

Testosterone Effects on Short-term Physical, Hormonal, and Neurodevelopmental Outcomes (TESTO) in Infants With 47,XXY

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Abstract

Context: 47,XXY/Klinefelter syndrome (XXY) is associated with impaired testicular function and differences in physical growth, metabolism, and neurodevelopment. Clinical features of XXY may be influenced by testosterone during the minipuberty period of infancy.

Objective: We tested the hypothesis that exogenous testosterone treatment positively affects short-term physical, hormonal, and neurodevelopmental outcomes in infants with XXY.

Design: Double-blind randomized controlled trial, 2017–2021.

Setting: US tertiary care pediatric hospital.

Patients: Infants 30 to 90 days of age with prenatally identified, nonmosaic 47,XXY (n = 71).

Intervention: Testosterone cypionate 25 mg IM injections every 4 weeks for 3 doses.

Main outcome measures: The *a priori* primary outcomes were change in percent fat mass z-scores and change in the total composite percentile on Alberta Infant Motor Scales assessment from baseline to 12 weeks.

Results: The between-group difference in change in percent fat mass z-scores was -0.57 (95% CI, -1.1 to -0.06 ; $P = .03$), secondary to greater increases in lean mass in the testosterone-treated group (1.5 ± 0.4 kg vs 1.2 ± 0.4 ; $P = .001$). Testosterone suppressed gonadotropins and inhibin B ($P < .001$ for all). In contrast, there were no significant group differences in short-term motor, cognitive, or language outcomes ($P > .15$ for all).

Conclusions: In this double-blind randomized controlled trial in infants with XXY, testosterone injections resulted in physical effects attributable to systemic androgen exposure; however, this dose suppressed the hypothalamic-pituitary-gonadal axis. Neurodevelopment outcomes were not impacted by treatment. These results do not support routine testosterone treatment in infants with XXY; however, long-term follow-up on physical health, neurodevelopment, and testicular function is needed.

Key Words: Klinefelter syndrome, testosterone, mini puberty period, critical window

Abbreviations: ABAS-3, Adaptive Behavior Assessment System Third Edition; AIMS, Alberta Infant Motor Scale; AMH, anti-müllerian hormone; Bayley-3, Bayley Scales of Infant and Toddler Development, 3rd edition; CHCO, Children's Hospital Colorado; FM, fat mass; FFM, fat free mass; %FM, percent fat mass; HPG, hypothalamic-pituitary-gonadal; INHB, inhibin B; PDMS-2, Peabody Developmental Motor Scales, version 2; SES, socioeconomic status.

The widespread adoption of noninvasive prenatal screening via cell-free DNA brought with it a substantial increase in the number of infants recognized to have sex chromosome aneuploidies (1). The most common sex chromosome aneuploidy, 47,XXY or Klinefelter syndrome, is estimated to affect in 1 in 600 males (2). Most infants born with XXY have no overt signs or symptoms, although some studies report subtle, nonspecific features in comparison to males without XXY including smaller birth

size, reduced penile growth in the first year of life, mild hypotonia, and a more passive temperament (3). As adolescents and adults, individuals with XXY typically have testicular dysfunction, which manifests as microorchidism, hypergonadotropic hypogonadism, and infertility (4). Neurocognitive and cardiometabolic manifestations are also well-described as prominent features of the phenotype contributing to increased morbidity throughout childhood and adulthood, as well as increased

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mortality (2, 5-11). Although testosterone deficiency can contribute to neurocognitive deficits and poor cardiometabolic health in men, the causal role in XXY is unclear as postpubertal testosterone treatment fails to normalize these findings. With these observations, in addition to more individuals being diagnosed earlier in life, there is a growing interest in prevention and/or early intervention opportunities to decrease morbidity in XXY.

In the first months of life, the hypothalamic-pituitary-gonadal (HPG) axis is transiently active, driving testicular testosterone production in males (12). Although the specific purpose of this minipuberty period of infancy is unclear, it is hypothesized to be a critical window in development with an essential role in sexual differentiation of multiple tissues and programming for future sex-specific cellular processes (3, 13-21). Several human studies have reported associations between infant testosterone production and sex differences in linear growth, lean mass accumulation, brain lateralization, language organization, and gender identity (17, 22, 23). Mouse models manipulating sex steroid exposure have bolstered these findings, illustrating that early postnatal testosterone exposure stimulates systemic epigenetic changes resulting in permanent impacts on neurocognition and energy metabolism (14, 16). Studies in infants with XXY confirm that the minipuberty testosterone surge does occur; however, systemic testosterone concentrations may be lower than average (24-27). Furthermore, retrospective reports of a clinical cohort of boys with XXY treated with testosterone in infancy describe higher cognitive, language, motor, and social communication abilities in childhood compared to boys without a history of testosterone treatment (19, 28). Previously, we reported differences in adiposity between infants with XXY who did and did not receive testosterone (29). However, there has not been a rigorously conducted study to inform the potential benefits or risks of infant testosterone treatment in XXY.

The aim of this double-blind, placebo-controlled, randomized prospective clinical trial was to test the hypothesis that exogenous testosterone treatment during the minipuberty period of infancy has positive effects on short-term physical, hormonal, and neurodevelopmental outcomes in boys with XXY. Additional aims were to inform the side effect profile of treatment, determine if any observed immediate effects are sustained, and explore whether the timing (age) of intervention matters. This study was designed to inform best clinical practice recommendations for the many infant boys now prenatally identified to have an additional X chromosome.

Methods

Overall Study Design

This was a randomized controlled trial assessing the efficacy and safety of testosterone injections in infants with XXY. Infants with prenatally identified, nonmosaic 47,XXY received either testosterone or placebo injections for 3 months followed by 3 months of crossover intervention. Outcomes were assessed by blinded investigators at baseline, after the first treatment period (12 weeks), and after the crossover period (24 weeks).

Setting, Recruitment, and Participants

The study took place at Children's Hospital Colorado (CHCO) in the outpatient Pediatric Clinical Translational Research Center. Participants were recruited through the

interdisciplinary eXtraordinary Kids Clinic at CHCO, local genetics and obstetrics offices, local and national advertisements, XXY social media groups, family support groups, and national pediatric endocrinology mailing lists. Infants between aged 31 and 90 days were eligible if they were prenatally identified and subsequently confirmed via chorionic villous sampling, amniocentesis, or postnatal blood/tissue to have a karyotype of 47,XXY. Exclusion criteria included >20% mosaicism for typical 46,XY cell line, gestational age <36 weeks, birth weight <2.5% or >97.5% for age, use of medications known to impact body composition (eg, insulin, GH), allergy to any of the components in testosterone cypionate, history of thrombosis in self or first-degree relative, and exposure to androgen therapy outside of the study protocol. The study was approved by the local institutional review board (COMIRB 17-1317), registered on [ClinicalTrials.gov](https://clinicaltrials.gov) (NCT03325647) with the full protocol, and the protocol was filed with the US Food and Drug Administration (Investigative New Drug file #124260). The parents of every participant provided written informed consent before any study procedures. An independent data safety and monitoring board provided additional study oversight.

Randomization

Following enrollment, participants were assigned to 1 of 2 groups through block randomization in blocks of 20 with a 1:1 allocation scheme determined from an automated list generated at the beginning of the study. To ensure allocation concealment, CHCO Investigational Drug Services generated and held the randomization list and dispensed the study drug. Study personnel, participants, and outcome assessors did not have access to the randomization list and were blinded to group assignments. The investigational drug and placebo were identical in appearance and packaging, preventing unblinding during dispensing and administration. Investigational Drug Services had no interaction with participants or study staff, ensuring blinding was maintained throughout the trial until the data were locked for analysis.

Intervention

Participants received a total of six 0.125-mL injections in the vastus muscle during the 6-month study period. Participants randomized to group A were given 1 injection of 25 mg testosterone cypionate (200 mg/mL concentration) every 28 days for a total of 3 injections, followed by placebo (saline) injection once every 28 days for a total of 3 injections during study weeks 12 through 24. Group B received placebo injections every 28 days for the first 3 months of the study, followed by 3 testosterone injections. This testosterone regimen was chosen based on its efficacy and safety in treating boys with micropenis (30).

The first and fourth injections were administered by a nurse at the end of the respective study visits. If it was not feasible to return for monthly injections, parents were trained to give subsequent injections themselves and were given a dosing calendar and log. A prepared syringe with study drug was mailed to the participant for each dose. An automated reminder email was sent to the parents 24 hours before each injection was due, and an electronic survey was sent 1 week following the injection to document administration details and any side effects. There were no differences in reported issues with administration, outcomes, or side effects based on who administered the injections (83% parent, 17% nurse).

Study Visits and Timeline

In-person study visits occurred at enrollment (baseline), 12 weeks (± 2 weeks), and 24 weeks (± 2 weeks). If an in-person study visit was not possible (eg, secondary to the COVID-19 pandemic), a limited study visit was conducted via video conference, primarily to assess for side effects and obtain parent-report measures. Enrollment commenced in November 2017 and final study visits occurred in May 2021.

Study Assessments

Prenatal, birth, medical, developmental, and feeding histories were obtained from parents at the initial study visit. Any updates, including new medications and any perceived adverse events, were collected at subsequent visits. In addition, parents subjectively rated their infant's temperament as "easy going," "average," or "difficult" at each visit. Physical examinations were conducted by a board-certified pediatrician blinded to participant treatment status and trained on accurate measurement techniques. Weight was measured to the nearest 0.01 kg. Stretched penile length, head circumference, arm span, waist circumference, and total body length (using an infantometer) were measured to the nearest 0.1 cm. Weight, length, head circumference, and weight-for-length age- and sex-specific z -scores were calculated from World Health Organization norms. Penile length z -scores were calculated from published norms (31). Parent(s) completed a sociodemographic survey that included self-reported race, ethnicity, education level, occupation, and family income; answers were used to calculate the Hollingshead index as a measure of socioeconomic status (SES).

Body composition was assessed using air displacement plethysmography for infants up to 10 kg (32). Using the measured body mass and total volume, fat mass (FM) and fat free mass (FFM) were estimated to the nearest gram, and percent fat mass (%FM) was then calculated by dividing the FM by the total body mass. Two independent air displacement plethysmography measurements were obtained, with a third measurement if the first two %FM differed by $>3\%$. Because of the vast differences in body composition expected in early infancy, sex- and age-specific %FM z -scores were calculated from published norms (33).

Standardized neurodevelopmental assessments were administered by trained psychometrists, child psychologists, or pediatricians blinded to participant treatment status. The Alberta Infant Motor Scale (AIMS) is a measure of gross motor maturation designed to evaluate gross motor development over the first year of life in the prone, supine, sitting, and standing positions, with total scores ranging from 0 to 55, which is then converted to an age-normed percentile (0-100) (34, 35). Because the AIMS is observational, it is feasible to conduct in person or via video conference. The Peabody Developmental Motor Scales, version 2 (PDMS-2), includes 6 subtests that assess gross and fine motor scales from birth to 5 years of age, yielding age-normed quotient scores (mean 100, SD 15) for total, fine, and gross-motor abilities and scaled scores (mean 10, SD 3) for each subdomain (36). The Bayley Scales of Infant and Toddler Development, 3rd edition (Bayley-3) is a well-validated standardized assessment spanning 3 domains: cognitive, language (expressive and receptive), and motor (fine and gross) (37). Raw scores were converted to age-normed composite scores (mean 100, SD 15) and subdomain scaled scores (mean 10, SD 3). In addition,

growth score values based on raw scores without age comparison were used to assess change in development relative to each individual (38). The PDMS-2 and Bayley-3 require in-person administration.

Parents completed the Adaptive Behavior Assessment System Third Edition (ABAS-3), a validated parent-report measure capturing adaptive skills across the life span. The ABAS-3 yields scaled scores in 7 subdomains that inform 3 domain standard scores (mean 100, SD 15) and an overall generalized adaptive composite. Parents also completed an electronic survey 1 week after every injection to document administration details, any deviations or problems with administration, and treatment emergent adverse events (from a provided list as well as open-ended). The severity and causality for all adverse events was determined by the study team; adverse events were regularly assessed by the data safety and monitoring board.

Infants had a venous blood draw in the morning following 3 hours of fasting at each study visit. Samples were collected in a serum separate tube, processed, and stored at -80°C until batch analysis without any freeze-thaw cycles.

Laboratory Methods

Total testosterone was measured using liquid chromatography-tandem mass spectrometry with a detection limit of 0.02 nmol/L; intra-assay coefficient of variation was $<5\%$ and inter-assay coefficients of variation were $<8\%$ throughout the range of observed values. LH (Roche Cat#11732234, RRID:AB_2800498) and FSH (Roche Cat#11775863, RRID:AB_280049) were measured by means of a sensitive electrochemiluminescent immunoassays with a quantification range 0.1-200 mIU/mL and cross-reactivity for similar hormones $<0.1\%$. Inhibin B (INHB; Ansh Labs Cat# AL-107, RRID:AB_2783661) and anti-müllerian hormone (AMH; Ansh Labs Cat# AL-105, RRID:AB_2783659, RRID:AB_2783659) were measured by solid-phase sandwich assays. The lower limit of detection for INHB was 4.6 pg/mL with a quantification range up to 1100 pg/mL. The lower limit of detection for AMH was 0.7 pmol/L with a quantification range up to 114 pmol/L. Detailed methods and quality control measures are available (39).

Statistical Analyses

Data were examined for outliers, missing values, and normality by visual inspection and applicable tests; following these tests, no outliers were removed, and the minimal missing data were treated as missing without imputation. Summary baseline data are reported as mean with SD, median with interquartile range, or number and proportion depending on data type. Baseline variables were compared between randomized groups to evaluate whether there were any intrinsic differences that would need to be accounted for in analyses.

The a priori primary outcomes were change in %FM z -scores from baseline to 12 weeks and change in the total composite percentile on the AIMS from baseline to 12 weeks. Secondary and exploratory outcomes included change scores from both baseline to 12 weeks and 12 to 24 weeks for other body composition variables, anthropometric measurements, and standard scores on the PDMS, Bayley 3, and ABAS 3; serum hormone concentrations; and number and type of adverse events. Change scores were chosen because we predicted the intraindividual correlation to be high for most of our outcomes of interest, allowing for a more precise

estimate as well as clinical applicability in determining the effect of testosterone treatment. Therefore, participants who did not have measures from both timepoints were not included in the analysis.

All outcomes were assessed between groups with 2-tailed *t*-tests or Wilcoxon tests along with computed difference in means (or medians) and 95% CI, with significance set at 5%, adhering to an intention-to-treat analysis. An initial sample size of 27 per group was determined based on the ability to detect a %FM *z*-score difference of 0.5 between groups with 80% power; because of the COVID-19 pandemic, we over-enrolled beyond this goal to account for anticipated missing longitudinal air displacement plethysmography data. Following the primary unadjusted outcome analysis, linear regression models were performed with treatment group, baseline measure, and potential covariates (eg, age of enrollment, duration between measures, feeding source, SES, baseline testosterone concentration) for all outcomes from both the 12- and 24-week visits as secondary/exploratory endpoints. No interim analyses for efficacy were conducted. Adverse events were summed and risk ratios with 95% CIs calculated based on whether the participant was receiving testosterone or placebo at the time of the adverse event. All analyses were conducted in R v.4.3.2.

Results

The CONSORT diagram for the 72 enrolled participants in the Testosterone Effects on Short-term Physical, Hormonal, and Neurodevelopmental Outcomes (TESTO) trial is shown in Fig. 1. The observed attrition was only 2.8%; however, not all outcome assessments could be completed due to the disruption caused by the COVID-19 pandemic. Demographics and baseline assessments were similar between the randomized groups (Table 1). Infants were low-average size for gestational age and enrolled at a mean age of 2 months. One third of

participants endorsed Hispanic ethnicity and/or non-White race; however, the majority of the sample were in households making >\$100 000 per year and had an average Hollingshead socioeconomic index that reflected a high level of parental education and occupational status. All but 1 had a prenatal cell-free DNA screening positive for 47,XXY. Approximately half elected prenatal diagnostic testing (chorionic villous sampling or amniocentesis), whereas the other half confirmed the 47,XXY diagnosis postnatally. The 1 infant who was not identified by cell-free DNA screening was diagnosed with elective amniocentesis for advanced maternal age.

The a priori primary outcome of change in %FM *z*-score at the 12-week visit was statistically different between the treatment groups, with the placebo-treated group gaining a %FM *z*-score of 0.56 more than the testosterone-treated group (Table 2). When examined by absolute %FM rather than *z*-score, this translated to a gain of $2.8 \pm 4.7\%$ in the testosterone-treated group compared to $4.8 \pm 4.4\%$ in the placebo group ($P = .081$). Body composition differences were entirely due to FFM gain: $+151 \pm 43$ g in the testosterone-treated group vs $+118 \pm 35$ g in the placebo group ($P = .001$), whereas FM gain did not statistically differ: $+61 \pm 36$ g vs $+71 \pm 36$ g ($P = .270$). Testosterone treatment was associated with a robust linear growth velocity of 35.1 ± 6.9 cm/y compared to 29.3 ± 5.9 cm/y for infants receiving placebo ($P < .001$). Stretched penile length increased by 1.1 ± 0.5 cm (*z*-score $+1.3 \pm 0.6$) with testosterone, compared to 0.1 ± 0.5 cm (*z*-score $+0.1 \pm 0.6$) in the placebo group ($P < .001$).

In contrast to these physical outcomes, the a priori outcome of AIMS total score assessing gross motor abilities was not different between treatment groups (change in AIMS percentile score 15.4 ± 29.5 vs 8.4 ± 29.6 ; $P = .319$). Cognitive, language, and motor composites and their respective subdomains on the Bayley-3 were not different between treatment groups at visit 2 (Table 2 and Fig. 2). Motor outcomes assessed by the PDMS-2 were also not different between treatment groups.

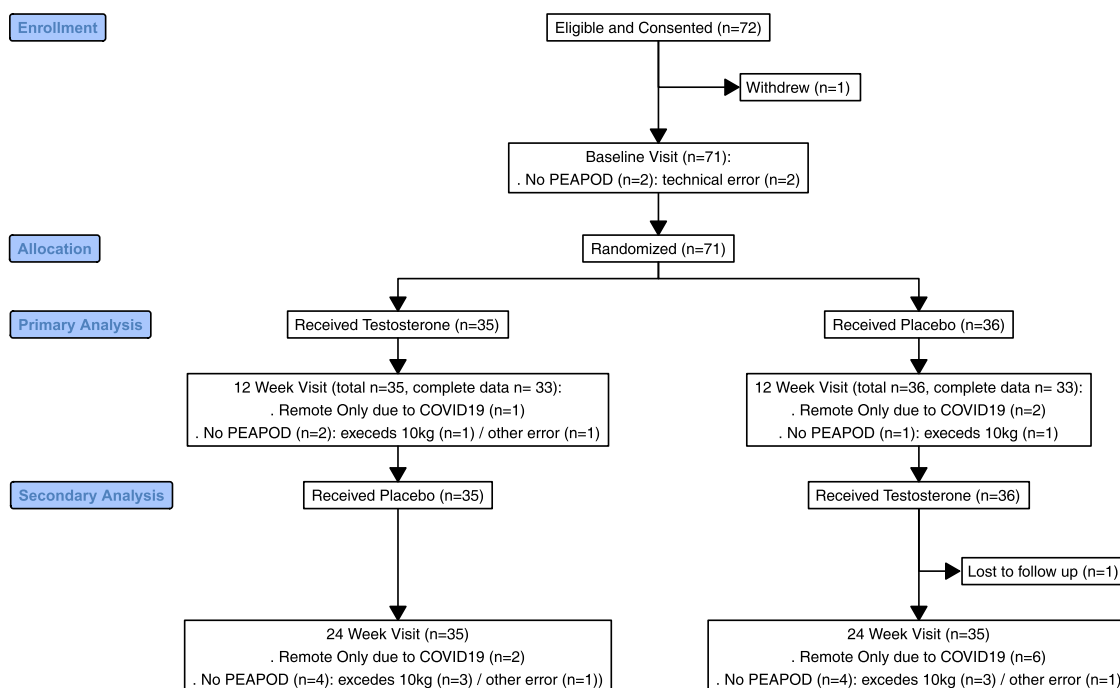


Figure 1. CONSORT diagram detailing participant allocation and completion of primary outcome measures for the TESTO trial.

Table 1. Baseline demographics and characteristics of the sample stratified by randomization group

	All (n = 71)	Group A (n = 35)	Group B (n = 36)	P value
Demographic variables				
Race				.508
White	63 (88.7%)	30 (85.7%)	33 (91.7%)	
Asian	3 (4.2%)	1 (2.9%)	2 (5.6%)	
Black	4 (5.6%)	3 (8.6%)	1 (2.8%)	
Native American	1 (1.4%)	1 (2.9%)	0	
Ethnicity				.396
Hispanic/Latino	16 (22.5%)	6 (17.1%)	10 (27.8%)	
Not Hispanic/Latino	55 (77.5%)	29 (82.9%)	26 (72.2%)	
Hollingshead SES Index	55.5 [49, 61]	55.5 [52, 61]	53.8 [41, 61]	.245
Household annual income				.217
<\$50 000	2 (2.9%)	0	2 (5.7%)	
\$50 000-75 000	9 (13.2%)	4 (12.1%)	5 (14.3%)	
\$75 000-100 000	10 (14.7%)	4 (12.1%)	6 (17.1%)	
\$100 000-150 000	17 (25.0%)	11 (33.3%)	6 (17.1%)	
\$150 000-250 000	18 (26.5%)	6 (18.2%)	12 (34.3%)	
>\$250 000	12 (17.6%)	8 (24.2%)	4 (11.4%)	
Gestational history variables				
Reason for genetic screening				.427
Elective	28 (39.4%)	12 (34.3%)	16 (44.4%)	
Advanced maternal age	41 (57.7%)	22 (62.9%)	19 (52.8%)	
Abnormal US/other	2 (2.8%)	1 (2.9%)	1 (2.9%)	
Prenatal confirmatory testing	37 (52.1%)	16 (45.7%)	21 (58.3%)	.408
Maternal prepregnancy BMI (kg/m ²)	25.8 ± 5.5	25.2 ± 6.0	26.4 ± 5.0	.376
Maternal pregnancy weight gain (kg)	13.1 ± 7.0	13.4 ± 7.2	12.8 ± 6.9	.722
Maternal age at birth (y)	35.1 ± 5.1	34.9 ± 4.1	35.3 ± 5.9	.713
Paternal age at birth (y)	35.9 ± 5.3	35.9 ± 5.1	36.0 ± 5.5	.965
Gestational age (wk)	39.3 ± 1.1	39.2 ± 1.0	39.4 ± 1.2	.598
Birthweight (kg)	3.26 ± 0.42	3.24 ± 0.46	3.29 ± 0.39	.613
Birth length (cm)	50.8 ± 2.5	50.6 ± 2.6	50.9 ± 2.4	.625
Visit 1 measures				
Infant age (d)	65.5 ± 15.7	65.8 ± 16.0	65.1 ± 15.6	.867
Feeding source				.931
Breast milk only	38 (53.5%)	18 (51.4%)	20 (55.6%)	
Formula only	14 (19.7%)	7 (20.0%)	7 (19.4%)	
Breast milk + formula	19 (26.8%)	10 (28.6%)	9 (25.0%)	
Parent-described temperament				.569
Easy-going	42 (59.2%)	21 (60%)	21 (58.3%)	
Average	28 (39.4%)	13 (37.1%)	15 (41.7%)	
Difficult	1 (1.4%)	1 (2.9%)	0 (0.0%)	
Weight z-score	−0.73 ± 0.92	−0.72 ± 0.96	−0.73 ± 0.88	.962
Length z-score	−0.59 (1.06)	−0.78 (0.94)	−0.41 (1.14)	.140
Weight-for-length z-score	−0.26 (1.05)	−0.02 (1.05)	−0.50 (1.02)	.057
Penile length (cm)	3.71 (0.49)	3.68 (0.45)	3.73 (0.53)	.667
Penile length z-score	−0.24 (0.61)	−0.28 (0.56)	−0.21 (0.67)	.667
%fat mass z-score	−0.77 (1.04)	−0.77 (1.18)	−0.77 (0.90)	.999
Fat free mass (kg)	4.19 (0.55)	4.22 (0.62)	4.15 (0.47)	.626
Fat mass (kg)	1.05 (0.37)	1.07 (0.42)	1.04 (0.32)	.728
Alberta Infant Motor Scale percentile	31.4 (24.0)	25.4 (23.9)	37.2 (23.0)	.038
Peabody gross motor quotient	96.6 (5.7)	96.3 (5.9)	96.9 (5.6)	.689
Peabody fine motor quotient	91.6 (7.1)	91.7 (7.4)	91.5 (6.8)	.904

(continued)

Table 1. Continued

	All (n = 71)	Group A (n = 35)	Group B (n = 36)	P value
Peabody total motor quotient	93.8 (5.4)	93.7 (5.4)	93.9 (5.4)	.853
Bayley-3 cognitive composite	105 (9.9)	106 (8.9)	104 (10.8)	.280
Bayley-3 language composite	101 (8.0)	102 (7.2)	100 (8.7)	.238
Bayley-3 motor composite	101 (9.1)	100 (8.3)	101 (10.0)	.483
LH (mIU/mL)	3.74 [3.04, 4.86]	3.71 [3.40, 4.51]	4.04 [2.84, 5.00]	.531
FSH (mIU/mL)	2.05 [1.66, 2.88]	1.89 [1.54, 2.43]	2.30 [1.78, 2.89]	.136
Total testosterone (ng/dL)	175 [132, 207]	173 [122, 207]	178 [138, 200]	.724
Inhibin B (pg/mL)	248 [201, 301]	242 [197, 300]	256 [218, 301]	.878
Anti-mullerian hormone (pmol/L)	839 [634, 1107]	877 [750, 1107]	833 [625, 1107]	.564

Data are represented as mean \pm standard deviation; median [25th percentile, 75th percentile]; or n (%). Group A received testosterone in the first half of the study and group B received placebo.

Abbreviations: BMI, body mass index; SES, socioeconomic status; US, ultrasound.

Table 2. Change scores for primary and select secondary outcomes at the primary endpoint (12 weeks)

	Group A testosterone	Group B placebo	Mean difference ^a	Effect size ^b	P value
%FM z-score	-0.07 \pm 1.0 n = 31	0.49 \pm 1.0 n = 30	-0.56 [-1.1, -0.06]	-0.57 [-1.1, -0.06]	.030*
Length z-score	0.65 \pm 0.67 n = 34	-0.02 \pm 0.69 n = 34	+0.68 [0.35, 1.01]	0.99 [0.49, 1.5]	<.001*
Stretched penile length (cm)	1.1 \pm 0.5 n = 34	0.1 \pm 0.5 n = 34	+1.0 [0.78, 1.25]	2.1 [1.5, 2.7]	<.001*
AIMS %ile	15.4 \pm 30 n = 35	8.4 \pm 30 n = 36	+7.0 [-7.0, 21.0]	0.24 [-0.23, 0.71]	.319
Bayley 3 Cognitive Composite	-3.4 \pm 9.8 n = 34	-1.4 \pm 13.5 n = 34	-2.0 [-7.8, 3.7]	-0.17 [-0.65, 0.31]	.484
Bayley 3 Language Composite	-7.0 \pm 10.1 n = 34	-3.7 \pm 9.7 n = 33	-3.4 [-8.1, 1.4]	-0.34 [-0.82, 0.14]	.166
Bayley 3 Motor Composite	-3.0 \pm 14.0 n = 34	-6.0 \pm 13.2 n = 33	+3.0 [-3.7, 9.6]	0.22 [-0.26, 0.7]	.375
PDMS-2 Gross Motor Quotient	1.8 \pm 7.4 n = 34	0.1 \pm 7.2 n = 33	+1.7 [-1.8, 5.3]	0.24 [-0.24, 0.72]	.335
PDMS-2 Fine Motor Quotient	9.0 \pm 9.5 n = 32	6.7 \pm 9.3 n = 34	+2.3 [-2.3, 6.9]	0.24 [-0.24, 0.73]	.325
ABAS-3 Generalized Adaptive Composite	3.5 \pm 8.1 n = 35	2.9 \pm 7.4 n = 36	+0.6 [-3.1, 4.3]	0.08 [-0.39, 0.55]	.747

Data are represented as mean \pm SD.

Abbreviations: ABAS-3, Adaptive Behavior Assessment System Third Edition; AIMS, Alberta Infant Motor Scales; FM, fat mass; PDMS-2, Peabody Developmental Motor Scales—version 2.

^aAbsolute mean difference and 95% CI of the mean difference between testosterone-treated vs placebo-treated groups.

^bCohen's d point estimate and 95% CI of the effect size.

*Denotes significance at alpha of 0.05.

There were also no differences in scores for any of the domains on parent-rated adaptive function assessment (ABAS-3).

Testosterone treatment suppressed reproductive hormone concentrations (Fig. 3). The change in LH between visits 1 and 2 for group A was -3.5 mIU/mL (-4.1 to -2.9) vs -1.8 (-3.3 to -0.8) in group B ($P < .001$). FSH (-1.5 mIU/mL [-2.0 to -1.0] vs -0.7 [-1.3 to -0.4]; $P < .001$) and INHB (-113 pg/mL [-167 to -66] vs 0.0 [-31.7 to 31.0]; $P < .001$) were also significantly suppressed with testosterone treatment. There was not a significant difference between groups for change in AMH (+128 pmol/L [0-401] vs +174 [-0.5 to 312]; $P = .669$).

Results for the second half of the study (changes from visit 2 to visit 3, blinded treatment crossover), were similar to the first half of the study (Table 3). Testosterone treatment increased FFM and both body and penile length with no

discernable effect on any neurodevelopmental outcomes. At visit 3, the group receiving testosterone in the second half of the study had a significantly greater penile length (5.6 ± 8.4 cm vs 4.8 ± 6.0 cm; $P < .001$), lower FSH (0.32 [0.21-0.41] vs 0.44 mIU/mL [0.37-0.73]; $P < .001$), and borderline significantly lower AMH (918.00 [609.50-1178.00] vs 1145.00 [910.00-1319.00]; $P = .038$); there were no differences in any other outcomes. Testosterone treatment was not associated with a difference in parent-reported temperament. At visits 2 and 3, respectively, 86% and 82% of infants were rated by their parent(s) as “easy-going” with the remainder being “average” temperament (none rated as “difficult”), with no differences based on testosterone treatment.

Results did not change when regression models were applied controlling for potential confounding variables (eg,

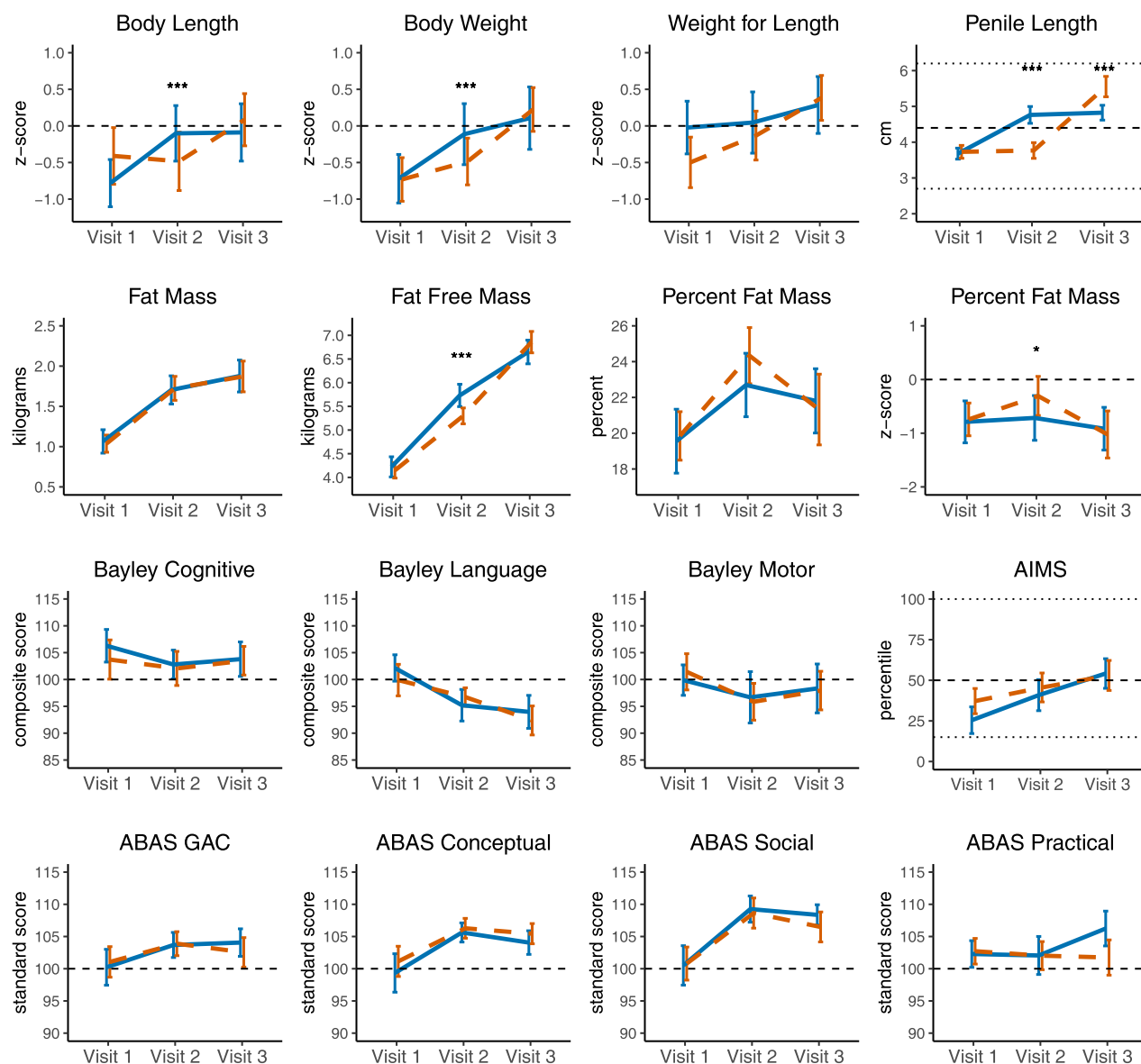


Figure 2. Mean and 95% CI of the mean by study visit for group A (solid line; testosterone first, placebo second) and group B (orange dashed line, placebo first, testosterone second) at visit 1 (baseline, ~2 months of age), visit 2 (~5 months of age), and visit 3 (~8 months of age). Outcomes from blinded physical examination (row 1), air displacement plethysmography (row 2), neurodevelopmental assessment (row 3), and parent-reported development (row 4). Where applicable, dashed line represents the average in the general population and normal ranges are depicted with a shaded background. Significant differences between groups is indicated by asterisks (* <0.05 , ** <0.01 , *** <0.001).

Abbreviation: ABAS, Adaptive Behavior Assessment System Third Edition; AIMS, Alberta Infant Motor Scales.

race, ethnicity, SES, breastfed status, maternal body mass index or pregnancy weight gain, birthweight). Similarly, neither baseline testosterone concentration nor baseline inhibin B concentrations influenced the relationship between testosterone treatment and any developmental outcomes.

During the 6-month study period, all infants experienced at least 1 adverse event for a total of 483 adverse events reported, with 297 occurring during testosterone administration and 210 during placebo administration (Table 4). Increased penile erection was the only adverse event occurring in significantly more individuals while on testosterone (62.9% of participants on testosterone vs 11.3% participants on placebo; $P < .001$). However, pubic hair (14.1% of participants) and acne (47.9% of participants) were also attributed to testosterone treatment for biologic plausibility. During testosterone

administration, 2 patients had emergency department encounters unrelated to the study, 1 surgery unrelated to the study, and no hospitalizations and no deaths. During placebo administration, there were a total of 7 emergency department encounters unrelated to the study and 2 surgeries unrelated to the study, and no hospitalizations and no deaths.

Discussion

In this randomized, double-blind, placebo-controlled clinical trial, we found that 3 doses of testosterone cypionate 25 mg given IM every 4 weeks to infants with 47,XXY induce anabolic changes in growth and body composition. These physical outcomes were overall favorable, as they normalized penile length, growth velocity, and lean mass in the short term. However, this

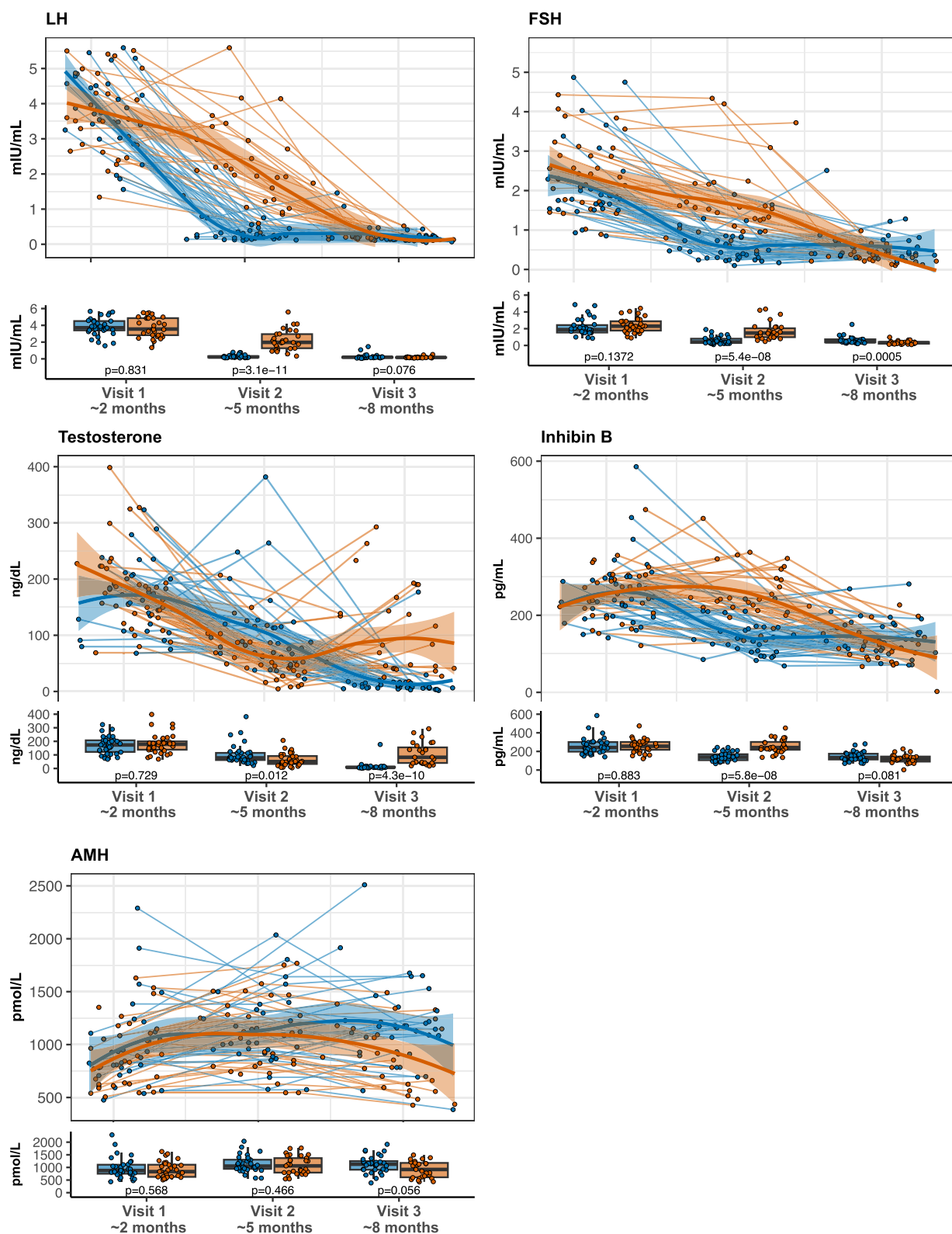


Figure 3. Spaghetti plots for longitudinal hormone concentrations by age (1-9 months) and corresponding box plots at each study visit for those in group A (blue) treated with testosterone followed by placebo, and group B (orange) treated with placebo followed by testosterone. Each circle is a value for an individual participant at a given time; lines of the spaghetti plot represent individual participants over time, and the bolder lines are smoothed loess curves with 95% CIs around the loess curves for each group. Box plots show the 1st and 3rd quartiles as the bottom and top of the box respectively with median in the middle and error bars representing 95% of the data.

Abbreviation: AMH, anti-müllerian hormone.

Table 3. Results for the second half of the study (12-24 wks)

	Group A (placebo)		Group B (testosterone)		Significance (P value)	
	Change v2-v3	Visit 3	Change v2-v3	Visit 3	Change	Visit 3
%FM z-score	0.07 ± 0.77	−0.86 ± 1.18	−0.68 ± 0.94	−1.0 ± 1.3	0.003*	.624
Length z-score	0.04 ± 0.67	−0.09 ± 1.14	0.61 ± 0.69	0.08 ± 1.05	0.002*	.537
Stretched penile length (cm)	0.06 ± 0.42	0.66 ± 0.75	1.63 ± 0.55	1.57 ± 1.06	<0.001*	<.001*
AIMS %ile	13.3 ± 31	54 ± 27	8.4 ± 20	53 ± 27	0.447	.854
Bayley 3 Cognitive Composite	1.1 ± 11.4	104 ± 9.4	0.7 ± 9.3	104 ± 7.9	0.881	.896
Bayley 3 Language Composite	−1.5 ± 11.7	94 ± 9.0	−4.6 ± 9.2	92 ± 8.0	0.260	.458
Bayley 3 Motor Composite	1.4 ± 15.6	98 ± 13	2.5 ± 11.4	98 ± 11	0.775	.896
PDMS-2 Gross Motor Quotient	2.7 ± 6.5	101 ± 7.3	5.5 ± 4.8	102 ± 6.9	0.073	.460
PDMS-2 Fine Motor Quotient	3.1 ± 7.4	104 ± 5.6	4.6 ± 7.7	104 ± 5.4	0.458	.933
ABAS-3 Generalized Adaptive Composite	0.5 ± 5.9	104 ± 6.2	−1.4 ± 6.1	103 ± 6.8	0.215	.347

Data are represented as mean ± SD. Change reflects the difference between values from visit 2 (~12 weeks) and visit 3 (~24 weeks), during which time group A was receiving placebo injections and group B was receiving testosterone injections. Visit 3 reflects the absolute values of the outcome measure from final visit assessment after both groups had received both treatments.

Abbreviations: ABAS-3 = Adaptive Behavior Assessment System Third Edition; AIMS, Alberta Infant Motor Scales; FM, fat mass; PDMS-2, Peabody Developmental Motor Scales—version 2.

*Denotes significance at alpha of 0.05.

intervention did not yield measurable beneficial effects on neurodevelopment, even in post hoc subgroup analyses. Additionally, our findings suggest potential adverse effects on the endogenous HPG axis, challenging the assumption that a short course of testosterone treatment is benign. Based on these results, there is no evidence on which to recommend testosterone treatment to modify the neurodevelopmental trajectory for infants with XXY; however, we cannot exclude a possible effect on neurodevelopmental outcomes emerging over time given the short evaluation time in this study. Longer term evaluation of cardiometabolic, neurodevelopment, and gonadal function outcomes is needed.

The physical effects of testosterone treatment that we observed align with our pilot study that found an increase in lean mass and corresponding lower adiposity (29). Multiple observational studies have found higher adiposity in XXY compared to male controls throughout the lifespan (29, 40, 41). In addition, adiposity early in life is associated with cardiometabolic disorders in adulthood, potentially because of lower lean mass, greater fat mass, or both (42, 43). The favorable results we observed on body composition may or may not persist and/or impact later metabolic health in this cohort and require further study. Similarly, testosterone treatment significantly increased penile size. A short course of testosterone is currently used in clinical practice to treat micropenis, resulting in increase in penile length with few reported side effects (44). For those enrolled in the current study, we found penile length to be slightly shorter than average; however, none of the boys enrolled in this study would qualify as having micropenis. Although treatment of micropenis with testosterone is accepted in both endocrinology and urology clinical practice, there are limited data informing short- or long-term outcomes of micropenis treatment beyond the initial gain in penile size, including the impact on future testicular function, childhood/adult wellbeing, or adult penile length. These longer-term outcomes are likely of more importance to patients and parents and warrant future study.

Our results are in contrast to existing literature proposing that testosterone treatment in the first year of life is associated with better neurodevelopmental outcomes in childhood in

XXY. Despite assessing multiple developmental domains with several standardized assessments and parent report, we did not appreciate any evidence supportive of neurodevelopmental benefits, despite multiple post hoc subanalyses. Samango-Sprouse et al compared a cohort of boys with XXY seen for clinical developmental evaluation who received infant testosterone with those who did not, reporting cognition, motor ability, language development, social skills, and behavior are all superior in the group with infant testosterone exposure (19, 45-47). However, there are multiple confounding factors that may have had an impact on these findings, including but not limited to baseline differences in families seeking off-label treatments (no randomization) and the lack of blinding of the assessors of subjective outcomes. Pursuing infant testosterone may have a positive effect on parents (eg, hope, empowerment) and subsequently parent-child attachment that supports development—we did not assess these outcomes. It is also possible that the many neurodevelopmental benefits found by Samango-Sprouse et al are not immediately apparent but emerge with time. Further study will be needed to answer these questions.

Treatment-emergent adverse events were generally anticipated, minor, and temporary, with the exception of suppression of the endogenous HPG axis that may have long-term effects on testicular function and future spermatogenesis. Future studies should also consider whether aiming for more physiologic supplementation with a lower dose of testosterone and/or alternative mode of administration (eg, transdermal, subcutaneous) would yield similar positive effects without HPG suppression. Although testicular dysfunction and infertility are nearly universal in XXY, most will spontaneously enter puberty and up to half of young men seeking biologic paternity can successfully retrieve sperm through testicular sperm extraction. Evaluation of testicular function in childhood, puberty, and beyond will be needed before we can consider infant testosterone a safe intervention for XXY or other indications. Testosterone treatment did not negatively affect infant temperament, which continued to be rated by parents as “easy going.”

Table 4. Adverse events

AEs	Entire study		On testosterone		On placebo		RR	95% CI	P value
	# of AEs n = 488	individual (%) n = 71	# of AEs n = 278	individual (%) n = 70	# of AEs n = 210	individual (%) n = 71			
Any AE	—	68 (95.8%)	—	64 (91.4%)	—	56 (80.0%)	1.87	1.03-3.93	.057
Increased fussiness	91	45 (63.4%)	54	34 (48.5%)	37	26 (36.6%)	1.28	0.91-1.77	.175
Penile erections	87	44 (62.0%)	71	44 (62.9%)	16	8 (11.3%)	2.90	2.08-4.14	<.001
Increased appetite	88	39 (54.9%)	43	27 (38.6%)	45	30 (42.3%)	0.93	0.65-1.29	.732
Acne or boils	62	34 (47.9%)	33	23 (32.9%)	29	20 (28.2%)	1.12	0.77-1.55	.586
Increased sleepiness	54	30 (42.3%)	28	19 (27.1%)	26	18 (25.4%)	1.05	0.70-1.47	.850
Eczema or another rash	30	26 (36.6%)	13	12 (17.1%)	17	16 (22.5%)	0.84	0.50-1.26	.527
Fever	16	11 (15.5%)	6	5 (7.1%)	10	8 (11.3%)	0.76	0.34-1.32	.562
Injection site reaction	13	9 (12.7%)	7	7 (10%)	6	3 (4.2%)	1.46	0.81-2.02	.208
Vomiting	12	9 (12.7%)	5	5 (7.1%)	7	6 (8.5%)	0.91	0.42-1.51	.999
Pubic hair	10	10 (14.1%)	6	6 (8.6%)	4	4 (5.6%)	1.23	0.63-1.82	.532
Change in stool pattern	7	6 (8.5%)	5	4 (5.7%)	2	2 (2.8%)	1.36	0.61-1.99	.441
Upper respiratory infection	7	6 (8.5%)	2	2 (2.9%)	5	5 (7.0%)	0.56	0.16-1.30	.441
Acute otitis media	4	4 (5.6%)	1	1 (1.4%)	3	3 (4.2%)	0.50	0.09-1.43	.620
Sleeplessness	4	2 (2.8%)	3	1 (1.4%)	1	1 (1.4%)	1.01	0.19-1.92	.999
Decreased appetite	2	2 (2.8%)	0	0 (0%)	2	2 (2.8%)	0.00	0.00-1.33	.497
Edema of foreskin	1	1 (1.4%)	1	1 (1.4%)	0	0 (0%)	2.03	0.42-9.60	.497

AEs could be reported at any time throughout the 6-month study period. Bolded AEs were attributed to testosterone treatment. Abbreviations: AE, adverse event; RR, risk ratio for adverse event occurring while on testosterone compared to while on placebo.

Although this study was robustly designed and adequately powered for short-term outcomes, there are several limitations to consider before applying these findings to practice. First, although we achieved diversity in terms of self-reported race and ethnicity, this study sample was highly educated and socioeconomically affluent, not only affecting generalizability to other populations but also potentially minimizing variability in outcomes that may mask smaller yet potentially significant benefits from testosterone. Next, although allowing parents to administer the injections was the most pragmatic approach for this national study, it does raise the possibility of inconsistent or improper intervention delivery affecting the accuracy of dosing, timing, adherence, and potentially outcomes. Regarding the neurodevelopmental outcomes, the measures used in this study may not be sensitive enough to detect changes in this population, although our inclusion of 3 different standardized direct assessments and a parent-report measure strengthens our confidence in our null findings for neurodevelopmental outcomes. Given we had very few boys with low baseline testosterone concentrations, it is still plausible those with an insufficient testosterone surge during the minipuberty period would have benefits from supplemental testosterone. Finally, this study was designed to assess short-term outcomes only and these outcomes do not necessarily have immediate clinical implications. Longitudinal follow up will be needed to determine the duration of the observed physical and hormonal effects of testosterone treatment in infancy, as well as establish whether neurodevelopmental benefits emerge over time.

Conclusion

In conclusion, in this double-blind randomized controlled trial, testosterone treatment in infants with XXY had clear

effects on physical outcomes and was well-tolerated clinically; however, no benefits or harms to short-term neurodevelopmental outcomes were noted. Intramuscular testosterone suppressed the HPG axis including the production of inhibin B, with unknown implications for future testicular function. The results of this study do not support universally adopting testosterone treatment for infants with XXY into clinical practice at this time; however, additional research potentially with lower doses and longer duration is warranted. It will be important to follow this cohort long term to determine if the altered hormone profile persists, if the advantageous effects on body composition are sustained, and whether neurodevelopmental benefits emerge with time or in a specific subset of the population.

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Data Availability

Deidentified individual-level data are deposited in the NIH Data and Specimen Hub (DASH), a controlled-access data repository and can be requested through the DASH Portal (dash.nichd.nih.gov).

Statement of Ethics

This study protocol was reviewed and approved by the Colorado Multiple Institutional Review Board, #17-1317. Written informed consent was obtained by a parent or legal guardian before any study procedures.

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