight, mono(2-ethylhexyl) phthalate (MEHP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP) to reflect overall DEHP exposure. We examined phthalate interactions for only those SNPs for which there was a statistically significant main effect with either AGD, ASD or PW. In order to account for multiple comparisons, we applied resampling procedures based on non-parametric bootstrap to obtain adjusted *P* values that control the family error rate at 0.05.[29]

We examined the CAG repeat length within the *AR* gene in relation to AGD, ASD and PW using multivariate linear regression models adjusting for infant weight and center. The CAG repeat was examined as a continuous variable and also categorized (<22, 22–25, >25) using cutpoints previously reported in the literature.[16]

RESULTS

The study population ($n=106$) included male infants from predominantly Minnesota and Missouri who were 2–34 months of age. Infant weight (5–18.5 kg) and length (57–97.3 cm) varied considerably because of the large infant age range. The majority of mothers were European American and between 25 and 34 years old (**Table 1**).

We submitted a total of 53 SNPs to KBiosciences for genotyping. For approximately 15 SNPs (one in *INSL3*, two in *RXFP2*, five in *ATF3*, three in *AR*, two in *ESR1*, one in *ESR2* and one in *CTGF*), no results could be obtained either because an assay could not be designed (and no SNP in tight linkage disequilibrium was available for replacement) or because a designed assay performed poorly (most likely due to poor DNA quality). Given the small sample size, we further excluded results from 14 SNPs (four in *INSL3*, two in *RXFP2*, one in *ATF3*, two in *AR*, one in *ESR1*, one in *ESR2* and three in *CTGF*) with a minor allele frequency of less than 10% within our study population. These 14 SNPs included three rare SNPs previously reported only in cases of cryptorchidism.[2] Therefore, a total of 24 SNPs were available for testing (**Table 2**).

Table 1 Demographic characteristics for 106 male infants, Study for Future Families II, 1999–2005

	n *(%)*
Center	
California	19 (17.9%)
Minnesota	49 (46.2%)
Missouri	38 (35.8%)
Maternal race	
Caucasion	87 (82.1%)
Non-White	18 (17.0%)
Unknown	1 (0.9%)
Maternal age category (year)	
18–25	21 (19.8%)
25–34	61 (57.5%)
35–43.5	23 (21.7%)
Unknown	1 (0.9%)
Infant age (month)	
2–10	22 (20.8%)
10–20	44 (41.5%)
20–34	40 (37.7%)
Infant weight (kg)	
5–8	19 (17.9%)
8–12	51 (48.1%)
12–18.5	36 (34.0%)
Infant length (cm)	
57–75	26 (24.5%)
75–85	37 (34.9%)
85–97.3	38 (35.8%)
Unknown	5 (4.7%)

The G allele of rs2077647 (located in a coding region of *ESR1*) was associated with a shorter AGD (−7.3 mm, 95% CI: −11.6 to −3.1) (**Figure 1**). In addition, the T allele of rs10475 (located in the 3′ untranslated region of *ATF3*) was associated with a shorter ASD (−4.3 mm, 95% CI: −7.2 to −1.4) (**Figure 2**). We observed no significant association between PW and any SNP tested (**Figure 3**). After resampling to control for multiple comparisons, we found that the adjusted *P* value for the association between the minor allele of rs2077647 and AGD was 0.02 and the adjusted *P* value for the association between the minor allele of rs10475 and ASD was 0.07.

We examined whether maternal phthalate exposure modified the relationship between the SNPs rs2077647 and rs10475 and AGD and ASD, respectively. The coefficients for the interactions terms were small in absolute value and not statistically significant in multivariate linear regression analyses (**Table 3**).

The mean CAG repeat length was 23 with a range of 10–29. We observed a 2.95-mm increase in ASD in infants with >25 CAG repeats as compared to infants with <22 CAG repeats ($P=0.80$). We did not observe an association between CAG repeat length and AGD or PW (**Table 4**).

DISCUSSION

We conducted a pilot study to test the hypothesis that common variation in genes previously reported to influence hormonally-mediated male genital development is associated with the intermediate phenotypes AGD, ASD and PW. We found two SNPs that were independently associated with AGD and ASD. *ESR1* is well known to play an important role in early genital development, and recent studies suggest that *ATF3* may affect differentiation of penile tissues but the extent to which *ATF* is involved in male reproductive development remains to be determined.

Table 2 Single nucleotide polymorphisms analyzed in 106 male infants from the Study for Future Families II, 1999–2005

Gene	*Minor allele*	*SNP rs number**	*Minor allele frequency (%)*	*Context*
Relaxin/insulin like family receptor (*RXFP2*)	A	rs7325513	49	Coding-synonymous
	G	rs17076657	13	Coding-nonsynonymous
	A	rs7331833	25	
Activating transcription factor (*ATF3*)	T	rs3125289	47	Intron
	T	rs1126526	18	mrRNA-untranslated
	T	rs10475	26	mrRNA-untranslated
Androgen receptor (*AR*)	T	rs1204038	17	Intron
	T	rs1204039	17	Intron
	C	rs2767564	16	Intron
	G	rs4827546	17	Intron
	C	rs4827547	15	Intron
	C	rs5918758	14	Intron
	G	rs5919395	14	Intron
	C	rs5919402	19	Intron
	G	rs7061037	15	Intron
Estrogen receptor 1 (*ESR1*)	G	rs2077647	49	Coding-synonymous
	A	rs1062577	10	mRNA-untranslated
	C	rs3798577	33	mRNA-untranslated
Estrogen receptor 2 (*ESR2*)	G	rs1255998	15	mRNA-untranslated
	G	rs928554	43	mRNA-untranslated
	C	rs1256120	16	mRNA-untranslated
Cysteine-rich angiogenic inducer (*CYR61*)	A	rs2297141	43	Intron
	C	rs9658584	21	Intron
Connective tissue growth factor (*CTGF*)	T	rs9399005	27	Intron

Abbreviation: SNP, single nucleotide polymorphism.
*dbSNP reference SNP.

Appropriate concentration and timing of hormonal signaling during fetal life are critical for normal male reproductive development.[10,30] Specifically, increased estrogen exposure during fetal life can lead to an increased incidence of hypospadias and cryptorchidism in mice.[31] Estrogen signaling is predominantly mediated by ESR1 and ESR2, both of which are expressed in fetal male genital tissues. Polymorphisms within *ESR1* have been associated with hypospadias, male infertility and sperm parameters including decreased sperm density and abnormal sperm motility and morphology.[13,32] We found that the rs2077647 G allele was associated with a shorter AGD. Several previous studies have found this allele to be associated with a variety of disease phenotypes including: increased risk of Alzheimer's disease,[33] increased risk of colon, bladder and prostate cancers,[34,35] and increase in child onset mood disorders.[36] All of these disorders are thought to be in part hormonally mediated, potentially through the actions of ESR1.

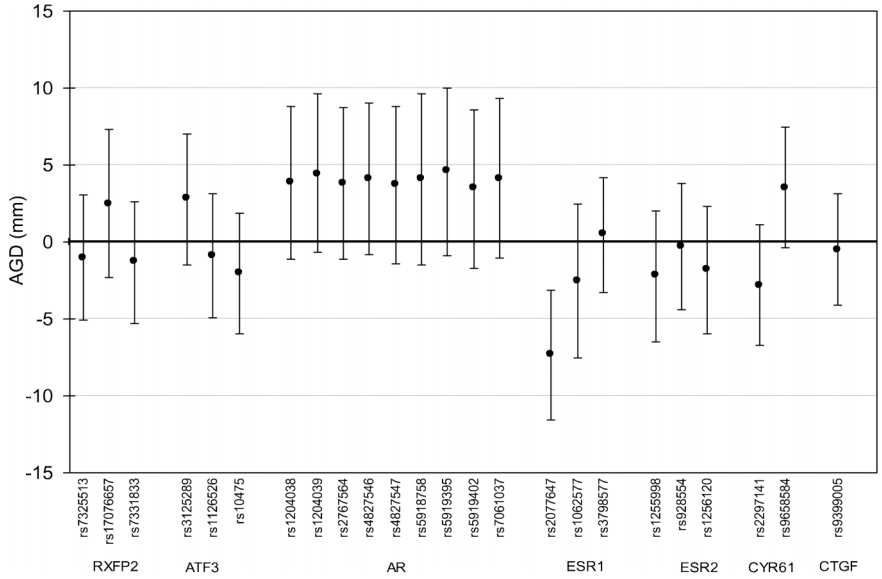

Figure 1 Difference in anogenital distance (AGD) (mm), 95% confidence interval, associated with minor allele carriership (adjusted for study center, age and infant weight percentile), Study for Future Families II, 1999–2005.

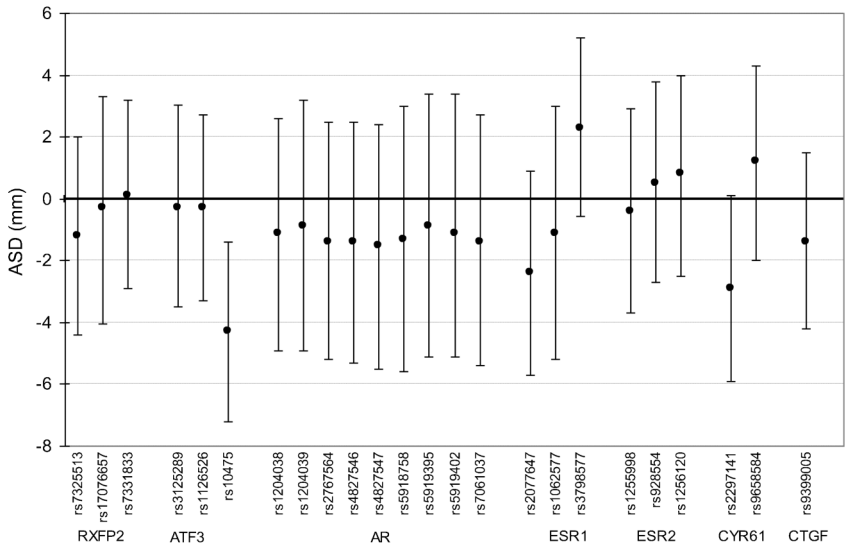

Figure 2 Difference in anoscrotal distance (ASD) (mm), 95% confidence interval, associated with minor allele carriership (adjusted for study center, age and infant weight percentile), Study for Future Families II, 1999–2005.

ATF3 is an estrogen responsive gene that is expressed within the genital tubercle during mouse sexual differentiation and has been found to be upregulated in foreskin tissue in human patients with hypospadias.[37,38] *ATF3* is involved in suppression of cell cycling, and hypothesized to inhibit cell growth in urethral formation.[11] In a cohort of 41 boys with hypospadias, 10% carried rare *ATF3* alleles as compared to none of thirty controls.[39] Beleza-Meireles *et al.*[40] found that the odds of having the individual minor alleles of three common, unlinked *ATF* SNPs (rs3125289, rs1877474, rs11119982) spanning a 16-kb region in intron 1 were increased in 330 cases of hypospadias boys as compared to 380 healthy controls in Sweden. No published studies have found an association between the rs10475 polymorphism and male reproductive outcomes. We found that ASD was decreased in infants with the rs10475 T allele, suggesting that *ATF3* may be involved in the formation of male reproductive tissues more generally and not limited to the urethral plate. This SNP is located in the 3′ untranslated region of *ATF3*, and could be functionally important if it alters the binding of regulatory miRNAs. We used the TargetScan database (http://www.targetscan.org) to assess whether this SNP, or a strongly correlated SNP, is located within a predicted miRNA binding site, but found no such evidence.

While our study supports a relationship between common genetic variants and male reproductive phenotypes, it has several limitations, the most notable of which is a relatively small sample size given the effect size for which we were looking. Additionally, a substantial fraction of genotypes failed to be called suggesting that the original

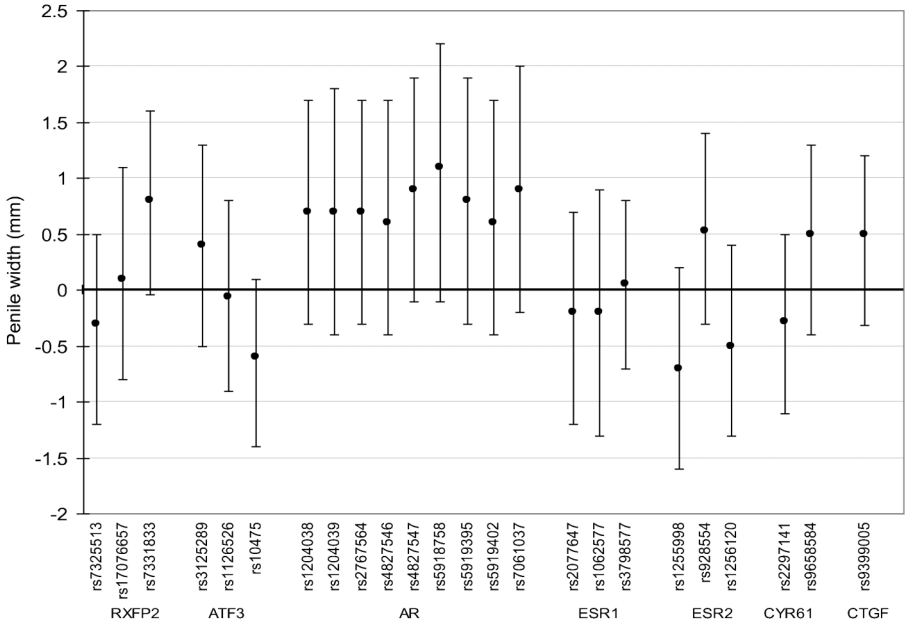

Figure 3 Difference in penile width (PW) (mm), 95% confidence interval, associated with minor allele carriership (adjusted for study center, age and infant weight percentile), Study for Future Families II, 1999–2005.

Table 3 Linear regression analysis of joint relationship of genotypes and maternal urinary phthalate with genital distance measures (adjusted for center, age and infant weight percentile), Study for Future Families II, 1999–2005

	Genotype	*Measure*	*Phthalate*	*Phthalate* genotype interaction term coefficient (95% CI)*	P *value*
rs2077647	AA or AG	AGD	Sum DEHP Metabolites (MEHP/MEOHP/MEHHP)	−1.3 (−6.0, 3.4)	0.58
			MEP	−2.0 (−4.8, 0.8)	0.16
			MBP	0.1 (−4.4, 4.5)	0.97
rs10475	TT or TC	ASD	Sum DEHP metabolites (MEHP/MEOHP/MEHHP)	−0.8 (−4.2, 2.7)	0.67
			MEP	0.01 (−2.1, 2.1)	0.99
			MBP	−0.7 (−4.2, 2.9)	0.71

Abbreviations: AGD, anogenital distance; ASD, anoscrotal distance; MBP, mono-butyl phthalate; MEHHP, mono-(2-ethyl-5-hydroxyhexyl) phthalate; MEHP, mono(2-ethylhexyl) phthalate; MEOHP, mono-(2-ethyl-5-oxohexyl) phthalate; MEP, mono-ethyl phthalate.
*Refers to a statistical interaction term to examine how phthalate modify the relationship between genotype and outcome.

Table 4 Associations (regression coefficients, 95% confidence intervals (CI)) between AR CAG repeat length and anogenital measures, Study for Future Families II, 1999–2005*

		AGD (mm)		*ASD (mm)*		*PW (mm)*	
	n	β	*95% CI*	β	*95% CI*	β	*95% CI*
AR CAG repeat length							
<22	53	Referent			Referent		Referent
22–25	33	−2.39	−6.56, 1.78	−0.34	−3.57, 2.88	−0.36	−1.23, 0.50
>25	16	0.48	−4.83, 5.80	2.95	−1.16, 7.06	0.21	−0.89, 1.32
Per one repeat increase		−0.14	−0.80, 0.52	0.06	−0.45, 0.57	0.003	−0.14, 0.14

Abbreviations: AGD, anogenital distance; ASD, anoscrotal distance; PW, penile width.
*All analyses adjusted for center, age and weight percentile.

samples, and thus, the DNA quality, had degraded. We conducted 27 independent tests for each of the intermediate phenotypes. We used a resampling procedure based on bootstrap to account for multiple comparisons and found that adjusted *P* values were very similar to those within the original analysis.[29]

We did not observe a statistically significant interaction between prenatal phthalate exposure, genetic variants and the three measures we examined. This result was to be expected given the small sample size. Nevertheless, this is an important environmental exposure to consider in future studies. Phthalates are synthetic chemicals produced in large volumes and used in a variety of industrial and common products.[41] It is well demonstrated that prenatal phthalate exposure decreases early life testosterone concentrations leading to a variety of adverse reproductive defects in animal offspring,[18] but the mechanisms by which this occurs are still unclear. DEHP and dibutyl phthalate have been found to decrease *INSL3* mRNA expression and decrease testosterone concentrations in pregnant dams,[42] but it is likely that other mechanisms are at play. These may involve a gene–environment interaction in which risk alleles increase an individual's susceptibility to phthalate exposure and subsequent health impacts. Future studies with larger sample sizes will be needed to adequately test if and how phthalates modify male reproductive development.

Our study is novel in that it is the first study to use AGD and ASD, intermediate phenotypes for male reproductive abnormalities, in an attempt to identify genetic risk variants that might also be important in birth defects such as hypospadias and cryptorchidism. Our results suggest that variation in *ESR1* and *ATF3* are associated with the hormone-sensitive male phenotypes, AGD and ASD. These results are consistent with the roles of *ESR1* and *ATF3* in estrogen mediated signaling of genital development, and suggest that development of estrogen responsive male reproductive tissues may be influenced by heritable factors. Using novel intermediate phenotypes to understand the genetic contribution to hormone male reproductive development will help elucidate the causes of abnormalities in male reproductive programming.

AUTHOR CONTRIBUTIONS

SS (first author) wrote the grant for the initial study, conducted the study with team members, conducted analysis and wrote the manuscript. SMS (last author) mentored the entire project and was involved in conception, study design, analysis and write-up and review. SHS was the PI on the parent grant that collected all of the specimens used for the study. She reviewed and edited the final manuscript. FMF and HWW were responsible for DNA extraction and genetic analyses. MB contributed to final review and edits of the manuscript. CZ contributed to statistical analysis of the data. RG contributed to project conception and reviewed/edited the final manuscript.

COMPETING FINANCIAL INTERESTS

The authors have no competing financial interests to declare.

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