TITLE: Effects of Testosterone and Genetic Factors on Psychological and Motor Function in Klinefelter syndrome

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PROJECT SUMMARY/ABSTRACT:

Klinefelter syndrome (KS/XXY) is the most common chromosomal abnormality in humans (1:650 males) and represents an excellent model in which to study the interplay between genetic factors and reproductive hormones on neurodevelopment. Males with KS have increased rates of verbal cognitive impairments, executive dysfunction, psychosocial problems, and motor skills deficits. Testosterone deficiency develops during adolescence in the majority of affected males, but objective data about the psychological and motor effects of testosterone replacement therapy in KS is lacking. Here we propose the first-ever placebo-controlled study of the psychological and motor effects of testosterone therapy in adolescents with KS. We hypothesize that testosterone therapy initiated in early puberty in KS/XXY will lead to improvements in executive function, psychosocial functioning, and motor skills, while externalizing behaviors will remain unchanged. We also hypothesize that genetic polymorphisms in the androgen receptor gene influence response to testosterone therapy.

In the proposed research project we aim to: (1) study the psychological and motor effects of testosterone therapy in early adolescent males with KS/XXY and (2) investigate genetic factors influencing the clinical phenotype and response to testosterone therapy in KS/XXY, including androgen-receptor (AR) polymorphisms and parent-of-origin of the extra X chromosome. Our preliminary studies suggest that testosterone therapy started in early adolescence improves attention and self-report of personal adjustment, and does not lead to increased negative behaviors, and that individuals with the short CAG-repeat polymorphism of the androgen receptor gene have an improved response to testosterone therapy compared to the long CAG polymorphism. To accomplish our aims, we will conduct a randomized, prospective, double-blind, placebo-controlled trial of testosterone replacement therapy in Tanner 2-3 males with KS/XXY, comparing psychological factors (executive function, attention/inhibition, verbal fluency), behavior (social adjustment, aggression) and motor skills (strength, coordination) in testosterone versus placebo after 6 and 12 months of therapy. We will also evaluate if polymorphisms in the AR gene and the parent-of-origin of the extra X chromosome are related to the clinical phenotype or response to testosterone treatment. Results will influence treatment guidelines for testosterone in patients with KS/XXY and will lead to improved understanding of the pathophysiology of KS.

As a subspecialist in Developmental-Behavioral Pediatrics, I am committed to becoming an independent investigator with a research program focused on understanding the role of hormonal and genetic factors on neurodevelopment and behavior in children with sex chromosomal disorders and other neurogenetic syndromes, and in conducting clinical trials to develop evidence-based treatments to improve medical and psychological outcomes of children. This application outlines five primary career development aims that will (1) lead to specialization in clinical trials design and execution for neurogenetic disorders, (2,3) enhance experience in neuropsychology and molecular diagnostic methods to enhance future research endeavors, (4) increase understanding of current neuroimaging and animal research on reproductive hormone effects on neurodevelopment, and (5) enhance abilities to design research in vulnerable populations of children with neurodevelopmental and neurogenetic disorders applying current bioethical principles. These aims will be reached through direct experience during the research project, mentoring sessions, personalized tutorials, and participation in related research discussion groups and research conferences. Supplementary didactic coursework in neuropsychology, behavioral genetics, neuroendocrinology, and biostatistics will also lead to a Masters degree in Clinical Science.

This research project will recruit subjects through a unique clinic called the *eXtraordinarY Kids Clinic*, and will take advantage of strong infrastructure for research and career development support at Children's Hospital Colorado and the UC-Denver Colorado Clinical & Translational Research Institute. I have a assembled a strong team of mentors and collaborators with broad and successful research careers in psychology, outcomes in sex chromosomal abnormalities, endocrinology, clinical trials, genotype-phenotype studies, neurogenetic syndromes, developmental disabilities, bioethics, and molecular biology. My institution has committed to providing protected time for research, additional research supports including research space, research pharmacy services, statistical and database support, bioethical consultation, tuition/fees for coursework, and any additional supports needed to successfully complete the research project and to enhance my career development into an independent investigator.

INTRODUCTION/SPECIFIC AIMS

Klinefelter syndrome (KS) is the most common chromosomal disorder in males (1:650), and is associated with elevated rates of psychological impairments (executive function, attention, and verbal cognitive deficits, social and emotional disorders) and motor skills deficits. KS is associated with primary hypogonadism that most often develops during adolescence, leading to the need for testosterone replacement therapy. Due to the lack of objective data about the effects of testosterone therapy during adolescence in KS, there is significant variability in clinical practice in the timing of and factors considered when initiating testosterone. Although KS treatment guidelines describe improvements in various psychological factors following testosterone therapy, there is only one 1988 case series in KS adults describing general improvements in mood, attention, and social relationships.[14] The specific psychological effects of testosterone therapy have not been studied in adolescent males with KS. We hypothesize that starting testosterone therapy in early adolescence will lead to improvements in psychological factors and motor skills. Our preliminary, uncontrolled data suggests that testosterone improves attention and self-report of personal adjustment, and does not lead to increased negative behaviors. A prospective, placebo-controlled study is needed to guide management.

Genetic factors involved in the variability of the KS phenotype have not yet been elucidated. Preliminary studies suggest that polymorphisms in the androgen receptor (AR) gene on the X-chromosome and the parent-of-origin of the extra X chromosome influence phenotypic severity.[15-17] The long CAG-repeat polymorphism of the AR is less responsive to testosterone, and males with KS with the long polymorphism show more significant features (taller stature, increased gynecomastia, decreased professional employment) compared to those with a short polymorphism. Our new preliminary data suggests that more significant improvements in psychological functioning occur in KS males with the more responsive, short polymorphism, indicating there may be different responses to testosterone therapy that are dependent on the genotype at the AR in males with KS. A paternal parent-of-origin of the extra X chromosome may also be associated with a more severe phenotype. This study will examine whether polymorphisms in the AR gene or parent-of-origin of the extra X chromosome influence the clinical phenotype and response to testosterone in KS.

Specific Aim 1: To determine if testosterone therapy started in early puberty has beneficial psychological and motor effects in adolescents with KS, we will conduct a randomized, prospective, double-blind, placebo-controlled trial of 12-months of transdermal testosterone therapy.

- Recruit 50 adolescent males with KS in early puberty (Tanner stage 2-3)
- Randomize sample to receive either Testosterone vs. Placebo
- Evaluate baseline functioning using a battery of standardized assessments carefully selected to evaluate psychological factors (verbal and nonverbal cognitive skills, attention/inhibition, verbal fluency), behavior (aggression, irritability, social functioning), and motor skills (strength, coordination).
- Compare changes in the Testosterone vs. Placebo groups after 3, 6, and 12 months.

Hypothesis 1a: Testosterone treatment will lead to improvements in executive function (specifically, attention/inhibition and verbal fluency).
Hypothesis 1b: Testosterone treatment will lead to improvements in psychosocial functioning (personal adjustment, social skills), while externalizing behaviors (aggression, irritability) will remain unchanged.
Hypothesis 1c: Testosterone treatment will lead to improved motor strength and motor coordination.

Specific Aim 2: To investigate genetic factors influencing the clinical phenotype and response to testosterone therapy in adolescents with KS, including androgen-receptor (AR) repeat length polymorphisms and the parent-of-origin of the extra X chromosome.

- Using the battery of assessments outlined in Aim 1, obtain clinical phenotype data on the 30 subjects from Aim 1, plus an additional 25 subjects with KS of a broader age range (8-18).
- Isolate DNA from blood samples (n=75 total) to determine AR repeat length and parent-of-origin.
- Compare the clinical phenotype to genetic findings, adjusting for confounding variables (e.g. SES).
- For subjects treated with testosterone, compare the change in psychological and motor functioning from baseline to 1 year in subjects with a short versus long AR polymorphisms.

Hypothesis 2a: Individuals with a maternally-inherited supernumerary X chromosome will have higher cognitive and motor scores compared to the paternally-inherited group.
Hypothesis 2b: Males with KS with a short AR polymorphism will have higher cognitive and motor scores compared to those with a long AR polymorphism.

Hypothesis 2c: After testosterone therapy, males with KS with a short AR polymorphism will show greater improvement in psychological and motor functioning than those with a long AR polymorphism.

RESEARCH STRATEGY:

11. SIGNIFICANCE:

11.A. Prevalence, Medical Features, and Psychological Features in KS

Klinefelter syndrome(KS)/XXY is the most common chromosomal disorder in males. With a prevalence of 1 in 650 male births, there are approximately 235,000 males with KS in the U.S.[21, 22][23] A recent study suggests that prevalence of KS is increasing due to increasing maternal age and environmental factors.[24] There is a need for research on clinical treatments in this very common genetic disorder.

KS is associated with characteristic physical features and medical problems. The presence of the extra X chromosome leads to tall stature, changes in body proportions, small testicles, and infertility.[25] Most males with KS start puberty with testicular enlargement at a typical age (11-13 years), but then fail to attain normal adult testicular size and testosterone (T) production, leading to the need for T replacement therapy.[26] This T therapy is important to complete pubertal development and for long-term health benefits such as improved muscle strength, normalization of sexual function, and promotion of bone mineralization.

Psychological studies in KS show verbal cognitive deficits, executive function deficits, attention problems, and psychosocial problems. The most comprehensive studies on psychological functioning in KS were prospective studies of males identified by newborn screening in the 1970's and 80's who were followed into adulthood.[27-29] Dr. Bender, a primary mentor, was the lead psychologist for the Denver site. Cognitive evaluations showed full scale IQ in the average range, however with verbal IQ significantly lower than performance IQ and approximately 10 points less than sibling controls. Language-based learning disabilities are present in 80%.[30-33] Verbal fluency is impaired in KS compared to controls, with greater deficits on time-dependent tasks. [27]

Walzer and colleagues [33] reported on the "behavioral style" of children with KS, noting lower activity levels than controls, increased distractibility, and social withdrawal. Social interactions have been described as impaired due to decreased self-esteem and anxiety.[34, 35] A recent study in a self-selected sample of 51 Dutch children showed ADHD in 63%, increased social difficulties, and autism spectrum disorders in 27%.[36]

Studies of adults with KS have found executive function impairments,[37] especially in areas of attention, working memory, and verbal executive function. In children, a small study of 3 patients noted problems with inhibition, but not concept formation, problem solving, or set shifting ability.[38] Another recent study described language deficits, and attention problems without impulsivity in a cohort of 50 children.[39]

Motor function is impaired in KS. Hypotonia typically presents in infancy, and early gross motor delays are common with an average age of walking independently of 14.5 months, compared to 12.4 months in typical children.[40] A controlled study of motor development in 14 males with KS showed deficits in overall gross motor composite scores, as well as upper extremity coordination, speed and dexterity.[41] A recent larger study of 50 children demonstrated deficits in both gross and fine motor skills, with significant deficits in upper limb speed and dexterity, running speed, and strength in the adolescent group.[39] Intention tremor is associated with KS and other sex chromosome disorders, which can also affect motor skills.[42, 43]

In summary, previous studies have shown neuropsychological, behavioral, and motor skill deficits in males with KS. Studies on interventions that impact psychological and motor problems are lacking.

11.B. Testosterone Treatment in KS

Testosterone (T) deficiency develops in adolescence in males with KS. Most individuals with KS develop T deficiency during adolescence or young adulthood. There is strong evidence and consistent recommendations for T replacement therapy for normalization of bone density, pubertal maturation, muscle development, and sexual function in KS.[25, 26, 44-47] In typical adolescents, puberty begins with increasing levels of pituitary follicle stimulating hormone (FSH) and luteinizing hormone (LH), and LH stimulates production of T from the testes. In males with KS, FSH and LH typically increase normally in early puberty, but then commonly rise into the elevated range after age 12 due to testicular unresponsiveness.[48, 49] In most cases, serum T increases above prepubertal levels, however total T levels either remain at the lower limits of normal or drop below the normal range as shown in **FIGURE 2.**

*There is variability in the recommendations for the **timing of** initiation of T therapy in KS*. Most sources support therapy when LH and FSH begin to rise in early puberty,[50-55] others support waiting for a plateau or decreasing T level,[48, 56] and others initiate for symptoms of T deficiency.[54] While Wikstrom et al. questions whether T is needed in early puberty prior to significant deficits in serum T (Time 1 in Figure 2 below),[56] these authors noted findings of gynecomastia and exaggerated GnRH responses in early puberty,[56] and describe that "a relative T deficiency from midpuberty onwards is obvious…Consequently, although it seems that androgen supplementation from midpuberty onwards is necessary, to date, placebo-controlled studies showing the benefits of early T substitution are lacking, especially regarding the positive effects of early T therapy on cognitive and behavioral parameters."[48] **If psychological and motor deficits in males with KS improve with early T, this would support earlier initiation of T replacement therapy.**

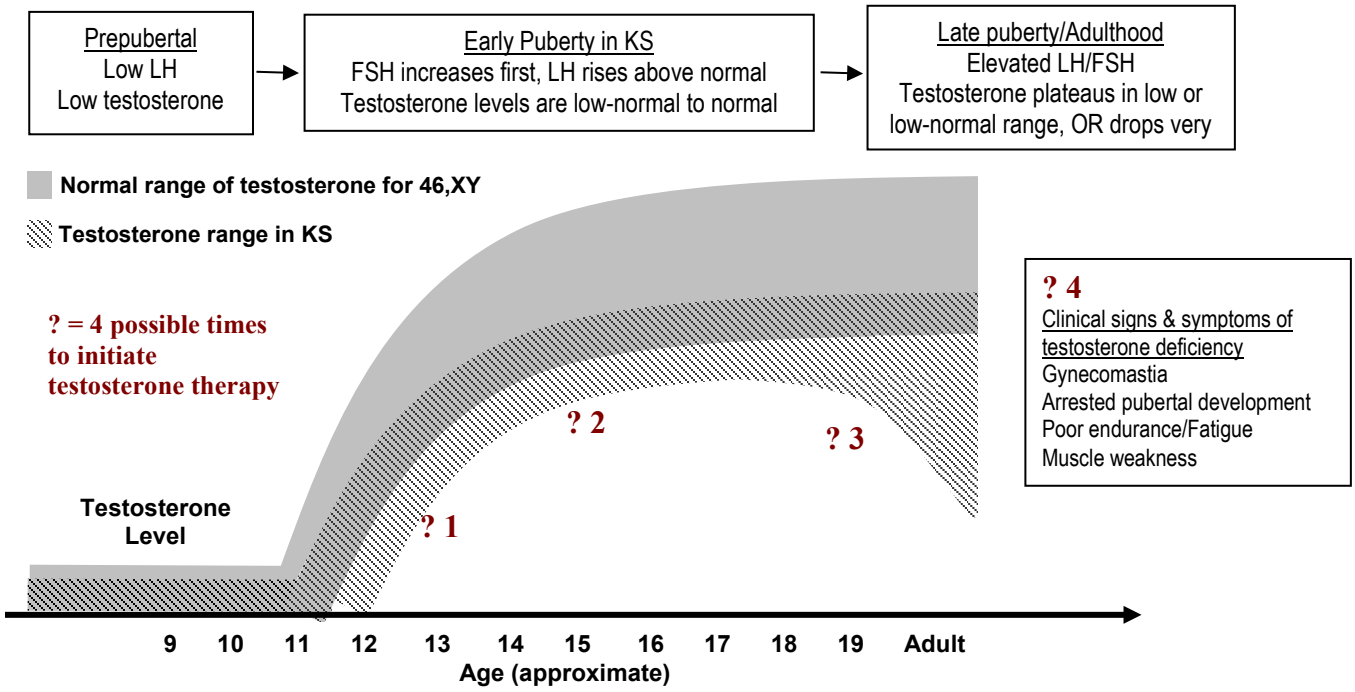

FIGURE 2. VARIABILITY IN TIMING OF TESTOSTERONE (T) TREATMENT. T levels in KS (dashed area) compared to normal males (gray). Question marks (?) indicate variability in practice in the timing of T therapy. Current recommendations are variable and include: (1) Beginning of puberty, (2) Mid-puberty at plateau or decrease in T with elevated LH/FSH, (3) fall in testosterone levels or (4) clinical signs/symptoms of deficiency.

Previous studies of psychological and motor effects of T in KS are limited and lack rigorous, controlled study design. However they suggest a positive effect on attention, executive function, motor skills, self-esteem, and verbal fluency. In a descriptive case series of adults with KS, T therapy was shown to have a positive impact on self-esteem, mood, energy level and general well-being in one long-term study.[57] A recent cross-sectional study of 50 males age 4-19 found a trend toward improvements in some motor skills (p=0.08 in agility, visual-motor control and running speed) in the 11 patients who had received androgen treatment compared to the untreated group.[39] Another study describes neuroimaging and verbal fluency in 10 XXY adults who had been identified by newborn screening, and 5 were currently or had been previously treated with T, while the other 5 had not. In the treated group, temporal lobe gray matter was increased and verbal fluency scores were higher than the untreated group.[58] These are the only studies that have examined the effects of T therapy on psychological or motor features specifically in KS. Other studies in hypogonadal adults described significant improvements in mood, verbal and spatial memory, and muscle strength after 2-6 months of treatment.[59-61]

Psychological effects of T Treatment in Other Adolescents with Hypogonadism. Studies on typical males with constitutional delay of puberty have shown mixed results. A double-blind, placebo-controlled trial of 24 boys with constitutional delay treated with T alternating with placebo for 2 years showed no significant effects on behavioral rating scales completed by parents. However, the boys in this study did not have baseline emotional or behavioral abnormalities, thus results support that worsening of behavior did not occur with T, but cannot address whether improvements occurred.[62] In the same cohort, adolescent self-report of aggressive

behaviors/impulses[63] and self-perceived competence[64] increased with treatment. Interestingly, parents did not report increased aggression, so perhaps changes in self-report reflect increased feelings of assertiveness by the adolescents. Other studies in androgen-treated adolescents with pubertal delays found improvements in adult social outcomes, self-esteem, and personal adjustment,[65] while others report no significant effects on adult outcomes,[66] spatial performance,[67] and behavior.[68] It is important to note that typical XY males do not have the neuropsychological phenotype of KS, so T may have different psychological effects.

In a group of males with Prader-Willi syndrome, a genetic disorder also associated with hypogonadism, cognitive deficits, and behavioral problems, there were no detrimental effects on behavior after T treatment.[69]

New evidence suggests that androgen deficiency may be present in infancy and early childhood in KS. Two studies have reported a lower neonatal T surge in prenatally diagnosed KS infants compared to XY controls.[16, 70] However, 1 study reports conflicting results with high-normal concentrations in 3 month old infants.[71] In 22 children less than 2 years old, Ross reported shorter phallus length, smaller testicular size, lower neonatal T, and hypotonia as evidence of early testicular failure.[16] Thus, if androgen deficiency is present in early child-hood, initiation of T therapy should be considered at an earlier age than current practice in late adolescence or adulthood. While the proposed study doesn't include prepubertal children, these recent findings support that T replacement may be needed at a younger age than current practice, supporting the need for the current study.

11.C. Genetic Factors in KS

The specific genes on the sex chromosomes and the genetic mechanisms involved in the variability of the phenotype of KS are not yet known. Proposed mechanisms have included gene dosage effects of sex chromosome genes that escape X-inactivation, polymorphisms of sex chromosome genes, and imprinted regions that would show differential phenotypes based on the parent-of-origin of the extra chromosomes [72].

Studies of polymorphisms in the androgen receptor (AR) gene on the X-chromosome suggest a correlation with the physical phenotype and social functioning in KS. The AR gene has a polymorphism in the CAG (cytosine-adenine-guanine) repeat number in the coding sequence, and the length of the CAG repeat (CAGn) inversely correlates with receptor responsiveness to T.[73] Adult males with KS with a high CAGn (long polymorphism, receptor less responsive to T) were shown to have more significant physical features of KS (taller adult height, more gynecomastia), and decreased employment and social outcomes.[74] A study in children with KS also found a correlation between CAGn and penile length.[75] Thus, a goal of our study is to examine if males with KS with long AR CAGn polymorphisms have more significant physical or psychological impairments, and to evaluate if there are differences in the response to T treatment. We hypothesize that those with a short AR CAGn polymorphism will have a better response to T, and our preliminary data supports this.

Parent-of-origin of the extra X chromosome may impact phenotype through mechanisms of imprinting. A study in 45,X showed that females with a maternally-inherited X were more likely to have social deficits and autistic behaviors compared to a paternally-inherited X.[76] In KS, the additional X chromosome is maternally inherited in approximately 50% of cases.[77, 78] Two studies suggest that a paternally-inherited extra X is associated with a more severe phenotype, including delayed puberty,[79] and impaired language and motor skills.[80] Other studies have not found associations with cognitive, motor, or psychiatric conditions.[81]

12. PRELIMINARY STUDIES:

12.A. Children's Hospital Colorado *eXtraordinarY Kids Clinic.* The *eXtraordinarY Kids Clinic* was started in 2007. Dr. Tartaglia is the clinic director, and team members include endocrinology (Dr. Phil Zeitler), 3 psychologists, genetic counseling, speech therapy, and occupational therapy. In addition to providing comprehensive clinical care for children with X&Y chromosome disorders, the clinic also serves as an excellent base for recruitment into clinical-translational research and represents a unique resource potentially unparalleled in other centers nationally. Referrals, clinic visits, and the waiting list have increased steadily since 2007. See **FIGURE 3.**

Dr. Tartaglia's previous research during fellowship training included over 200 subjects in 3 years with different X&Y chromosome disorders. She is involved as a speaker and advocate for this patient group. The strong infrastructure and her reputation with this population optimize the chances of successful recruitment.

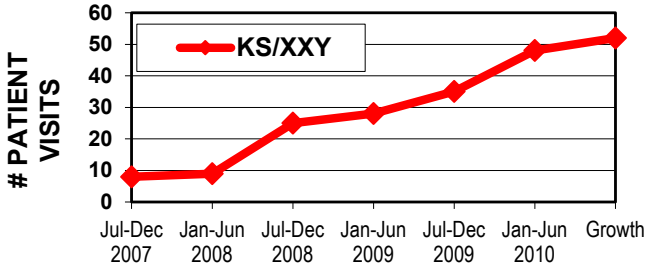

FIGURE 3 – Number of patient visits for KS in the *eXtraordinarY Kids Clinic* during each 6 month period since clinic opened in 2007.

	XXY	Other (Triple X, XXYY, etc)
Total visits	153	365
Ethnicity		
Caucasian	84.9%	86%
Hispanic	8.5%	6.6%
Black	3.3%	3.0%
Asian	3.3%	4.4%

Table 2 – Total numbers and ethnicity of patients since clinic opening

12.B. This topic has been identified as a research priority by the national advocacy group for KS (KS&A) and by international experts. In 2003, an NIH meeting co-sponsored by KS&A (www.genetic.org) and March of Dimes identified a research priority to "determine the relationship between hormonal therapy, neurodevelopmental outcome, and time of onset of treatment."[82] This priority was reiterated at the May 2010 International Meeting for Klinefelter Syndrome in Copenhagen, where international researchers across disciplines discussed research priorities. KS&A supports our efforts and will aid in recruitment. (Appendix A).

12.C. Pilot study of Psychological Effects of Testosterone Therapy in Males with KS. Preliminary, prospective data was obtained in a convenience sample of 16 adolescents with KS starting on T therapy and followed for 12 months. For this pilot study, the T treatment regimens varied due to differences in clinical care by individual endocrinologists. Patients and parents each completed a standardized questionnaire (Behavioral Assessment System for Children–2) at baseline and after 12 months of therapy. The BASC-2 parent-report includes subscales of externalizing behaviors (aggression, hyperactivity, conduct) and Attention problems. The self-report (completed by the adolescent) includes a score of Personal Adjustment, which includes domains of relations with parents, interpersonal relations, and self-esteem. Scores are reported as T-scores, with a mean of 50 (s.d.10). Scores <40 or >60 are in the "at-risk" range. Results were analyzed by paired t-test and are shown in **FIGURE 4**. There were no differences in externalizing behaviors after 12 months of T, however attention problems significantly decreased and personal adjustment significantly increased. Significant changes were detected after 12 months of T in this project. Studies of T in hypogonadal adults show statistically significant changes in psychological domains after 2-6 months.[59, 61]

Molecular studies were conducted to establish the molecular techniques for analysis of the AR CAGn polymorphism, to establish a median for data analysis, and to determine if response to T was dependent on CAGn. We conducted AR CAGn analysis on 40 males with KS, including the 16 in our T pilot study as per the protocol described in 13.E.2. Our findings of a median repeat length of 21-22 are consistent with the general population and previous studies in KS.[73-75]. The pilot study sample was subgrouped into short (CAGn≤21) and long (CAG≥22) polymorphisms. Results in **FIGURE 4** show that improvements in attention and personal adjustment were significantly higher in the subgroup with the short, more-responsive AR polymorphism.

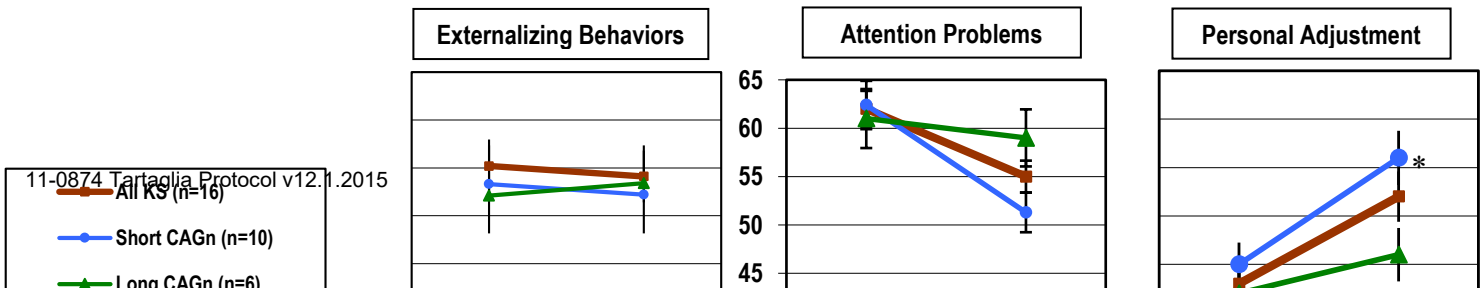

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FIGURE 4 – Behavioral effects of 12 months of testosterone therapy in males with KS (n=16)

12.D. Building on Pilot Research and Previous Experiences. My previous studies have been either descriptive or pilot studies conducted to obtain preliminary data for this application. The preliminary results presented are based on parent-report questionnaires from an uncontrolled study of a convenience sample of patients starting on T. The aims of the following proposed project build on pilot data and previous experience by including direct assessment of psychological and motor domains. Conducting a double-blind, placebo-controlled trial is new to this investigator and more rigorous in design than the pilot study. The proposed study also builds on previous experience by including an additional level of genetic analysis. Further study of these preliminary findings in a rigorous are indicated since these results may change management of KS.

13. RESEARCH APPROACH:

13.A. Overall Study Design (FIGURE 5)

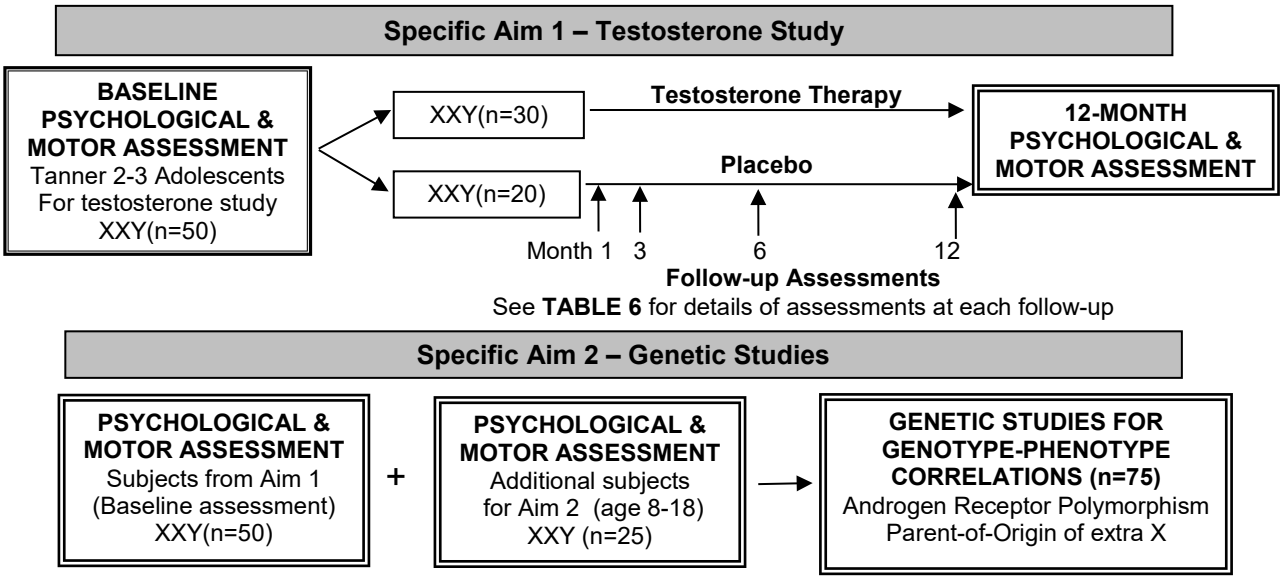

FIGURE 5. Overall Study Design

13.B. Recruitment. Participants will be recruited through: (1) the *eXtraordinarY Kids Clinic*, (2) subspecialty clinics including endocrinology, adolescent medicine, and genetics, (3) regional genetics outreach clinics (4) postings on websites and in newsletters of SCA organizations, and (5) presentations at national KS meetings.

A total of 75 subjects with KS will be included. For Aim 1, this will include 50 subjects in early adolescence (Tanner stage 2-3). We will also include their biological parents if available, for up to a possible 150 subjects in this parent of the study. This is a very motivated population and we anticipate good participation and low drop-out rates. However, to be conservative we have calculated sample size so that power will still be 80% for the Aim 1 primary outcomes with a 15% drop-out rate. An additional 25 subjects age 8-18 will be included to increase power for Aim 2. **TABLE 3** presents recruitment goals. We aim to recruit 85% of Aim 1 patients by the end of Year 3. We will need to enroll 66 patients in the first 3.5 years, or approximately 1.5 patients/month.

	Year 1	Year 2	Year 3	Year 4	Year 5
Aim 1	12	15	15	8	Final assessments, Run genetic studies, Final data analysis
Aim 2	5	5	5	10	
Total	**17**	**20**	**20**	**18**	**TOTAL = 75**

TABLE 3. KS participant recruitment goals per year

13.C. The Psychological & Motor Assessment Battery for Aim 1 and Aim 2 (TABLES 4 and 5).
To control for interrater reliability, assessments will be administered by the same professionals in the same order. All research team members are board certified and have experience administering the assessments.

Domain Assessed	Measure	Administered by
Cognitive/Adaptive	Wechsler scales (WISC-IV, WAIS-III) [83]	Dr. Boada / Neuropsychology
	Vineland Adaptive Behavior Scales–2 (parent interview)[84]	Dr. Tartaglia
	CGI-I / S	Dr. Tartaglia
Executive Function	Delis Kaplan Executive Function System (DKEFS) [85]	Dr. Boada / Neuropsychology
	Tower of London-Drexel [86], Wisconsin Card Sorting Test (WCST) [87]	Dr. Boada / Neuropsychology
	Wechsler Memory Scale Spatial Span Subtest [88]	Dr. Boada / Neuropsychology
	Test of Variables of Attention (TOVA) [89, 90]	Dr. Boada / Neuropsychology
Language/Memory/Learning	CTOPP Phoneme Awareness and Rapid Naming subtests [91]	Dr. Boada / Neuropsychology
	California Verbal Learning Test [92]	Dr. Boada / Neuropsychology
	Test of Word Reading Efficiency (TOWRE)	Dr. Boada / Neuropsychology
	Test for Reception of Grammar - Version 2 (TROG-II)	Dr. Boada / Neuropsychology
	Clinical Evaluation of Language Functioning (CELF)- formulated sentences sub-test	Dr. Boada / Neuropsychology
Motor	Grip Strength (Jamar dynamometer), Grooved Pegboard [93]	Dr. Tartaglia
	Beery Visual Motor Integration-5th Ed. (VMI and Motor Coordination) [94]	Syd Martin / Occupational Therapy
	Bruininks-Oseretsky Test of Motor Development–2nd Ed.(BOT-2) [95]	Syd Martin / Occupational Therapy

TABLE 4. Psychological and Motor Assessment Battery

Domain Assessed	Measure	Completed by:	Scored by:
Executive Function	Behavioral Rating Inventory of Executive Function (BRIEF) [96, 97]	Parent/Caregiver and Teacher	Dr. Tartaglia
	Conners' Parent and Teacher Rating Scales (ADHD) [98, 99]	Parent/Caregiver and Teacher	
Behavior and Social/Emotional	Behavior Assessment System for Children–2nd Edition [100, 101]	Parent, Teacher & Patient	Dr. Tartaglia
	Social Responsiveness Scale [101, 102]	Parent/Caregiver	
	Likert Scale of Mood [59]	Parent/Caregiver and Patient	
	Abberant Behavior Checklist	Parent/Caregiver	Dr. Tartaglia
Communication	Children's Communication Checklist-2	Parent/Caregiver	Dr. Tartaglia
Socioeconomic status	Hollingshead 2-factor questionnaire [103]	Parent/Caregiver	Dr. Tartaglia

TABLE 5. Questionnaire Battery

Rationale for selection of psychological and motor assessment battery: The above battery was chosen because it provides a comprehensive assessment of relevant areas of neuropsychological, behavioral, and motor function. Specifically, it includes verbal and nonverbal cognitive abilities (Wechsler scales), reading (TOWRE), and both verbal and nonverbal executive functioning (EF) tasks (Verbal Fluency, DKEFS Color Word Interference vs. Design Fluency, WCST, Wechsler Memory Spatial Span, Tower of London, TROG-II and CELF subtest). Attention and EF will be measured directly, as well as through subscales on parent and teacher questionnaires (Conners', BRIEF). Daily living communication skills will be measured using the Children's Communication Checklist- 2. Motor tasks have been chosen to focus on areas previously shown to be affected in KS, including motor coordination, dexterity, and strength.[39, 41] Adaptive functioning will be measured using the Vineland-II, including communication and social subscales. To characterize behavioral and social domains commonly affected in KS, we will assess externalizing behavior problems (BASC-2 externalizing subscales, Abbarant Behavior Checklist questionnaire), ADHD symptoms (Conners' subscales),

and psychosocial functioning (BASC-2 self-report, Vineland-2 Social Domain, Social Responsiveness Scale) and the Clinical Global Impression Scale by severity and improvement. Likert Scales of Mood Parameters identified significant differences in mood and energy in studies of T in hypogonadal adults.[59] The BASC-2, SRS, and VMI have been used in previous KS research to identify clinically significant differences in behaviors, ADHD symptoms, and motor skills.[11, 104, 105] Parental education and occupation will be collected to control for SES.[103] All tests are normed on appropriate age-stratified populations, and show acceptable reliability and validity. See Appendix B for all testing protocols and questionnaires being administered.

13.D. AIM 1 METHODS – Randomized, double-blind, placebo-controlled trial of testosterone in KS

13.D.1. Inclusion and Exclusion Criteria

Inclusion Criteria: Adolescent males of all ethnic and racial groups with nonmosaic KS in pubertal stage Tanner 2 or 3 will be included in Aim 1. *Rationale***:** We decided to use pubertal stage as entry criteria instead of chronological age because of the variable age of pubertal onset in typical males and in KS (there can be significant differences in pubertal development between two individuals of the same age). With the entire study group sharing physiologic changes of early puberty, there will be less variability in pubertal development within and between groups at baseline, and less concern of differential responses in prepubertal vs. pubertal males.
Exclusion Criteria: Exclusion criteria include non-English speaking individuals because many psychological questionnaires are not available in other languages. Individuals with a history of deep vein thrombosis, cancer or other medical problems that would interfere in their ability to complete the assessment are also excluded.

13.D.2. Study Protocol

Randomization/Blinding: Participants will be randomized to either receive Testosterone (T) (n=30) or Placebo (P) (n=20) as shown in **FIGURE 5.** A coded randomization scheme will be provided from the CTRC. All individuals (patient, parent, evaluators) will be blinded, except for the treating endocrinologist (Dr. Zeitler) and research pharmacist. Dr. Zeitler will remain unblinded in order to adjust the dose of the T group to achieve target serum T levels (see below), and so that he can monitor for P patients who meet criterion to "escape" to treatment (see Placebo section). *We considered the option of having Dr. Zeitler remain blinded, however it is important for him to be unblinded to ensure the intended differences in serum T levels between groups.* There is individual variability in the absorption of topical T, and the T dose needed to obtain target serum levels will different between subjects. For the P group, a plan for 'dose' changes has been developed to maintain blinding (see Placebo). To maintain blinding, Dr. Zeitler will not be informed of results of psychological assessments, and evaluators will not be told of details (i.e. dose changes) that may compromise blinding. A coding system will be developed for interim data safety monitoring. Blinding will be maintained until study completion.

Treatment group: T therapy will be delivered using transdermal gel (Androgel; Abbott Pharmaceuticals). Androgel that is typically dispensed via a pump system that delivers 1.25g per pump. The starting dose will be 1.25g (equivalent to 1 pump per day). For this study, the gel will be repackaged into syringes (to allow for delivery of a placebo gel in an identical delivery system). The syringes will deliver 17.5g of Testosterone per syringe. This is equivalent to a 2 week supply if the subject is on the starting dose of 1.25g per day, or a 1 week supply if the dose is increased to 2.5g per day. The subjects and their parents will be instructed how to dispense a single dose with each visit and understanding will be confirmed during each phone callThis treatment protocol has been developed based on publications by various experts in KS.[106, 107]. The gel was selected over injections because it is the formulation most likely to result in steady blood levels of T (compared to peaks and troughs of injections), it allows delivery of a noninvasive placebo formulation to a pediatric population, and it can be discontinued easily in case of adverse events. Initial T dose will be 1.25g/day and increased at 1 month intervals until target levels are obtained. Target levels for the T group will be the mid-range for pubertal stage as defined by laboratory norms:

Tanner 2 goal=150 ng/dL+/-50 (Normal 2-300) Tanner 4 goal=400 ng/dL+/-75 (Normal 90-840)
Tanner 3 goal=200 ng/dL+/-75 (Normal 30-390) Tanner 5 goal=500 ng/dL+/-100 (Normal 280-1000)

If a change in the treatment protocol needs to be made for unanticipated clinical concerns, Dr. Zeitler will make decisions preserving the best clinical care, and alterations in the protocol will be noted. We will continue to collect data in a blinded manner, and changes will be taken into consideration during data analysis.

Placebo Group: The Placebo (P) group will receive topical gel vehicle delivered in an identical container as the T group starting at 1 pump per day. To maintain blinding, the 'dose' of the P group will be increased at variable rates as determined by the research pharmacy so there are no clear differences in the volume of gel between T and P groups. To maintain blinding, both groups will follow the same education and monitoring plan (see below) so administration and side effect monitoring are equivalent. We developed a plan for patients in the P group to "escape" to the treatment arm if there are negative clinical consequences of delaying T (see **FIGURE 6** in Human Subjects). The criterion for 'escape' to treatment is the development of persistent gynecomastia (>90 days). In adolescents with KS, persistent gynecomastia is a clinical indication for initiation of T because T may minimize gynecomastia and associated psychological distress in adolescent males. Based on clinical experience, we anticipate that this will occur in 1-2 patients, and we have accounted for this loss in our power analysis. These patients will be followed for the duration of the study and included in Aim 2.

Medical History and Physical Examination: An interview detailing medical, psychological, and family history will be conducted by Dr. Tartaglia at baseline, using a datasheet created for the study. Examination will include assessment of height, segment ratios, BMI, Tanner pubertal staging, grip strength and neurological examination. We will request that psychiatric disorders are stable prior to enrollment, and that no changes to psychiatric medications are made during the study unless there is decompensation requiring clinical treatment.

	Baseline	1 month*	3 months	6 months*	12 months
Psychological/Motor Assessment					
Cognitive/Adaptive/Language Assessments	X				X
Executive Functioning Assessments	X			X	X
Motor Assessments					
Questionnaire Battery (Table 5)	X		X	X	X
Lab Draws/Xrays					
Testosterone, Luteinizing Hormone (LH)	X	X	X	X	X
Follicle Stimulating Hormone (FSH)	X			X	X
DNA for molecular studies (Aims 1,2)	X				
Complete Blood Count, AST/ALT	X	X	X	X	X
Lipid panel	X			X	X
Bone Age X-ray	X				X
History / Physical Examination	X	X	X	X	X
Adverse Events Monitoring Form		X	X	X	X

TABLE 6. Components of Study Visits and Safety Monitoring Plan*

*Telephone follow-up will also occur at 2 weeks and 9 months to monitor for adverse effects and to answer questions

Plan for Subject Education and Monitoring Compliance: Upon enrollment, each subject and their parents will receive an education session on the application of the gel and will be required to demonstrate proper technique in the dispensing and applying the gel. They will receive verbal and written instructions about timing of application, guidelines around use (i.e. showering, swimming) after application, potential side effects, and care to prevent transmission to others. The possibility of acne in early adolescence with or without T therapy will be discussed to maintain blinding. Lab draws for serum T levels will be drawn consistently in the morning before the daily dose. A fixed quantity of gel (T or P) will be dispensed at 2-month intervals, and the remaining gel will be measured by the research pharmacy after each 2 month interval to monitoring compliance.

Monitoring Plan for Adverse Events: Possible risks with T treatment include acne, a skin reaction at the application site, priapism, headaches, and a risk of hormonal effects in caregivers or partners with improper application techniques. If supraphysiologic levels are reached, behavioral agitation, polycythemia, and liver function abnormalities are additional risks. The current protocol provides T at conserv-ative dosages and with close monitoring as outlined in **TABLE 6.** The safety monitoring plan, including responsibilities of the Data Safety Monitoring Board (DSMB), is described in the Human Subjects section. A copy of the finalized DSMB protocol has been added as Attachment 1.

13.D.3 Aim 1 Outcome Measures and Data Analysis Plan

Hypothesis 1a: T will lead to improvements in executive function (specifically, attention and verbal fluency).

Primary Outcome Measures: Attention/Inhibition: Conners' Parent & Teacher Rating Scales ADHD DSM-IV subscale; Verbal Fluency: Delis-Kaplan Executive Function System Verbal Fluency Tasks
Secondary Outcome Measures: Attention/Inhibition: TOVA Continuous Performance Task, BRIEF, BASC-2 Attention subscale; Verbal Fluency: Vineland-2 Communication Domain
Analysis Plan: The primary outcomes of interest are the changes in scaled scores from baseline to 12 mos in the Delis-Kaplan EF scores, and the change in raw scores on the Conners' ADHD scales. We will test the patient specific change in a 2-sided, 2-sample t-test, comparing T to P groups. The mean scores for the Delis-Kaplan subtests in the normal population are 10 with a standard deviation of 3. We will assume a clinically significant average change of 4.5 (1.5 s.d.) with a range of 0–9. Allowing for 15% drop-out, we will have greater than 80% power to detect a change of 4.5 units with a significance level of 0.05. To analyze the difference in the Conners' ADHD raw scores at 12 months, we will compare patient specific change in the T and P groups by a 2 sample t-test. Using published data in normal controls compared children with ADHD, we have 80% power to detect differences of 8 points with current sample sizes. [98, 99]

A multivariate mixed model will also be employed to incorporate the 3 measurements (baseline, 6 months, and 12 months) on each child, allowing for the inclusion of covariates including age, race/ethnicity, and socioeconomic status.[108] Exploratory analyses will be performed to determine if baseline cognitive function or the secondary measures are associated with response to therapy.

Hypothesis 1b: Testosterone will lead to improvements in psychosocial functioning (personal adjustment, social skills), while externalizing behaviors (aggression, irritability) will remain unchanged.
Primary Outcome Measures: Psychosocial: BASC-2 personal adjustment scale (self-report), Social Responsiveness Scale (parent); Externalizing behavior: BASC-2 Externalizing Composite (parent)
Secondary Outcome Measures: Psychosocial: Vineland-2 Socialization scale; Externalizing behavior: Conners' Emotional Lability Index
Analysis Plan: For psychosocial functioning, power considerations and analysis plan are similar to Aim 1. Since we hypothesize no change in externalizing behavior scores, an equivalence test of means will be used. The current sample will achieve 80% power to conclude that the T and P groups are within one s.d.

Hypothesis 1c: Testosterone treatment will lead to improved motor strength and motor coordination.
Primary Outcome Measures: Motor Strength: Bruininks Test of Motor Development-2 (BOT-2) Strength subscale; Motor Coordination: BOT-2 Motor Coordination subscales
Secondary Outcome Measures: Motor Strength: Grip strength; Motor Coordination: Beery VMI Motor score
Analysis Plan: We will use analyses similar to those in Aim 1a.

13.E. AIM 2 METHODS – Investigating genetic factors influencing the phenotype and response to T.

13.E.1. Inclusion and Exclusion Criteria

Inclusion Criteria: An additional 25 males age 8-18 with KS of all ethnic and racial groups will be included.
Exclusion Criteria: Exclusion criteria are the same as Aim 1 but males with KS who are already on androgen treatment will also be excluded (to prevent confounding variables).

13.E.2. Methods. DNA will be extracted from venous blood samples and analyzed for CAG repeat length in the AR gene (HUMARA).[109] The CAG motif in exon-1 of AR is a hypervariable short tandem repeat of 9-36 repeats. , Amplification at the HUMARA locus is achieved by PCR of a 280bp fragment flanking the CAG repeat and two restriction sites. PCR products and the Genescan 500 ROX size standard will be analyzed by automated sequencer to determine CAGn by GeneScan software. The weighted CAGn mean of the 2 X chromosomes will be calculated as per the protocol described in Zitzman et. al.[74]

For parent-of-origin of the X chromosome, DNA will be extracted from cheek swab, blood or saliva samples. Pyrosequencing (PSQ) assays will be used to identify a set of 13 polymorphic sites on the X chromosome. The sequencing of these sites will be compared between the subject and parent samples to determine parent-of-origin.

13.E.3. Outcome Measures and Data Analysis Plan

Hypothesis 2a: Males with a maternal extra X will have higher cognitive/motor scores compared a paternal X.
Outcome Measures: Wechsler verbal and nonverbal IQ, BOT-2 Motor Scales

Analysis Plan: T-tests will be used to test for differences between outcome measures in the maternal vs. paternal groups. Assuming approx. 50% maternal and 50% paternal, we have 80% power to detect a difference of at least 0.75 s.d. between groups. Additional multivariate analyses will be performed to adjust for age, race/ethnicity and SES.

Hypothesis 2b: Males with short CAGn will have higher cognitive and motor scores compared to a long CAGn.
Outcome Measures: Wechsler verbal and nonverbal IQ, BOT-2 Motor Scales
Analysis plan: Spearman correlation coefficients will be calculated and p-values reported. The group will be split in short vs. long CAGn based on median value in preliminary data (21) and outcomes compared by t-tests. Additional multivariate analyses will be performed to adjust for age, race/ethnicity and SES.

Hypothesis 2c: After T, males with a short AR CAGn will show greater improvement than the long AR CAGn.
Outcome Measures: Post-treatment change in Wechsler verbal and nonverbal IQ, Vineland-2 adaptive composite, Executive function composite score, BOT-2 Motor Scales
Analysis plan: The change from baseline in psychological and motor function scores will be modeled using repeated measures regression techniques, with an interaction term for treatment arm and AR polymorphism length. P patients will be included as controls. Linear contrast will be used to test slopes in specific groups. We have 80% power to identify response differences between the short vs. long groups.

13.F. ANTICIPATED CHALLENGES AND ALTERNATIVE STRATEGIES. We have taken great care to emphasize our strong infrastructure, experience, and patient numbers to support our abilities to recruit target numbers for this project. If goals are not met during the first 2 years, we will expand recruitment to regional medical centers in Arizona, New Mexico, and Utah, and other professionals involved in the KS community. Recruitment of minorities has been a challenge because of primary recruitment from internet-based sources. We will also broadly recruit from regional genetics and community clinics where more minorities are seen.

Since there is variability in the natural production of T in early pubertal males with KS, it is possible that there will not be significant differences in the serum T levels between T and P groups (i.e. some P patients will have endogenous serum T levels that are equivalent to the T group). We will compare serum T levels between groups at 6 and 12 months. If there are not significant differences, we will also analyze data based on serum T levels to determine if T levels correlate with outcomes. Aim 2 does not depend on the results of Aim 1. Thus, genotype-phenotype correlation studies in Aim 2 stand alone as significant contributions to the literature in KS.

13.G. FUTURE DIRECTIONS. This project will generate results that will lead to important follow up studies and new hypotheses. If results show that there are positive changes in the T group compared to P, this will support earlier initiation of T therapy, and results will need to be confirmed in a multicenter design that broadens the physical outcome measures (i.e. bone density, gynecomastia) and also more deeply explores psychological domains that change with T using more specific neuropsychological constructs and other modalities such as neuroimaging. If there are differential effects of T based on the AR polymorphism, then future studies will be needed to establish if there are different dose response curves based on AR polymorphism, and if higher T dosages in patients with the less responsive receptor can lead to similar physical, psychological, and motor improvements (i.e. personalized medicine). If our hypotheses are not supported, then studies investigating genetic, medical, neuropsychological, and social factors that predict improved psychological outcomes in KS are needed so that interventions other than androgen treatments can be developed and tested. Future directions also include development of a protocol for XXYY and XXXXY where there is often reluctance to start T treatment due to more significant cognitive and behavioral difficulties. Training and experience in design and execution of clinical trials will also allow investigation of other interventions, including psychological/academic therapies or psychopharmacologic medications.

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HUMAN SUBJECTS SECTION
P.I. Nicole Renee Tartaglia, MD

14. Protection of Human Subjects:

14.1 Risks to Human Subjects

14.1.a Human Subjects Involvement, Characteristics, and Design
Seventy-five males with Klinefelter syndrome (KS)/XXY will be included in this protocol. All subjects will be between the ages of 8 and 18. Review of previous genetic testing results showing 47,XXY karyotype will be confirmed prior to participation in the study.

Inclusion criteria include English speaking male adolescents age 8-18 with 47,XXY syndrome. For Aim 1, 50 adolescents in early puberty (Tanner 2-3) will be included. Participants in Aim 1 will be randomized through a randomization scheme developed by the CTRC to 2 groups, either Testosterone (T; n=30) or Placebo (P; n=20). The T group is larger to allow for improved power to analyze for differences in response to testosterone depending on subgrouping by the polymorphism of the Androgen Receptor gene. For Aim 2, 25 individuals age 8-18 will be included. All ethnic groups and racial groups will be included.

Exclusion criteria include non-English speaking individuals because many of the standardized psychological questionnaires are not available in other languages. Individuals who are already on testosterone therapy will be excluded from Aim 2. Individuals with medical problems that would interfere in their ability to complete the assessment are also excluded from the study. Individuals with a medical history of deep vein thrombosis (DVT) or cancer are also excluded. Mosaic karyotypes will also be excluded because they often show milder neuropsychological problems, and we would like to optimize potential effect sizes for this study.

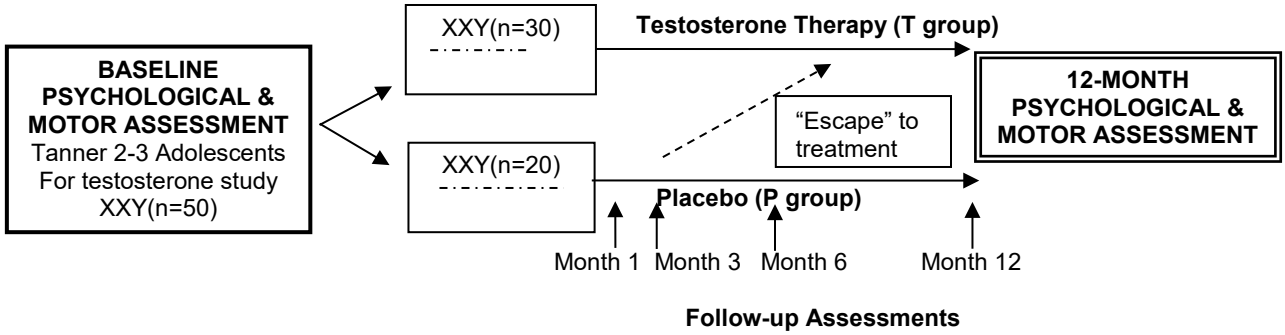

FIGURE 6. Study design for Aim 1. Diagram includes 'escape' to treatment group if testosterone becomes clinically indicated during the study (persistent gynecomastia >3 months).

For subjects in Aim 1 in the T group, transdermal testosterone will be initiated using transdermal gel (Androgel) that is typically dispensed via a pump system that delivers 1.25g per pump. The starting dose will be 1.25g (equivalent to 1 pump per day). For this study, the gel will be repackaged into syringes (to allow for delivery of a placebo gel in an identical delivery system). The syringes will deliver 17.5g of estosterone per syringe. This is equivalent to a 2 week supply if the subject is on the starting dose of 1.25g per day, or a 1 week supply if the dose in creased to 2.5g per day. The subjects and their parents will be instructed how to dispense a single dose with each visit and understanding will be confirmed during each phone call. This starting dose has been developed based on publications by various experts in endocrinologic treatment of KS.[87, 88] Topical preparation was selected over injections to minimize risk, to allow delivery of a noninvasive placebo formulation to a pediatric population, and to administer a formulation most likely to result in steady blood levels of T (compared to peaks and troughs of injectable preparations). Also a topical formulation can be discontinued easily if there are adverse events. Dosage

will be increased at 1 month intervals until target levels are obtained. Target levels for the T group will be the mid-range for pubertal stage as defined by laboratory norms:

Tanner 2 goal=150 ng/dL+/-50 (Normal 2-300) Tanner 4 goal=400 ng/dL+/-75 (Normal 90-840)
Tanner 3 goal=200 ng/dL+/-75 (Normal 30-390) Tanner 5 goal=500 ng/dL+/-100 (Normal 280-1000)

Appreciation for Participation**:** Patients will receive gift cards of $5 per lab draw, $15 for psychological assessments, $20 at the 12-month evaluation. Teachers will receive a $10 gift card once for appreciation of their time at the beginning of the study period.

14.1.b Sources of Materials

Research material collected in this study includes psychological and motor assessment results, standardized behavioral questionnaires completed by the subject, his parent(s) and teacher. Teacher questionnaires will be sent directly to the teacher with a letter providing parent consent to the teacher participation (see attachment 4). Blood samples will also be collected at each study visit for safety monitoring (laboratory testing), serum testosterone levels to determine dosage changes, and DNA analysis. Cheek swab samples will be collected at the baseline visit to collect DNA samples for parent-of-origin analysis. Only members of the research team and the data safety monitoring board will have access to identifiable private information about the subjects. For patient safety, all data will be kept in a research file in a locked drawer, in a locked office. All computer files will be password-protected on an encrypted server.

These materials will be collected by the research team at the study visits specifically for the proposed research project.

14.1.c Potential Risks

There is an increase over minimal risk to the patients due the study population being children, and a portion of the study population having cognitive impairments. The psychological and motor assessment protocol includes assessments that cause little increased risk to the participant above standard medical care, since psychological assessments are also often clinically indicated in this age group in this population.

Testosterone therapy is being initiated in this protocol in adolescents in early puberty. There is significant variability in the published recommendations about when to initiate testosterone replacement therapy in KS/XXY, and some sources recommend initiation of testosterone in early adolescence (as it is being done in this study), while others recommend waiting for more significant symptoms later in adolescence or adulthood.

As with any medication, there are risks and potential side effects associated with treatment. Possible common risks with testosterone treatment include acne, a skin reaction at the application site, and headaches. There is a small risk that topical testosterone preparations can produce hormonal effects in female caregivers or partners if proper application techniques are not followed or if there is vigorous skin-to-skin contact after application. If excess testosterone is applied and supraphysiologic levels are reached, behavioral agitation, abnormalities in liver function, priapism, and polycythemia are additional increased risks. There is also a potential risk that supraphysiologic levels of testosterone will lead to fusion of the growth plates and decreased final adult height. Close monitoring will occur to follow for elevated serum testosterone levels as shown in the monitoring plan in TABLE 6 below (copied from the research plan).

	Baseline	**1 month***	**3 months**	**6 months***	**12 months**
Psychological/Motor Assessment					
Cognitive/Adaptive/Language Assessments	X				X
Executive Functioning Assessments	X			X	X
Motor Assessments	X				X
Questionnaire Battery (Table 5)	X		X	X	X
Study Procedures					
Testosterone, Luteinizing Hormone (LH)	X	X	X	X	X

Follicle Stimulating Hormone (FSH)	X			X	X
DNA for molecular studies (Aims 1,2)	X				
Cheek swab for molecular studies (Aims 1,2)	X				
Complete Blood Count, AST/ALT	X	X	X	X	X
Lipid Panel	X			X	X
Bone Age X-ray	X				X
History / Physical Examination	X	X	X	X	X
Adverse Events Monitoring Form		X	X	X	X

TABLE 6. Components of Study Visits and Safety Monitoring Plan*

*Telephone follow-up will also occur at 2 weeks and 9 months to monitor for adverse effects and to answer questions

There are 5 venous blood draws associated with the study which expose the subject to risk, as well. The venipuncture procedures are only slightly beyond what would occur in standard medical practice for children with KS/XXY in early puberty (where they would typically receive approximately 1-2 blood draws per year to monitor for serum testosterone levels).

At the baseline visit, cheek swab samples will be collected from the participant and at least 1 parent. Samples from both parents will be collected if they are both present for the visit. The subject or their parent(s) may experience mild discomfort however there are no additional risks associated with this procedure.

At baseline and 12 months, a bone age X-ray will be obtained to monitor for whether testosterone treatment leads to any changes in bone age. There is a risk of increased exposure to radiation. To minimize risk, if a subject has had a previous bone age X-ray within 2 months of the base line visit, and if the X-ray is available for review, then we will not repeat the baseline X-ray.

The psychological assessment measures are standard assessments used by psychologists or developmental-behavioral pediatricians in clinical practice. The full research assessment will take approximately 6 hours to complete, depending on the skill level of the participant (those with higher cognitive functioning take longer to assess. However, those with higher cognitive functioning are generally more likely to tolerate a longer assessment period, as well). Some of these hours will involve interview of the parent or completion of standardized questionnaires by the parent, decreasing the demand placed upon the child. These assessments put the subject at risk for frustration and fatigue.

Loss of confidentiality is a risk to all research participants. Please see below for further discussion of protections against risk.

Alternative treatment would be to withhold testosterone replacement therapy in early adolescence and monitor serum for physical exam changes (gynecomastia), lack of pubertal development, or low serum testosterone levels to determine when therapy should be initiated. This is essentially being done in the placebo arm of the current study to better answer the question of when testosterone replacement therapy should be initiated.

14.1.2 Adequacy of Protection Against Risks

14.1.2a Recruitment and Informed Consent
Participants will be recruited through postings of a COMIRB-approved recruitment flyer on internet websites and in newsletters of KS/XXY support organizations, and postings in endocrinology, child development, and genetics clinics at Children's Hospital Colorado and regional private and community clinics. The study will also be posted on clinicaltrials.gov website as required.

We will also recruit patients through a web-based clinical research recruitment site called Recruitsource.com. Many patients with XXY have registered with Recruitsource and indicated the types of trials they are interested in participating in. This database allows researchers to search for

patients who meet their inclusion criteria in their database. The researcher then provides the list of potential subjects from database to the Recruitsource administrators, and these subjects are contacted through Recruitsource with a link to the clinicaltrials.gov website to review the study information and provides our contact information if they would like more information about the trial.

If interested in participating, study subjects initiate contact with Susan Howell (genetic counselor and research coordinator in *eXtraordinarY Kids Clinic*) for further information and instructions. This initial contact with occur by phone or email. Susan Howell will contact eligible families separately from clinical visits to discuss the study, and will emphasize the differences between clinical care and research. Dr. Tartaglia will be available to answer questions as needed. Ms. Howell will not inform Dr. Tartaglia of families who have declined participation as to not affect the clinical relationship or influence or clinical care.

Consent will be obtained in a private room in the CTRC Research Clinic. The CTRC is a research clinic that is in a different building than the clinical visits for the *eXtraordinarY Kids Clinic*, further distinguishing research from clinical care. At the first study visit, the consent and assent form will be reviewed with the subject and their parents by Susan Howell, and they will be given the opportunity read the protocol, and to ask additional questions about the protocol. Comprehension and autonomy will be assessed by asking questions about the study and assessing their response., and participants will be given as much time as they feel is necessary to have the study goals and methods explained to them, and have their questions answered prior to signing the consent form.

Since all participants in the study are under 18, additional research procedure for research in children will be followed. Each child participant will also be assented using language and content appropriate for their developmental level. This will include information about the research goals, methods, risks, benefits, and confidentiality delivered using developmentally-appropriate language. Since participants in this study will be children over age 7, the official assent form will be reviewed and signed with a witness present. For adolescents whose capacity to understand resembles that of adults, the assent procedure will include the information similar to what would is provided for informed consent by adults. All children will have the opportunity to ask questions about the study, and the child will be asked to explain the goals and procedures of the study in their own words. During the assent process, the child will be advised that they can terminate their participation in the study at any time without affecting their clinical care. Assent will be obtained in a private room in the CTRC and participants will be given as much time as they feel is necessary to have the study goals and methods explained to them, and have their questions answered prior to signing the assent form.

The subject will be asked to provide consent if they turn 18 during the course of the study. Upon enrollment in the study, it will be determined if they will turn 18 during the study period. If this is the case, when they provide assent we will inform them that we will discuss their study participation with them again when they reach age 18. For these subjects, upon turning 18 they will first be contacted by telephone by a familiar member of the research team and sent a consent form addressed directly to them in the mail. At their next study visit, the consent form will be reviewed with the subject and informed consent will be obtained prior to any further study procedures being performed.

For rare cases of XXY associated with intellectual disability, there may be concerns about decisional capacity to participate in research. To determine if they have the capacity to consent for research, we will first determine if their decisional capacity has been assessed in the past through court proceedings. If this has not occurred, we will review any previous cognitive assessments to determine if cognitive impairments may be present that may influence decisional capacity. We will assess decisional capacity by asking the participant to restate in their words the purpose of the research and the steps of the research project. If there is a concern about the cognitive abilities of the subject or their overall ability to understand the research, the research team will consult with the child psychologist and ethicist of the Data Safety Monitoring Board (DSMB), who are very experienced in assessment of mental health and developmental disabilities. If decisional capacity is assessed to be impaired during the psychological testing after the consent process, consultation with the parent/guardian and the (DSMB) will be sought to determine whether individual has the decisional capacity to provide assent.

After all questions are answered and the consent and assent has been obtained, the subject and their parent/guardian, and the investigator will sign the consent form, and copies will be provided to the family.

14.1.2b Protection against Risk

a. Neuropsychological Assessment

The protocol includes a battery of tests evaluating developmental milestones, cognitive functioning, social-emotional functioning and motor skills. Associated risks can include:

1) Fatigue or frustration by the child/research participant

Plan to minimize risk: Assessments will be administered by experienced professionals who commonly encounter these emotions in testing situations in clinical settings, and who have been trained in techniques to prevent fatigue and frustration in the assessment of individuals with typical development and developmental disabilities. Examples include motivational strategies and rewards, offering and taking breaks as needed, providing snacks, and making the assessment as enjoyable as possible. If these strategies are not successful, participants and their parents/caregivers will be given the option to postpone or discontinue the assessment.

2) Discomfort or sadness by the parent/caregiver of the research participant

The parent/caregivers of the subject may experience distress or discomfort because some of the questions and questionnaires will be about sensitive topics such as the child's developmental skills and behaviors, including questions about maladaptive behaviors. Sometimes talking or answering questionnaire questions about a child's difficulties makes parents feel sad or overwhelmed.

Plan to minimize risk: The professionals involved in this study are trained and experienced in this situation, since it also occurs in our clinical practices. This risk will be discussed during the consent process, and parents/caregivers will be told that they can refuse to answer any questions and to postpone or stop the interview at any time. They will also be offered the opportunity to talk further with members of the research team about their questions and concerns, which will also minimize risk.

b. Medical Treatment/Procedures

1) Testosterone therapy

Testosterone therapy is being initiated in this protocol in adolescents in early puberty. There is significant variability in the published recommendations about when to initiate testosterone replacement therapy in KS/XXY, and some sources recommend initiation of testosterone in early adolescence (as it is being done in this study), while others recommend waiting for more significant symptoms later in adolescence or adulthood. However, as with any medication, there are risks and potential side effects associated with treatment as described above. Possible common risks with testosterone treatment include acne, a skin reaction at the application site, increased erections and libido, and headaches. There is a small risk that topical testosterone preparations can produce hormonal effects in female caregivers or partners if proper application techniques are not followed or if there is vigorous skin-to-skin contact after application. If excess testosterone is applied and supraphysiologic levels are reached, behavioral agitation, abnormalities in liver function, and polycythemia are additional increased risks. There is also a potential risk that supraphysiologic levels of testosterone will lead to fusion of the growth plates and decreased final adult height.

Plan to minimize risk: The current protocol provides testosterone at very conservative dosages to avoid potential complications from supraphysiologic levels. Research participants will be informed of the possible associated risks with testosterone therapy. All subjects and their parents/caregivers will participate in a session at the beginning of the study to review proper application techniques and how to minimize risk of spread to caregivers or female partners (rubber gloves, avoiding skin-to-skin contact at application site for 6 hours after application, etc.) Parents/caregivers will also be provided with a one-page instruction sheet with directions on

how to administer the gel with proper techniques and minimizing spread (see Attachment 2 for a copy of this instruction sheet provided to families). In addition, families will be provided with a dosing diary to track the amount of gel administered daily and identify any problems leading to missed doses (see attachment 3 for a copy of the dosing diary provided. Phone follow-up will be conducted 2 weeks after starting the study, and follow-up monitoring visits including medical history, physical examination, and blood draws will be conducted at 1, 3, 6, and 12 months after starting treatment. Levels will be monitored at these times to ensure that levels are maintained at a therapeutic dose but well below the supraphysiologic range. If supraphysiologic levels are identified, testosterone therapy will be discontinued for 2 weeks and additional monitoring will be performed at that time to determine serum testosterone levels and whether testosterone therapy at a lower dosage should be initiated.

TABLE 6 above outlines the plan for follow-up medical history, physical examination, and laboratory testing developed for the purpose of safety monitoring, as well as follow-up phone call to monitor for side effects and answer questions about the treatment. Assessment of adverse events will occur at each study visit for both Testosterone and Placebo groups using a standardized AE form.

2) Venous blood draws

There are five venous blood draws associated with the study, which exposes the subject to risk of pain, bruising and infection as a result of the venipuncture. However, this risk is not elevated above the risk with a clinical venipuncture.

Plan to minimize risk: All venipunctures will be performed by experienced phlebotomists at Children's Hospital Colorado using standard precautions.

3) Cheek swab collection

During the cheek swab collection from the participant and the parent(s), they may experience mild discomfort however there are no additional risks associated with this procedure.

c. Loss of Confidentiality and Privacy

1) Violation of privacy and loss of confidentiality are both risks to which research participants are exposed. The possibility of these risks increases when protected health information is collected.

Plan to minimize risk: All study evaluations will be conducted in a room that is private so as to not increase this risk. Any research that is conducted about a specific genetic condition may be considered stigmatizing since there is the potential for discrimination related to the genetic disorder. We are very sensitive to these concerns due to our previous work with this population. In addition to the standard safeguards, we protect privacy by limiting opportunities for this privacy to be breached. For example, after initial contact is made by the family/individual with KS/XXY, our correspondence by phone and email does not use the genetic diagnosis. Instead, wording such as "We are calling to confirm your appointment with Dr. T tomorrow," or "….research study at Children's Hospital…." are used. Since some parents and adults are often private about the genetic diagnosis, we discuss this with each family or individual participating in the study to determine the most appropriate wording for the research report, correspondence, etc. to respect their privacy and minimize risk.

Confidential ID numbers will be developed for each subject, and this number will be used for all study questionnaires and testing results. Personal information will not be included in the computer data files, and only a confidential ID number will be used for computerized data. A database for study results will be developed through RedCap with the help of the CTRC Data Management Team, and this file will be on a secure network, password-protected, and backed up according to standard accepted CTRC protocols. Paper research files containing other medical records that are pertinent to the study will be kept in a locked file cabinet in Dr.

Tartaglia's locked office. All members of the research team will be COMIRB certified to ensure full education about research risks.

d. Risk to the Investigators

There is no known risk to the investigators.

14.1.3 Potential Benefits of the Proposed Research to Human Subjects

The potential benefits for the patient is testosterone treatment by a team with experience in their specific genetic disorder with careful monitoring and follow-up. The information gained from the psychological assessment protocol may be very useful in a clinical setting to identify cognitive strengths and weaknesses that may inform educational interventions, may identify challenges that would respond to various interventions such as speech or occupational therapy, or may identify important psychological diagnoses that would benefit from treatment such as anxiety disorders or ADHD. These assessments are also conducted by a team of professionals with experience and background in KS/XXY. This expertise is rare in the U.S., and allows participants and their parents to ask questions related to the specific diagnosis, and to receive feedback from professionals with experience treating individuals with these conditions.

14.1.4 Importance of Knowledge to be Gained

Results of the study will directly affect clinical care of individuals with KS/XXY by identifying the positive and/or negative psychological and motor effects of therapy. Even if no significant improvements in psychological or motor factors are identified, documentation of either the presence or absence of worsening behavioral problems with testosterone treatment will be an important contribution to the medical literature. In this population, parents of children with KS and associated behavioral difficulties often experience reluctance to start therapy by clinicians due to concerns of worsening behavior, and literature resulting from this study will help clinicians and parents be more informed about testosterone treatment in this population. Additionally, results of this study may show differences in the response to testosterone that are dependant on the polymorphism of the androgen receptor gene. If this occurs, this would influence the clinical care of males with KS and would suggest that testosterone replacement doses may need to be adjusted depending on the genotype at the AR gene in males with KS.

4.1.4 Data and Safety Monitoring Plan

Data will be reviewed every 1 month for safety of the treatment protocol and side effects by Dr. Tartaglia, Dr. Zeitler, and the research team. In addition, since this is a blinded clinical trial in which some individuals will be receiving testosterone treatment at a time that is not standard of care by some recommendations, we will also establish a Data Safety Monitoring Board to review the study as described below. The endocrinologist (Dr. Zeitler) in the study is not blinded to the treatment, and thus he will be able to determine if the treatment or placebo protocol is clinically harmful to an individual and if there is a clinical indication for a different treatment.

Also, if statistical significance favoring one treatment method over the other is identified prior to completion of the study, and we feel there is harm to those not in that treatment arm, we will discontinue the study and publish results on the significant findings.

All adverse events will be reported to the IRB, and to other agencies as required including the FDA as per their protocol.

Table 6 above (copied from the research proposal), outlines the plan for follow-up medical history, physical examination, and laboratory testing developed for the purpose of safety monitoring, as well as follow-up phone call to monitor for adverse events.

Data Safety Monitoring Board: The study will be monitored by an independent DSMB under the auspices of the Colorado Clinical Translational Sciences Institute (CCTSI) CTRC which adheres to standard practices to assure human participant protection and data integrity. Members of the DSMB will include a statistician, endocrinologist, child psychologistand human subject advocate. The DSMB will monitor the numbers of participants being followed in each of the two arms (treatment and placebo) to assure the planned numbers per group, and will advise the PI and study team as necessary. The DSMB will monitor the occurrence of laboratory abnormalities, medication side effects, and increases in externalizing or negative behaviors in participants to assure that negative effects of testosterone are minimized. If side effects, laboratory abnormalities, or behavioral changes are deemed intolerable or negatively impact health of the study participant, the participant will be removed from the study and provided with appropriate medical or psychological follow-up. See Attachment 1 for a copy of the finalized DSMB protocol.

4.1.5 ClinicalTrials.gov Requirements

If funded, this trial will be registered at ClinicalTrials.gov.