Protocol #: **17-1317**
Project Title: **TESTO: Testosterone Effects on Short-Term Outcomes in Infants with XXY**
Principal Investigator: **Shanlee Davis, MD, MSCS**
Version Date: **9/11/2020**

I. Hypotheses and Specific Aims:

Karyotype 47,XXY, also known as Klinefelter syndrome, is the most common chromosomal abnormality in boys, affecting 1/600 male births or nearly 6 million individuals worldwide.[1] XXY is associated with testosterone (T) deficiency from primary testicular failure, motor and neurocognitive deficits, and cardiometabolic disorders.[2] Men with XXY have higher mortality, with cardiovascular disease being the leading cause of death (standardized mortality ratio 5.8).[3] An essential question in XXY is whether the observed phenotype is solely attributable to the gene dosage effect from the extra X chromosome, or whether T insufficiency contributes to the neurocognitive deficits and metabolic dysfunction and therefore could be mitigated by T treatment.

The hypothalamic-pituitary-gonadal axis is activated twice in post-natal life: the mini-puberty period of infancy and true puberty early in the second decade.[4] Although understudied, the T surge of mini-puberty in the first few months of life may play a role in long-term metabolic programming of energy metabolism and neurocognition.[5-7] Infants with XXY have a blunted T surge and giving T to infants with XXY has recently been proposed as an intervention.[8-10] However, the only data to support this practice are retrospective reports of better cognitive scores in XXY boys who had received T in infancy.[11,12] With the rapidly increasing rate of XXY diagnoses from non-invasive prenatal testing, it is crucial to study the efficacy and safety of infant T in XXY now.[13] Recognizing this clinical equipoise, we conducted a pilot study to evaluate body composition (a surrogate marker of cardiometabolic health) as a short-term outcome of T treatment. Building on these preliminary results, we propose further studying the short-term effects of T on cardiometabolic and developmental outcomes. Our central hypothesis is that T treatment positively influences cardiometabolic and neurodevelopmental outcomes in XXY infants (Figure 1). To test this hypothesis, we will undertake the following specific aims.

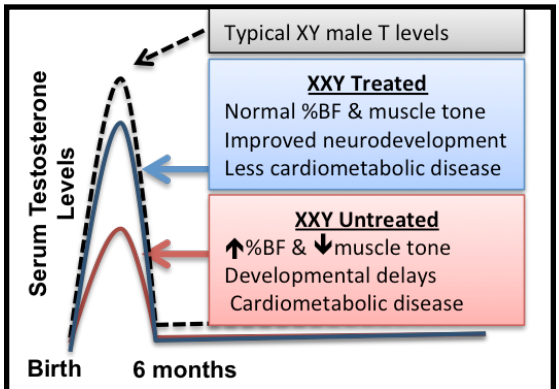

Figure 1. We hypothesize T treatment will improve outcomes in infants with XXY. BF=body fat.

Specific Aim 1: To quantify the short-term effect of T in infants with XXY on cardiometabolic outcomes.

Hypothesis: Percent body fat (%BF) will follow a normal trajectory in treated infants, while infants not receiving T will have greater %BF. *Approach:* In the TESTO: Testosterone Effects on Short-Term Outcomes trial, infants with XXY (n=75) age 4-12 weeks will be randomized 1:1 to receive 25mg intramuscular T injections every 4 weeks for 3 doses or placebo.. %BF will be assessed by air displacement plethysmography at baseline, 12 weeks, and 24 weeks. The primary outcome is change in %BF z-scores at 12 weeks between groups (powered to detect a difference of 0.5SD). Infants initially receiving placebo will get T in the second half of the study (12-24 weeks). Secondary outcomes include change %BF z-scores from 12-24 weeks, growth parameters, and serum leptin..

Specific Aim 2: To quantify the short-term effects of T in infants with XXY on neurodevelopment, gonadal function and safety outcomes.

Hypothesis: (*1)*: XXY infants receiving T will have greater gains in standard scores on early motor skills, emerging language, and adaptive functioning. (*2*): T treatment will not impair gonadal function, as measured by gonadotropins and biomarkers of Sertoli cell function. *(3)*: There will be no serious adverse outcomes associated with T treatment in infants with XXY. *Approach:* Infants from Aim 1 will be given standardized neurodevelopment assessments at baseline, 12 and 24 weeks. Serum hormone biomarkers of gonadal function will also be obtained at these times. Adverse events will be monitored throughout the study.

Specific Aim 3 (exploratory): To measure metabolite changes induced by T in infants with XXY.

Hypothesis: Circulating metabolites involved in energy metabolism, specifically long chain acylcarnitines (LCAC) and branched-chain amino acids (BCAA), will be lower due to improved mitochondrial function in T treated boys; T will favorably alter pathways involved in energy metabolism. *Approach:* LCAC and BCAA will be quantified at baseline and 12 weeks in the first 20 subjects. An unbiased platform of over 4,000 metabolites will also be performed to identify pathways that are differentially regulated with T treatment.

Assessing short-term safety and efficacy of T treatment in infants with XXY will provide the first evidence for establishing standard of care practices, with a planned extension for long-term outcomes. These results will generate preliminary data on mechanisms of the effects of T during the critical mini-puberty period for the development of future work, such as sex steroid-induced epigenetic modifications. Mentors with expertise in neurodevelopment, cardiometabolic outcomes, metabolomics, and clinical trials will optimize career development and foster expertise in gonadal and cardiometabolic function in children with sex chromosome aneuploidies.

II. Background and Significance:

XXY/Klinefelter syndrome is common and the diagnosis in childhood is increasing. An extra X chromosome is present in ~1/600 boys, therefore over 6 million boys and men worldwide are affected by this very common, but under-diagnosed, genetic condition known as XXY or Klinefelter syndrome (KS).[1] XXY is characterized by a high degree of phenotypic heterogeneity throughout the lifespan but nearly universal testicular insufficiency and infertility in adulthood.[2,14] Our knowledge of the natural history of XXY in infancy and childhood is largely limited to birth cohort studies from the 1970's and more recent cross-sectional studies limited by ascertainment bias. Historically, less than 10% of males with XXY are diagnosed before adolescence, however the rate of prenatal diagnosis is increasing exponentially as testing of free-fetal DNA in maternal blood evolves to become standard screening in prenatal care.[15,16] Furthermore, there is evidence the actual incidence of XXY is increasing as well.[17,18] With greater recognition in infancy and potentially increased prevalence of XXY, there is a high demand for evidence-based research, particularly investigation of early determinants that contribute to increased morbidity and decreased quality of life in this population.[19]

XXY results in universal testicular dysfunction and hypogonadism. While many features of the XXY phenotype are variable, testicular dysfunction is nearly universal.[20] In adults, this manifests as small testes, infertility, and hypergonadotropic hypogonadism (elevated gonadotropins and low testicular hormone concentrations).[2] The Leydig cells in the testes produce testosterone (T) and insulin-like hormone 3 (INSL3), while the Sertoli cells produce inhibin B (INHB) and anti-mullerian hormone (AMH). All of these testicular hormones have been demonstrated to be lower in XXY when compared to typical males from mid-puberty through adulthood.[21-23] Microscopically, the testes of men with XXY are fibrotic with rare germ cells and Leydig cell hypertrophy.[20]

Clinical evidence of testicular insufficiency in infants with XXY includes smaller testes size, reduced penile growth over the first months of life, mild hypotonia, and a passive temperament.[10] Testicular biopsies have shown a lower number of germ cells in infants and children with XXY, however the histological appearance of Sertoli and Leydig cells is typically normal.[24-28] Five studies (total N=83) of hormones during infancy in XXY conclude activation of the pituitary-gonadal axis in the first months of life, known as the mini-puberty, does occur, however serum T is lower in XXY in the majority.[20] The only study to quantify T concentrations with liquid chromatography/mass spectrometry (gold standard) was done by our collaborator Dr. Lahlou. She found 8/38 XXY infants (21%) had serum T below the normal range during the mini-puberty period, and 83% were below the median (Figure 2).[29] In summary, many XXY infants have evidence of testosterone insufficiency.

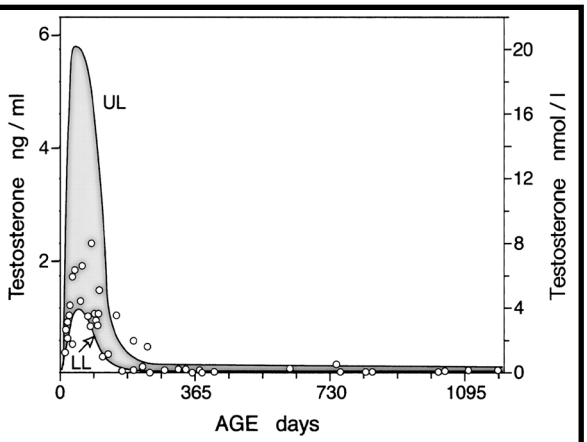

Figure 2. Serum testosterone in males with XXY (open circles) is low or low-normal during the mini-puberty period. Normal ranges in gray with Upper and Lower Limit (UL, LL). Lahlou et al. 2004.

Testosterone insufficiency increases cardiometabolic risk, and cardiovascular disease is the leading cause of morbidity and mortality in men with XXY. Adult men with XXY have a high prevalence of type 2 diabetes, nonalcoholic fatty liver disease and cardiac dysfunction, with a standardized mortality ratio of 5.8 from these conditions.[2,3,30-34] Many precursors of these conditions, such as insulin resistance, abdominal adiposity, and vascular dysfunction, have been shown to inversely correlate with T concentrations, and it is presumed the increase in cardiovascular-related morbidity and mortality in XXY is secondary to chronic hypogonadism.[32,35-39] In multiple populations including men with XXY, T has been shown to improve body composition – an early surrogate marker of altered metabolism associated with the development of disorders of insulin resistance.[30,40-42] However, there is a lack of randomized controlled trials of T in XXY and observational studies suggest that late treatment of hypogonadism does not reverse existing cardiovascular disease.[38] Furthermore, two recent studies have found evidence of high body fat and serum markers of insulin resistance in children with XXY.[43,44] Therefore, to reduce cardiovascular morbidity and mortality in XXY, we need to focus on prevention of early precursors. Body composition is a particularly intriguing outcome measure as it can be assessed throughout the lifespan, has been shown to be an early and modifiable risk marker of metabolic disease, and is directly altered by T.[30,45] Body composition is also a clinically meaningful outcome measure in infancy as high body fat percentage early in life may contribute to the decreased strength, endurance, and athleticism seen in XXY. A gap exists in our knowledge of body composition in infants with XXY and the effect of T on this important outcome measure.

The testosterone surge during the mini-puberty period in the first months of life likely plays a critical role in lifelong metabolic programming. We have known about the mini-puberty period for decades, however the purpose of this brief activation of the hypothalamic-pituitary-gonadal axis remains quite speculative. Many tissues are sexually dimorphic and exposure to T during sensitive time points is required for masculinization of these organs. Mouse models that manipulate exposure to sex steroids in the neonatal period have found that early T exposure induces epigenetic modifications and results in lifelong effects on neurocognition and energy metabolism[5,46] Blocking the neonatal T surge in male mice results in higher leptin concentrations and greater fat to muscle ratio for life, while a single dose of T given to neonatal female rodents results in differential gene expression in the liver in a masculinized pattern.[6,47] Exposure to T in neonatal mice also leads to decreased DNA methylation in the brain, resulting in the sexually dimorphic differences observed in brain structure and function.[48] In humans, several studies have reported associations between T concentrations during the mini-puberty period and brain lateralization, language organization, and gender identity.[7,49,50] These findings are particularly relevant in XXY given the high risk of neurodevelopmental impairment and cardiometabolic disease, and suggest the mini-puberty period is a critical time for intervention.

Studying tissue-specific gene expression and epigenetic modification in human infants is invasive and labor-intensive, however we can investigate circulating metabolites that reflect systemic pathway regulation. Pathways involved in energy metabolism are of specific interest in XXY. Long chain acylcarnitines (LCAC) are intermediate metabolites of fatty acid oxidation within the mitochondria.[51] Obesity and insulin resistance are strongly associated with mitochondrial dysfunction and accumulation of LCAC.[52] Branched chain amino acids (BCAA) also build up in the presence of mitochondrial dysfunction and are regarded as an excellent biomarker for obesity and insulin resistance.[53] Work by one of my mentors, Dr. Baker, has found that LCAC and BCAA are elevated in infants exposed to maternal obesity in utero, demonstrating abnormal metabolism from a very young age. We know maternal obesity is associated with insulin resistance in adult offspring and these metabolite differences in infancy represent the early metabolic programming that underlies childhood determinants of adult disease.[54,55] This work and the work of others highlight the critical importance of *timing* of exposures on lifelong metabolic programming, and how metabolites can serve as early biomarkers of this process.[56,57]

Finally, T treatment for infants with XXY is becoming common despite a lack of scientific evidence to support this practice.[8] Samango-Sprouse et al reported that a cohort of boys with XXY who had received T in infancy had better neurodevelopment in early childhood.[11,12] Largely because of this single study, many parents are requesting T for their XXY sons. These proactive parents are often successful in obtaining this treatment, reinforcing disparities in care without establishing if T is effective or safe in this population. A randomized and blinded trial of T is needed to fill these critical gaps and provide evidence for establishing standard of care.

In summary, testicular dysfunction is universal in XXY and likely contributes to the clinical phenotype, including abnormalities in metabolism and neurodevelopmental deficits. The mini-puberty period of infancy represents a biologically plausible time period for intervention that may not only have immediate effects, but play an important role for lifelong metabolic programming. *A gap currently exists in our understanding of short and long-term outcomes of T treatment in infants with XXY. This study will investigate short-term outcome measures of T, including cardiometabolic, neurodevelopmental, and gonadal function, as well as explore altered regulation of energy pathway metabolites as a probable mechanism underlying metabolic programming in infancy.*

This study is novel and the time is now. T treatment in infants with XXY has emerged as an off-label treatment in the US in the past few years, however there is no evidence to support this practice and considerable variability exists among providers. Our **extensive experience** through the eXtraordinarY Kids clinic, parent support groups, and ongoing research efforts in this population has proven this is a highly motivated group of parents who strongly desire T treatment for their sons. Despite the biologic plausibility, **no study has rigorously investigated the efficacy or safety of T in infants with XXY.** We have carefully designed this study with family engagement to allow us to answer important scientific questions on the short-term effects of T while ensuring successful recruitment and retention. Our research team has the expertise and ideal environment to successfully conduct this study and advance our understanding of the efficacy and safety of this practice. Recent changes in the clinical and research environments make now the **opportune time to conduct this study**:

- Technological advances in non-invasive prenatal screening have resulted in exponentially more infants being diagnosed with XXY. The May 2016 American College of Obstetrics and Gynecology Practice Bulletin recognized cell-free fetal DNA testing is a superior screening test that can be offered in **all** pregnancies.[13] Implementation of this guideline means **10 times as many infants will be diagnosed with XXY**. In addition, many proposed genomic-based newborn screening tools would incidentally diagnose sex chromosome aneuploidies. The demand for knowledge in infants with XXY is great.
- At the 2nd International Workshop on Klinefelter Syndrome (Germany, March 2016) the lack of randomized controlled trials of T in XXY, and the need for these studies to be conducted with patient-centered outcomes, was emphasized and nearly unanimously agreed upon by experts in KS.[58]
- The field of metabolomics offers an opportunity to investigate the mechanisms of these changes.

The outcome measures chosen for this study are not only surrogate markers for future disease and disability, but are **meaningful patient-centered outcomes in infancy and childhood**.

- High body fat with poor muscle tone may limit an infant's ability to engage with his environment.
- Delays in motor, language, and social development affect 75% of boys with XXY and strongly correlate with future cognition, behavior, and academic performance.
- This study will be the first to test the hypothetical mechanism demonstrated in animal models that T induces immediate metabolic and epigenetic modifications that in turn confer lifelong effects on metabolism and neurocognition.

III. Preliminary Studies/Progress Report:

Preliminary data to support the study concept: We have found testicular and cardiometabolic function are inversely related in pre-pubertal XXY boys, and androgen supplementation improves cardiometabolic markers. In a study of 93 boys with XXY age 4-12 (NCT00348945), 20% had evidence of testicular failure (low INHB) at baseline, as well as elevated percent body fat (%BF, z-score +1) and high prevalence of metabolic syndrome features.[59,60] Low INHB was associated with greater cardiometabolic risk including higher blood glucose, triglycerides, and low-density lipoprotein (LDL), and lower high-density lipoprotein (HDL), all $p<0.05$. This is evidence that both testicular and cardiometabolic function is impaired before puberty in boys with XXY. Furthermore, two years of treatment with a weak androgen improved body composition (Figure 3) and cardiometabolic markers.[61]

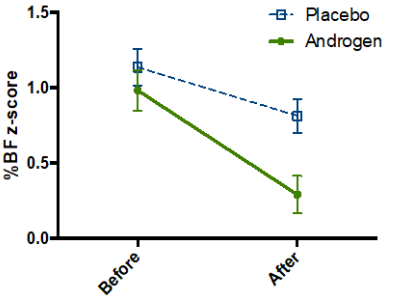

Figure 3. %BF is high in XXY boys 4-12 yrs old. Androgen therapy improves body composition (p=0.04).

Preliminary data to support the study design: Our pilot study suggests this regimen of T is well tolerated and does affect body composition. In preparation for this study, I designed a feasibility study to evaluate the short-term effects of T on body composition (COMIRB 14-7240, NCT02408445) in infants. We enrolled 18 infants with XXY between 6-15 weeks of age and randomized subjects to receive testosterone cypionate 25mg intramuscularly every 4 weeks x 3 or no treatment. I hold an Investigational New Drug (IND 124260) file for the use of T in infants with XXY, and this file will be updated with this protocol. There have been no serious adverse events and the only side effects were an increase in appetite (n=2) and acne (n=2). %BF at baseline was normal, however increased by a z-score of +0.86±0.65 over 12 weeks in infants not receiving T, while the change in %BF z-scores for infants who received T was -0.24±0.67 (Figure 4). XXY infants who did not receive T had 4% more body fat at 5 months of age than normal male infants in the local Healthy Start Study.[62]

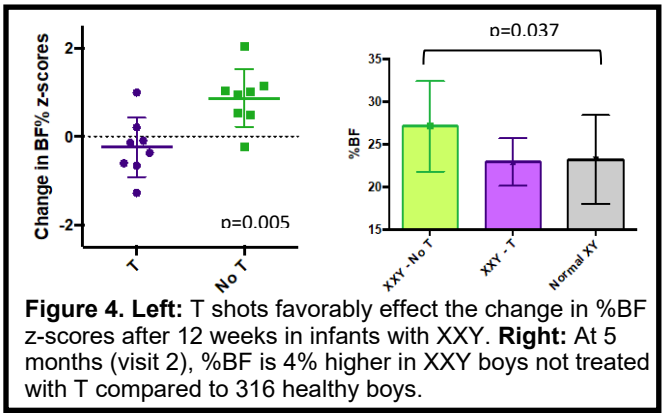

Figure 4. Left: T shots favorably effect the change in %BF z-scores after 12 weeks in infants with XXY. **Right:** At 5 months (visit 2), %BF is 4% higher in XXY boys not treated with T compared to 316 healthy boys.

Preliminary data to support feasibility of recruitment: Our pilot study exceeded recruitment goals and allowed us to adapt our study design based on limitations. We had excellent recruitment with an average one new subject per month and 100% retention rate without funds for recruitment or travel reimbursement. Informal focus groups and surveys have been conducted with parents to identify their reasons for or against participation. The greatest limitations in recruitment/retention have been 1) costs incurred due to travel to Denver and 2) unwillingness to participate in a trial where they may potentially be randomized to not receive T (as all perceived high benefits and low risks). After designing the current study, we surveyed 34 parents of XXY infants anonymously using a link posted on targeted social media groups. 67% reported they "definitely" would enroll their child if eligible in this study design, 21% said they "maybe" would enroll, and only 12% indicated they would not enroll. These pilot findings were critical in designing this study.

IV. Research Methods

A. Outcome Measure(s):

Primary for Specific Aim 1

Change in %BF z-scores from baseline to 12 weeks (per PeaPod)

Primary for Specific Aim 2

Change in motor composite percentile on the AIMS assessment from baseline to 12 weeks

Primary for Specific Aim 3

Change in C14:1 long chain acylcarnitine (LCAC) from baseline

Secondary outcomes: Change in %BF z-scores from 12 to 24 weeks; change in anthropometric measurements from baseline to 12 and 24 weeks; change in gross and fine motor scaled scores from the PDMS; cognitive and language scores on the Bayley; adaptive functioning from the ABAS; serum hormones, lipids, BCAA, LCAC, and pathway analysis of untargeted metabolomics; urine metabolites; and number and type of adverse events.

B. Description of Population to be Enrolled:

A total of 75 infants with XXY will be enrolled. We will include all races, ethnicities, and languages.

Inclusion criteria: Male infants with 47,XXY karyotype identified prenatally who are 4-12 weeks old (31 to 90 days of age). 47,XXY must be from a diagnostic test such as CVS, amniocentesis, or post-natal blood/tissue. Non-invasive prenatal screening results alone will not be accepted.

Exclusion criteria:

1) >20% mosaicism for a normal cell line
2) Gestational age at birth <36 weeks
3) Birth weight <2.5th%ile or >97.5%ile for age (small or large for gestational age)
4) History of thrombosis in self or a first degree relative
5) Exposure to androgen therapy outside the study protocol
6) Use of medications known to affect body composition, such as growth hormone or insulin
7) Known allergy to the testosterone cypionate solution components including benyl benzoate, benzyl alcohol, or cottonseed oil

Involving children is imperative as boys <18 years of age with XXY are much a different population than adult men with XXY. Men with XXY have been shown to have a high prevalence of type 2 diabetes, metabolic syndrome, fatty liver disease, and psychosocial difficulties. Clearly these processes begin in childhood. The potential to intervene in a preventative fashion, prior to the onset of disease, is more promising and productive than trying to treat pre-existing disease. Infant boys are exposed to endogenous testosterone in the first few months of life known as the “mini-puberty period of infancy.” Animal and human studies suggest this is a critical period for biologic programming, and therefore cannot be replicated at any other point in life. Including children in this study design allows us to investigate testosterone as a targeted intervention at this critical time point with evaluation of early markers of disease and disability. The ultimate goal is to improve the lives of children and adults with this condition.

The age of the infants at enrollment was chosen 1) to allow assessment of testicular function during the expected mini-puberty period of infancy, 2) to assure visits 2 and 3 will be completed before the infant is 10kg, as this is the upper weight limit for the PeaPod (our body composition assessment tool), and 3) feedback from parents.

C. Study Design and Research Methods

Overall study design. TESTO is a randomized controlled trial assessing the efficacy and safety of T injections in infants with a prenatal diagnosis of XXY. Body composition, neurodevelopment, serum hormones, safety parameters, and metabolomics will be assessed at baseline, 12 and 24 weeks into the

study. Infants will be randomized 1:1 to receive T vs placebo for the first 12 weeks of the study with the primary outcomes assessed after 12 weeks (Figure 5). The second half of the study will cross the treatment groups allowing us to evaluate if timing makes a difference in response and if initial benefits are sustained. This plan will optimize enrollment, as many parents will only participate if their son will get treatment, and allow us to maintain the blinded design.

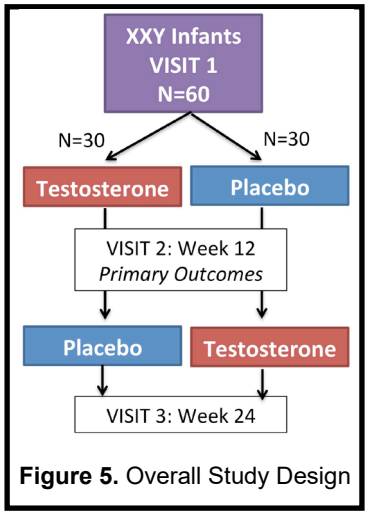

Figure 5. Overall Study Design

Setting. The study will be conducted at Children's Hospital Colorado (CHCO) in the outpatient Pediatric Clinical Translational Research Center (CTRC). CHCO is home to the eXtraordinarY Kids Clinic, the first comprehensive interdisciplinary clinic for sex chromosome aneuploidies.[63] The unique collaborative model and our expertise has led to national referrals and a high demand for clinical evaluations. We have successfully recruited hundreds of boys with XXY to participate in longitudinal and interventional research studies through close collaboration with family support groups and national/international experts in XXY. Our investigative team has expertise in child development, infant body composition, childhood disorders of insulin resistance, metabolomics, clinical trial recruitment, robust data acquisition and data analysis, making this the ideal setting.

Recruitment and Retention. Methods of recruitment will include 1) approaching new and existing patients seen at CHCO clinics including the eXtraordinarY Kids Clinic, Genetics Clinic, and Endocrinology Clinics, 2) outreach to the genetic counselors and obstetricians in the Denver metropolitan area who are ordering and interpreting prenatal testing results, 3) genetic counselors at CHCO who may receive calls/referrals for infants with XXY diagnoses, 4) Email and mailings to pediatric endocrinologists around the country (existing listserv) and through posting on the Pediatric Endocrinology Society active research website, 5) advertisements through the parent support group AXYS including websites, social media, emails, flyers, and mailings, 6) Email and mailings to prenatal genetic counselors throughout the country (existing listserv), and 7) study information on websites including Clinical Trials .gov, University of Colorado and Children's Hospital of Colorado. To facilitate dissemination of information about our study, we will design study-specific webpage linked off the existing eXtraordinarY Kids website.

The goal for recruitment will be 15 subjects per year in years 1-4. Based on our pilot study this goal is easily achievable. Modest travel expenses will be covered for out-of-state participants.

In between study visits, families may receive newsletters from the eXtraordinarY Kids Clinic with updates from our ongoing research and periodic phone calls to stay in touch. Families will be invited to our eXtraordinarY Party in conjunction with the bi-annual AXYS family conference.

Enrollment in multiple clinical trials. Enrollment in this protocol does not exclude participation in other clinical studies, with the exception of studies involving an intervention of androgen and/or other treatment that may impact body composition. Our team is concurrently enrolling for other studies involving infants with XXY, including the eXtraorindarY Babies Study (COMIRB 17-0118). These studies are complementary and participants can enroll in either or both studies. If participants enroll in both studies, visits will be coordinated for convenience of the participant's family and duplicate measures (ie blood draw, developmental testing, etc) will be combined.

Consent. When a child is identified through clinic or other method identified above, a member of the study team will contact the parent to inform them of the opportunity to participate in a study. In many cases, the parents will contact the PI, PRA or genetic counselor of the eXtraordinarY Kids Clinic after hearing about the study from one of the above sources. Prior to any information being collected, verbal consent to ask questions pertaining to the inclusion/exclusion criteria will be obtained. A member of the study team will describe the study and will offer to email/mail the consent form to the parents so the parents have ample time to review prior to deciding whether to schedule the first visit and enroll in the study.

Prior to any research procedures being performed, a study team member will review the consent form in person in a private location. Both verbal and written consent will be required from a parent or legal guardian to enroll in the study. All of the subjects in this protocol are infants and therefore too young to assent/consent to research participation themselves. To minimize coercion, parents will not be paid for enrolling their child in the study and only reimbursement for reasonable travel will be provided. We will emphasize to parents their child's participation is entirely voluntary and can be withdrawn at any time. As part of the consent process, parents will be asked to summarize the study in their own words to ensure comprehension, and parents will have the opportunity to ask questions. If the consenting parent does not speak English, an interpreter will assist with the consent process and a short-form consent document in the parent's preferred language will be completed. If the consenting parent is blind, illiterate, or with reading limitations, the entire consent form will be read to the subject and witnessed by someone outside of the study team.

Randomization. Subjects will be randomized 1:1 using block randomization in groups of 20 to 1) receive T treatment in the first 12 weeks followed by placebo, or 2) placebo for the first 12 weeks followed by T. Investigational Drug Services at CHCO will generate the automated randomization list and dispense the study drug to maintain allocation concealment. All investigators and parents will remain blind to the intervention group.

Study Procedures.

1. Study Visits. There will be a total of three in-person study visits:
Visit 1, week 0 (4-12 weeks of age)
Visit 2, week 12 ± 10 days (~5 months of age)
Visit 3, week 24 ± 10 days (~8 months of age)

	Visit 1: Week 0			Visit 2: Week 12			Visit 3: Week 24
Group A n=30		T	T	T	P	P	P	
Group B n=30		P	P	P	T	T	T	
Study Activity								
Medical History	X			X			X
Physical Exam	X			X			X
PeaPod	X			X			X
Developmental Eval	X			X			X
Blood draw	X			X			X
Urine/stool collect.	X			X			X
Safety Assessment	X	X	X	X	X	X	X

Figure 6. Overall study design, timeline and assessments. T=testosterone, P=placebo

Procedures during the study visits will take a total of approximately 3 hours and will usually be completed on the same day. If all procedures cannot be scheduled for the same day they will be scheduled for two days in a row. The study visits will include all of the following:

- Infant and maternal history collection including medications, therapies, infant feeding method, and adverse reactions (~30 minutes)
- Physical examination including length, weight, head circumference, arm span, waist circumference, stretched penile length, Tanner staging, and testicular size by Prader orchidometer (~30 minutes)
- Quantified body composition assessment by the PeaPod (~30 minutes)
- Standardized neurodevelopmental assessment (~30 minutes)
- Morning venous blood draw following a minimum of 180 minutes fasting (~30 minutes)
- Non-invasive urine and stool sample collection (~10 minutes)
- Visit 1 only: intramuscular injection and teach

2. Injections/Intervention. Participants in this study will receive a total of 6 injections. 25 mg testosterone cypionate (200 mg/mL) or placebo (saline) will be given via intramuscular injection every 4 weeks (study weeks 0, 4, 8, 12, 16, and 20). The total volume of each injection will be 0.125 mL. The first shot for all participants will be administered at CHCO before the end of study visit #1 and the infant will be observed for 15 minutes. Parents will elect to either have the 5 subsequent injections given by a nurse at CHCO or to give them at home themselves. If they elect to give them at home, a nurse in the Pediatric CTRC or in Endocrinology will teach the parents how to administer the shots. Parents will receive an automated email reminder 24 hours before the next injection is due. Parents will keep an administration log to document the injection was given.

Although our intent is to give all injections intramuscularly, if the parent inadvertently gives an injection subcutaneously it should not alter our validity. A recent study found subcutaneous testosterone injections are well tolerated and effective at raising serum testosterone levels,[64] although pharmacodynamics may differ from intramuscular injections therefore this method will not be encouraged.

3. Interim surveys. A parent will complete a REDCap survey one week after each injection to capture pertinent information about the injection and potential side effects. One week out was chosen as most side effects attributable to the medication will be present by then and waiting longer may increase recall bias. This survey will serve as a drug administration log as well as interim safety monitoring. All survey results will be review by study staff and any potential unanticipated events or severe adverse events will be followed up with a phone call to the parent.

Safety Considerations. This testosterone dosing and duration is accepted practice for treatment of micropenis in this age group without reported side effects.[65] We had no serious adverse events in our pilot study using this protocol. Intramuscular injections include the risk of inflammation and/or pain at the site of injection, sterile abscess, infection, and hypersensitivity reactions. Potential low-risk side effects of testosterone therapy include acne, pubarche, and increased erections. High doses and/or prolonged duration of androgen therapy in some populations has been associated with bone age advancement, venous thromboembolism, water retention, anaphalactoid reactions, suppression of clotting factors II, V, VII, and X, polycythemia, and priapism. Testosterone also suppresses spermatogenesis, however infants do not undergo spermatogenesis. The effect of early testosterone on later fertility is unknown.

Likelihood of experiencing any risk: moderate (temporary pain at injection site)

Likelihood of experiencing serious risk: very low

Alternatives: There are multiple formulations of testosterone, however all deliver much higher doses than proposed in this protocol. Injections are routinely administered as accepted standard of care in infants with micropenis, and are the choice of individuals currently advocating for testosterone in XXY infants. We have experience with testosterone gel in older populations, however even if we were able to deliver gel in an appropriate dose, risks of gel include unintentional contact, frequent application, skin reactions, and many of the possible side effects of testosterone therapy. Oxandrolone, an alternative androgen, is available as an oral formulation but has not been studied in this age group and appropriate dosing would be unknown. Therefore injections are the logical choice to study.

Protection against risk: Standard procedure for reducing infection risk will be utilized including sterile single use needles and skin preparation. Parents will be present and/or giving the injections. Infants will be observed for at least 15 minutes after their first injection to insure there are no immediate adverse reactions. Any subjects with a known allergy to any ingredients in the study drug or with a first-degree relative with a history of a blood clot will not participate in the study. Testosterone can be stored at room temperature, therefore no special precautions are required to ensure the safety of the medication.

Monitoring for reactions: One week after every testosterone injection, parents will be sent a brief REDCap survey via email to query for possible reactions. Parents will be given an optional log to record any side effects they notice at home to better help them complete the survey. At every study visit, a study team member will ask about all side effects, hospitalizations, surgeries, new diagnoses, and new medications. Parents will also be encouraged to report serious side effects to the study team immediately.

The PI will discuss any serious adverse events with Drs. Tartaglia and Zeitler and all these events will be reported to regulatory bodies as required. A Data and Safety Monitoring Board (DSMB) will be comprised of a minimum of three clinicians/scientists with expertise in clinical trial conduct and methodology, XXY syndrome/development, and biostatistics. The DSMB will meet after every 20 subjects are enrolled. Members will review data on all adverse events, changes in growth parameters, and testicular function. There will not be any predetermined criteria for altering the study protocol or discontinuing the study, therefore these options will be at the discretion of the DSMB.

Study Timeline. The timeline for major study activities are in Table 1.

Table 1. Study Timeline

Study Year		Year 1				Year 2				Year 3				Year 4				Year 5				
Year	2017				2018				2019				2020				2021				2022			
Quarter	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
IRB/IND/startup			■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Recruitment & Enrollment					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■				
Final Study Visits							■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
Metabolomics analysis					■	■	■	■	■	■	■	■					■	■	■	■	■	■	■	
DSMB Meetings							■	■			■	■			■	■								
Data analysis							■	■			■	■					■	■	■	■	■	■	■	
R03 (metabolomics); R01											■	■							■	■				
Manuscripts							■	■					■	■			■	■	■	■	■	■	■	■

D. Description, Risks and Justification of Procedures and Data Collection Tools:

1. **BODY COMPOSITION ASSESSMENT: The PeaPod** provides a reliable, non-invasive, quantitative assessment of fat mass and non-fat mass utilizing air-displacement plethysmograph technology in infants weighing less than 10 kilograms.[66] This instrument has been validated with other methods of body composition assessment, such as deuterium dilution and dual-energy X-ray absorptiometry, and normative values for healthy, term, average-gestational age males are published from birth through 6 months.[67] The PeaPod is safe and comfortable for infants and takes less than 10 minutes to perform, with the actual test less than 2 minutes. The chamber is heated and has white noise that distracts most infants. The infant can move, cry, and urinate during the assessment, therefore it is very "baby friendly". However, the PeaPod may cause temporary distress to the infant as they are unclothed and not swaddled. A blinded study investigator will perform this assessment at all study visits. Age-specific Z-scores for %BF will be calculated from published data.[67]

   Likelihood of experiencing any risk: low

   Likelihood of experiencing serious risk: very low

   Alternatives: Body composition can be measured by dual-energy x-ray absorptiometry (DXA), however DXA does involve low amounts of radiation exposure in addition to laying on a flat surface similar to the PeaPod. DXA also is best when the subjects lie still, which is challenging for young infants. Body fat can also be estimated with skin fold calipers, however this is generally not as accurate as the PeaPod and does have the risk of temporary discomfort as well. Therefore, we have determined the PeaPod has the least risk to obtain our outcome of interest.

   Protections against risk: When possible, we will perform the PeaPod assessment when the infant is awake and content having recently eaten. We will encourage the parents to bring a familiar toy that may distract the infant if he becomes distressed. We would encourage comforting/consoling after the procedure if temporary distress was experienced. Parents will be present during the PeaPod.

2. **NEURODEVELOPMENTAL ASSESSMENT:** The Alberta Infant Motor Scale (AIMS), Peabody Developmental Motor Scales -2 (PDMS-2), and the Bayley Scales of Infant Development – 3rd edition will be performed by a psychometrist trained to administer these standardized neurodevelopmental assessments. The **AIMS** is a performance-based and norm-referenced measure of infant gross motor maturation from birth to 18 months. It is intended to evaluate gross motor development over repeated assessments and has good reliability and predictive validity for later gross motor development.[68,69] In a systematic review of neurodevelopmental tests, the AIMS had the best psychometric properties and clinical utility.[70] The **PDMS-2** is a standardized assessment for infant development and includes six subtests to assess gross and fine motor scales from birth to 5 years. It is one

of the most common tools for discriminating normal from abnormal infant motor development.[71] This instrument was chosen as a broad assessment of motor development with normative reference data, and strongly correlates with other motor assessments. The **Bayley 3** is a comprehensive standardized assessment for infant development containing three domains: Cognitive, Language, and Motor.[72] The Bayley-3 is widely used to diagnosis developmental delays up to 3.5 years of age. Finally, a parent will complete the Adaptive Behavior Assessment System, 3rd edition (**ABAS 3**), a validated measure of adaptive skills across the lifespan, including infancy.[73] The ABAS incorporates early developmental skills in areas such as feeding, sleep, play, self-regulation, and social development. The primary risk of the developmental evaluation is identification of developmental delays that may warrant further evaluation and/or intervention. This may cause psychological distress to the parent.

Likelihood of experiencing any risk: low
Likelihood of experiencing serious risk: very low
Alternatives: None identified.
Protections against risk: Parents will be made aware of the high prevalence of at least mild developmental delays in XXY. Parents will be present during the evaluation. If delays are identified, the study team will disclose this to the parent(s) in a private area with specific recommendations for next-steps, potentially including referrals and/or resources.

3. **VENIPUNCTURE.** Venous blood samples will be drawn in the morning a minimum of 180 minutes after eating (fasting for an infant.) The collection of blood samples may result in temporary discomfort, bruising, bleeding, and on rare occasions, infection. Standard hospital precautions for venipuncture will be followed. Anemia is also a risk; the routine guidelines in our Pediatric CTRC are 2.5 ml/kg per blood draw. We will draw 8 ml of blood per visit. If the subject is <3.2kg on the first visit we will reduce the amount of blood to be drawn.
Likelihood of experiencing any risk: moderate (temporary discomfort).
Likelihood of experiencing serious risk: very low

Hormones. Serum will be processed and stored at -80 degrees until batch analysis for quantification of LH, FSH, total and free T by liquid chromatography tandem mass spectroscopy (LC/MS), AMH, INHB, estradiol and leptin. Serum testosterone, inhibin B, AMH, estradiol and gonadotropins at baseline will allow us to assess endogenous testicular function. Hormone concentrations after testosterone exposure will serve as a short-term safety measure of testicular function. Exploratory aims may include looking at whether baseline serum testosterone predicts change in our outcomes after testosterone exposure. Leptin is biomarker of adiposity and will be used as a secondary measure to support our Peapod findings. Our previous work has shown that leptin concentrations are higher in boys with Klinefelter syndrome and this study will be the first to assess this in infants and explore how testosterone supplementation alters leptin in this population. Finally, we will measure serum lipids (triglycerides and HDL) and insulin as markers of insulin resistance using Trig/HDL ratio and 1/insulin as estimates. There are very limited validated assessments of cardiometabolic health in infants, therefore these data will largely be exploratory. If blood volume is limited, the order of priority for serum measurements will be as follows: Testosterone > inhibin B > leptin > gonadotropins > AMH > lipids > insulin > estradiol. Additional serum may be saved for future analyses.
Metabolomics. Plasma will be processed and stored until batch analysis using electrospray tandem mass spectroscopy per standard protocols in the Reisdorph Lab.[74,75] Concentrations of each metabolite of interest will be quantified: acylcarnitines (short, medium, and long-chain) and BCAA (leucine/isoleucine and valine). Non-targeted measurements of metabolites using liquid chromatography/mass spectroscopy will be analyzed using a well-established platform for comprehensive unbiased metabolomics resulting in the relative quantification of over 4,000 metabolites, including amino and organic acids, glycerolipids, sphingolipids, and sterols.[76,77] Compound identification will be done through an in-house database. Principal components analysis will be used to consolidate the raw metabolite variables into clusters. We will use these data to determine pathways that are up or down regulated with exogenous testosterone. Additional plasma may be saved for future analyses.
DNA and RNA storage. Samples will be collected, processed, and stored at -80 degrees for future analyses.

4. **URINE AND STOOL COLLECTION**. Urine will be collected by placing cotton balls in the diaper immediately after arriving at the study visits. Cotton balls become saturated when the infant urinates, and the urine will be squeezed out into a cryovial for storage to later measure hormones and metabolites. If the infant stools during the study visit, a stool sample will be collected from the diaper and stored for future microbiome analysis. Both urine and stool samples will be stored as part of this protocol, with future plans to use these stored specimens to measure hormones and metabolomics (urine) and assess the microbiome (stool) both to look for differences unique to XXY infants and to assess acute changes with testosterone exposure on these measures. No invasive methods for obtaining urine or stool will be used in this protocol, therefore no risk to the participant is identified.

Likelihood of experiencing any risk: very low

Likelihood of experiencing serious risk: very low

Alternatives: There are no less-invasive alternatives to assess the microbiome or metabolites.

5. **HISTORY AND PHYSICAL EXAM.** A medical history for the participant including intrauterine history, birth history, medical and developmental history, and feeding history will be obtained from the parent at the first study visit by a member of the study team. Subsequent study visits will collect interim/updates to these histories as well as any potential study adverse events. A physical exam will be completed by the PI (in her absence, one of the other physicians on the protocol). Weight will be recorded to the nearest 0.01 kg. Length measurements will be recorded to the nearest 0.1 cm. Testicular volume to the nearest mL will be assessed using a Prader orchiometer. If testes are significantly less than 1mL, an estimate of 0.5mL will be used.

E. Potential Scientific Problems:

Recruitment and retention of subjects with rarer pediatric conditions can be problematic, however with our successful feasibility study and accommodation for participant travel in this budget, we do not anticipate enrollment as an obstacle. Utilizing a cross-over-type design reduces our major parental reservation of the study, which is the possibility of being randomized to not receive T, and still allows us to evaluate short-term effects of T and observe the metabolic pathways that have been altered. As with all clinical research, we acknowledge there will be ascertainment bias and strict inclusion criteria that may limit the generalizability of our results. Our proposal only includes prenatally diagnosed infants, however our previous work demonstrated no differences in cardiometabolic or gonadal function between pre- or postnatally diagnosed XXY boys therefore results from these outcomes should be generalizable.[78]. Finally, to further assess the external validity of our study we will collect and compare basic demographic data from families who decline study participation, and will also compare our study cohort to our historic data of infants who are seen in our eXtraordinarY Kids Clinic.

Results and interpretation from the second half of the participant protocol (weeks 12-24) does require certain assumptions, including that this larger study finds similar differences in body composition between treated and untreated infants as our pilot study did. There will not be a control group as part of this study for the second half of the study, but importantly these outcomes are all secondary. In addition, we are concurrently recruiting for a natural history study in infants with X&Y chromosome variations (eXtraordinarY Babies) that includes 100 infants with XXY who will have body composition assessments (PeaPod and later DEXA). Some of these will be infants participating in this study, but presumably an important subset will not be in the TESTO study and will not have received testosterone clinically. Finally, data from the Health Start Study of healthy local infants can serve as a non-XXY control group for normal adipose accumulation in infancy.

Our study does not address the large phenotypic variability in XXY. It is possible we will fail to find a difference in outcomes with T treatment because some boys with XXY have adequate gonadal function or have polymorphisms in their androgen receptor gene.[79,80] If we fail to find a difference in our primary out comes, we will perform post-hoc sub-analyses controlling for age and baseline testosterone concentrations. In addition, DNA will be extracted and saved for future genomic analysis, including sequencing of the androgen receptor gene and other polymorphisms that have been associated with phenotypic variability in XXY.[79-82]

Finally, we acknowledge there are many factors that influence body composition. These potential confounders will be minimized due to our randomized design and inclusion/exclusion criteria. We will collect data on variables known to contribute to body composition, including race, maternal BMI, maternal weight gain during pregnancy, presence of gestational diabetes, birth weight and gestational age, infant feeding method, and physical therapy interventions. We anticipate our randomized design will minimize differences in these potential confounding variables, however if significant differences are found we will adjust for them in the analyses.

F. Data Analysis Plan:

The primary outcome for **specific aim 1** is the change in %BF z-scores between baseline and 12 weeks between treatment groups. Z-scores will be calculated using published norms for age,[67] and the difference between groups will be assessed with a two-sided t-test using an alpha of 0.05. Allowing for a 10% drop-out rate, a sample size of 27 in each group gives us 80% power to detect a difference down to 0.5 z-score change between groups, and >99% power to detect the change score we observed in our pilot study (1.0 z-score change) assuming a variance of 0.65 per our pilot data. We will next use regression models with treatment group, baseline %BF, age of enrollment and feeding method as explanatory variables to assess absolute %BF at 12 weeks. Secondary analyses will include change in %BF z-scores from 12 to 24 weeks 1) in the group receiving T first (are the effects of T maintained or lost?) and 2) in the group receiving T second (does later T have the same effect?). We will analyze anthropometric measurements and serum biomarkers similarly.

For **specific aim 2,** the change in standard scores on developmental assessments will be analyzed in a similar fashion as in specific aim 1. Based on our pilot data, our sample size will yield 80% power to detect a difference between groups of 23 percentile points on the AIMS composite. Secondary analysis will include the changes in scaled scores for the individual Peabody domains and AIMS subtests, the Bayley composite scores, and the ABAS composite at 12 and 24 weeks. Gonadal function and safety outcomes will be compared between subjects on T and placebo using descriptive statistics.

For **specific aim 3 (exploratory)**, we will compare C14:1 concentrations (an easily identified LCAC) between treatment groups at 12 weeks using two-sided t-tests, hypothesizing C14:1 will be lower in the T treated group (representing improvement in fatty acid oxidation and energy metabolism). This aim is exploratory; with 10 subjects per group we will have 80% power to detect a difference of 1.3 SD with a two-sided alpha of 0.05. Then, paired t-tests will be used to compare C14:1 before and after T treatment (5 paired samples), with similar power. We will analyze secondary outcomes including other LCAC and BCAA (leucine, isoleucine, and valine) in a similar fashion. Next, we will use principle component analysis on the entire dataset from the comprehensive untargeted analysis to identify patterns and pathways altered with T treatment. The spectra data file from the non-targeted metabolomics panel will be uploaded to MetaboAnalyst, and the software will be used for Metabolite Set Enrichment Analysis as well as Pathway Enrichment Analysis. We will have >80% power to detect at least 2-fold changes between T treated and untreated using a significance level of 0.0001 (less than two false positives based on the conservative Bonferroni correction for multiple comparisons). We will also input the analytes into known KEGG pathways of energy metabolism to look for pathway enrichment.

Table 2. Summary of primary and secondary outcome measures for each Specific Aim (SA).

		Weeks		
	Primary Outcome	12	24	**Measurement Tool**
SA 1: Cardiometabolic	Change %BF z-scores from baseline	X		PeaPod
	Secondary Outcomes			
	Change %BF z-scores from baseline		X	PeaPod
	Change in height, weight, weight-for-length and waist circumference z-scores from baseline	X	X	Physical exam measurements
	Serum leptin, lipids, and insulin	X	X	Immunoassays in CCTSI core laboratory
SA	**Primary Outcome**			

	Change in motor composite from baseline	X		AIMS percentile composite score (all domains)
	Secondary Outcomes			
	Serum LH, FSH, INHB, AMH, E2 and Total T	X	X	Immunoassay and LC/MS (Lahlou lab)
	Change in gross and fine motor scaled scores	X	X	Scaled scores on all PDMS-2 domains, AIMS
	Cognitive and Language composite scores	X	X	Bayley III: cognitive and language domains
	Adaptive functioning	X	X	ABAS III standard scores
	Number of adverse events	X	X	Verbal questionnaire, parent-report
SA 3: Metabolomics	**Primary Outcomes**			
	Change in C14:1 (LCAC) from baseline	X	X	Targeted metabolomics
	Secondary Outcomes			
	BCAA, other LCAC	X	X	Targeted metabolomics
	Pathway analysis	X		Unbiased metabolomics

G. Summarize Knowledge to be Gained:

This will be the largest assessment of cardiometabolic health, neurodevelopment and testicular function biomarkers in infants with XXY to date and the only longitudinal or interventional study. Management of KS in infancy and childhood is particularly important as screening for fetal aneuploidy is becoming standard of care in all pregnancies through non-invasive prenatal testing. Thousands more infants will be diagnosed with KS with very limited evidence-based management guidelines currently available. Providers and parents alike are seeking more information and options to prevent complications and co-morbidities in this condition. Our results will inform the clinical management of testosterone therapy in infants with XXY, with the promise to provide an early intervention during a critical period that may prevent cardiometabolic and neurodevelopment deficits. If testosterone therapy positively effects body composition and development in infants and does not have safety issues, there would be support for giving this therapy as well as doing long-term follow-up studies. If no short-term benefits are shown following testosterone administration, this would provide evidence for not treating these infants. This study also sets up a unique opportunity to evaluate changes in the metabolome in response to exogenous testosterone, revealing underlying mechanisms involved in the regulation of metabolism and neurodevelopmental pathways during a critical period of infancy.

Furthermore, results of this work may be generalizable beyond KS in understanding the understudied relationship of hypogonadism, body composition, and insulin resistance characteristic of obese boys, a problem of increasing significance secondary to the pediatric obesity epidemic. Other hypogonadal populations including Turner syndrome, Prader-Willi syndrome, and Down syndrome, will also benefit from the results of this study as they too have a blunted mini-puberty period of infancy.

***Future Directions*.** Based on the metabolomics results from Aim 3, I will seek funding for the targeted and untargeted metabolomics analysis on stored specimens from all subjects in this study and potentially be able to compare to normal control infants and infants with other high risk factors for cardiometabolic dysfunction, such as maternal obesity and diabetes. We will consent for future contact to be able to follow this cohort longitudinally and compare their long-term neurodevelopmental outcomes to infants who do not receive T but have identical services through the eXtraordinarY Kids Clinic. Depending on our results, my future R01 proposals may include long term follow up of this cohort and using additional –omic studies to understand the programming mechanisms occurring during the mini-puberty period or other critical times in development. Another future direction is long-term longitudinal assessment of boys with XXY compared to XY controls, evaluating the many covariates (genetic, epigenetic, environment, T treatment, and other interventions) that influence phenotypic variability in XXY. My work will contribute to the development and refinement of evidence-based guidelines to ultimately improve cardiometabolic and neurocognitive outcomes in this population.

H. References:

1. Coffee B, Keith K, Albizua I, et al. Incidence of fragile X syndrome by newborn screening for methylated FMR1 DNA. *American journal of human genetics.* 2009;85(4):503-514.
2. Pacenza N, Pasqualini T, Gottlieb S, et al. Clinical Presentation of Klinefelter's Syndrome: Differences According to Age. *International journal of endocrinology.* 2012;2012:324835.
3. Bojesen A, Gravholt CH. Morbidity and mortality in Klinefelter syndrome (47,XXY). *Acta paediatrica.* 2011;100(6):807-813.
4. Rey RA. Mini-puberty and true puberty: differences in testicular function. *Annales d'endocrinologie.* 2014;75(2):58-63.
5. Ghahramani NM, Ngun TC, Chen PY, et al. The effects of perinatal testosterone exposure on the DNA methylome of the mouse brain are late-emerging. *Biology of sex differences.* 2014;5:8.
6. de Mello WG, de Morais SR, Dornelles RC, Kagohara Elias LL, Antunes-Rodrigues J, Bedran de Castro JC. Effects of neonatal castration and androgenization on sexual dimorphism in bone, leptin and corticosterone secretion. *Bone.* 2012;50(4):893-900.
7. Pasterski V, Acerini CL, Dunger DB, et al. Postnatal penile growth concurrent with mini-puberty predicts later sex-typed play behavior: Evidence for neurobehavioral effects of the postnatal androgen surge in typically developing boys. *Hormones and behavior.* 2015;69:98-105.
8. Wosnitzer MS, Paduch DA. Endocrinological issues and hormonal manipulation in children and men with Klinefelter syndrome. *American journal of medical genetics Part C, Seminars in medical genetics.* 2013;163C(1):16-26.
9. Lahlou N, Fennoy I, Carel JC, Roger M. Inhibin B and anti-Mullerian hormone, but not testosterone levels, are normal in infants with nonmosaic Klinefelter syndrome. *The Journal of clinical endocrinology and metabolism.* 2004;89(4):1864-1868.
10. Fennoy I. Testosterone and the child (0-12 years) with Klinefelter syndrome (47XXY): a review. *Acta paediatrica.* 2011;100(6):846-850.
11. Samango-Sprouse C, Stapleton EJ, Lawson P, et al. Positive effects of early androgen therapy on the behavioral phenotype of boys with 47,XXY. *American journal of medical genetics Part C, Seminars in medical genetics.* 2015;169(2):150-157.
12. Samango-Sprouse CA, Sadeghin T, Mitchell FL, et al. Positive effects of short course androgen therapy on the neurodevelopmental outcome in boys with 47,XXY syndrome at 36 and 72 months of age. *American journal of medical genetics Part A.* 2013;161A(3):501-508.
13. Practice Bulletin No. 163: Screening for Fetal Aneuploidy. *Obstet Gynecol.* 2016;127(5):e123-137.
14. Nakamura Y, Kitamura M, Nishimura K, et al. Chromosomal variants among 1790 infertile men. *International journal of urology : official journal of the Japanese Urological Association.* 2001;8(2):49-52.
15. Lo JO, Cori DF, Norton ME, Caughey AB. Noninvasive prenatal testing. *Obstetrical & gynecological survey.* 2014;69(2):89-99.
16. Bianchi DW, Wilkins-Haug L. Integration of noninvasive DNA testing for aneuploidy into prenatal care: what has happened since the rubber met the road? *Clinical chemistry.* 2014;60(1):78-87.
17. Morris JK, Alberman E, Scott C, Jacobs P. Is the prevalence of Klinefelter syndrome increasing? *Eur J Hum Genet.* 2008;16(2):163-170.
18. Herlihy AS, Halliday JL, Cock ML, McLachlan RI. The prevalence and diagnosis rates of Klinefelter syndrome: an Australian comparison. *The Medical journal of Australia.* 2011;194(1):24-28.
19. Herlihy AS, Gillam L, Halliday JL, McLachlan RI. Postnatal screening for Klinefelter syndrome: is there a rationale? *Acta paediatrica.* 2011;100(6):923-933.
20. Davis SM, Rogol AD, Ross JL. Testis Development and Fertility Potential in Boys with Klinefelter Syndrome. *Endocrinology and metabolism clinics of North America.* 2015;44(4):843-865.
21. Aksglaede L, Christiansen P, Sorensen K, et al. Serum concentrations of Anti-Mullerian Hormone (AMH) in 95 patients with Klinefelter syndrome with or without cryptorchidism. *Acta paediatrica.* 2011;100(6):839-845.

22. Overvad S, Bay K, Bojesen A, Gravholt CH. Low INSL3 in Klinefelter syndrome is related to osteocalcin, testosterone treatment and body composition, as well as measures of the hypothalamic-pituitary-gonadal axis. *Andrology.* 2014;2(3):421-427.
23. Christiansen P, Andersson AM, Skakkebaek NE. Longitudinal studies of inhibin B levels in boys and young adults with Klinefelter syndrome. *The Journal of clinical endocrinology and metabolism.* 2003;88(2):888-891.
24. Aksglaede L, Petersen JH, Main KM, Skakkebaek NE, Juul A. High normal testosterone levels in infants with non-mosaic Klinefelter's syndrome. *European journal of endocrinology / European Federation of Endocrine Societies.* 2007;157(3):345-350.
25. Muller J, Skakkebaek NE, Ratcliffe SG. Quantified testicular histology in boys with sex chromosome abnormalities. *International journal of andrology.* 1995;18(2):57-62.
26. Edlow JB, Shapiro LR, Hsu LY, Hirschhorn K. Neonatal Klinefelter's syndrome. *American journal of diseases of children.* 1969;118(5):788-791.
27. Mikamo K, Aguercif M, Hazeghi P, Martin-Du Pan R. Chromatin-positive Klinefelter's syndrome. A quantitative analysis of spermatogonial deficiency at 3, 4, and 12 months of age. *Fertility and sterility.* 1968;19(5):731-739.
28. Ratcliffe SG. The sexual development of boys with the chromosome constitution 47,XXY (Klinefelter's syndrome). *Clinics in endocrinology and metabolism.* 1982;11(3):703-716.
29. Cabrol S, Ross JL, Fennoy I, Bouvattier C, Roger M, Lahlou N. Assessment of Leydig and Sertoli cell functions in infants with nonmosaic Klinefelter syndrome: insulin-like peptide 3 levels are normal and positively correlated with LH levels. *The Journal of clinical endocrinology and metabolism.* 2011;96(4):E746-753.
30. Gravholt CH, Jensen AS, Host C, Bojesen A. Body composition, metabolic syndrome and type 2 diabetes in Klinefelter syndrome. *Acta paediatrica.* 2011;100(6):871-877.
31. Jiang-Feng M, Hong-Li X, Xue-Yan W, et al. Prevalence and risk factors of diabetes in patients with Klinefelter syndrome: a longitudinal observational study. *Fertility and sterility.* 2012;98(5):1331-1335.
32. Andersen NH, Bojesen A, Kristensen K, et al. Left ventricular dysfunction in Klinefelter syndrome is associated to insulin resistance, abdominal adiposity and hypogonadism. *Clinical endocrinology.* 2008;69(5):785-791.
33. Salzano A, Arcopinto M, Marra AM, et al. MANAGEMENT OF ENDOCRINE DISEASE: Klinefelter syndrome, cardiovascular system and thromboembolic disease. Review of literature and clinical perspectives. *European journal of endocrinology / European Federation of Endocrine Societies.* 2016.
34. Swerdlow AJ, Higgins CD, Schoemaker MJ, Wright AF, Jacobs PA, United Kingdom Clinical Cytogenetics G. Mortality in patients with Klinefelter syndrome in Britain: a cohort study. *The Journal of clinical endocrinology and metabolism.* 2005;90(12):6516-6522.
35. Bojesen A, Kristensen K, Birkebaek NH, et al. The metabolic syndrome is frequent in Klinefelter's syndrome and is associated with abdominal obesity and hypogonadism. *Diabetes care.* 2006;29(7):1591-1598.
36. Foresta C, Caretta N, Palego P, et al. Reduced artery diameters in Klinefelter syndrome. *International journal of andrology.* 2012;35(5):720-725.
37. Seth A, Rajpal S, Penn RL. Klinefelter's syndrome and venous thrombosis. *The American journal of the medical sciences.* 2013;346(2):164-165.
38. Pasquali D, Arcopinto M, Renzullo A, et al. Cardiovascular abnormalities in Klinefelter syndrome. *Int J Cardiol.* 2013;168(2):754-759.
39. Ishikawa T, Yamaguchi K, Kondo Y, Takenaka A, Fujisawa M. Metabolic syndrome in men with Klinefelter's syndrome. *Urology.* 2008;71(6):1109-1113.
40. Blouin K, Boivin A, Tchernof A. Androgens and body fat distribution. *The Journal of steroid biochemistry and molecular biology.* 2008;108(3-5):272-280.
41. Traish AM. Outcomes of testosterone therapy in men with testosterone deficiency (TD): part II. *Steroids.* 2014;88:117-126.
42. Wang C, Swerdloff RS, Iranmanesh A, et al. Transdermal testosterone gel improves sexual function, mood, muscle strength, and body composition parameters in hypogonadal men. *The Journal of clinical endocrinology and metabolism.* 2000;85(8):2839-2853.

43. Bardsley MZ, Falkner B, Kowal K, Ross JL. Insulin resistance and metabolic syndrome in prepubertal boys with Klinefelter syndrome. *Acta paediatrica.* 2011;100(6):866-870.
44. Aksglaede L, Molgaard C, Skakkebaek NE, Juul A. Normal bone mineral content but unfavourable muscle/fat ratio in Klinefelter syndrome. *Archives of disease in childhood.* 2008;93(1):30-34.
45. Ibanez L, Ong K, Dunger DB, de Zegher F. Early development of adiposity and insulin resistance after catch-up weight gain in small-for-gestational-age children. *The Journal of clinical endocrinology and metabolism.* 2006;91(6):2153-2158.
46. Swift-Gallant A, Coome LA, Ramzan F, Monks DA. Nonneural Androgen Receptors Affect Sexual Differentiation of Brain and Behavior. *Endocrinology.* 2016;157(2):788-798.
47. Dkhil MA, Al-Quraishy S, Abdel-Baki AA, et al. Epigenetic modifications of gene promoter DNA in the liver of adult female mice masculinized by testosterone. *The Journal of steroid biochemistry and molecular biology.* 2015;145:121-130.
48. Nugent BM, Wright CL, Shetty AC, et al. Brain feminization requires active repression of masculinization via DNA methylation. *Nat Neurosci.* 2015;18(5):690-697.
49. Alexander GM. Postnatal testosterone concentrations and male social development. *Frontiers in endocrinology.* 2014;5:15.
50. Friederici AD, Pannekamp A, Partsch CJ, et al. Sex hormone testosterone affects language organization in the infant brain. *Neuroreport.* 2008;19(3):283-286.
51. McCoin CS, Knotts TA, Adams SH. Acylcarnitines--old actors auditioning for new roles in metabolic physiology. *Nature reviews Endocrinology.* 2015;11(10):617-625.
52. Aguer C, McCoin CS, Knotts TA, et al. Acylcarnitines: potential implications for skeletal muscle insulin resistance. *FASEB J.* 2015;29(1):336-345.
53. Newgard CB. Interplay between lipids and branched-chain amino acids in development of insulin resistance. *Cell Metab.* 2012;15(5):606-614.
54. Desai M, Jellyman JK, Ross MG. Epigenomics, gestational programming and risk of metabolic syndrome. *Int J Obes (Lond).* 2015;39(4):633-641.
55. Barbour LA. Changing perspectives in pre-existing diabetes and obesity in pregnancy: maternal and infant short- and long-term outcomes. *Curr Opin Endocrinol Diabetes Obes.* 2014;21(4):257-263.
56. Simeoni U, Yzydorczyk C, Siddeek B, Benahmed M. Epigenetics and neonatal nutrition. *Early Hum Dev.* 2014;90 Suppl 2:S23-24.
57. Vickers MH. Early life nutrition, epigenetics and programming of later life disease. *Nutrients.* 2014;6(6):2165-2178.
58. Nieschlag E, Ferlin A, Gravholt CH, et al. The Klinefelter syndrome: current management and research challenges. *Andrology.* 2016;4(3):545-549.
59. Davis S, Lahlou N, Bardsley MZ, Temple M, Kowal K, Ross JL. Longitudinal Study of Boys with Klinefelter Syndrome Gives Evidence of Prepubertal Testis Defect. The Endocrine Society; 2015; San Diego, CA.
60. Davis S, Lahlou N, Bardsley MZ, Temple M, Kowal K, Ross J. Gonadal Function is Associated with Cardiometabolic Health in Prepubertal Boys with Klinefelter Syndrome.
61. Davis S, Cox-Martin M, Bardsley M, Kowal K, Zeitler P, Ross J. Effects of Oxandrolone on Cardiometabolic Health in Boys with Klinefelter syndrome: A Randomized Controlled Trial. *Journal of Clinical Endocrine Metabolism.* 2016.
62. Sauder KA, Kaar JL, Starling AP, Ringham BM, Glueck DH, Dabelea D. Predictors of Infant Body Composition at 5 Months of Age: The Healthy Start Study. *J Pediatr.* 2017;183:94-99 e91.
63. Tartaglia N, Howell S, Wilson R, et al. The eXtraordinarY Kids Clinic: an interdisciplinary model of care for children and adolescents with sex chromosome aneuploidy. *J Multidiscip Healthc.* 2015;8:323-334.
64. Spratt DI, Stewart I, Savage C, et al. Subcutaneous Injection of Testosterone is an Effective and Preferred Alternative to Intramuscular Injection: Demonstration in Female-to-Male Transgender Patients. *The Journal of clinical endocrinology and metabolism.* 2017.
65. Carrillo A. Disorders of Sexual Differentiation. In: Lifshitz, ed. *Pediatric Endocrinology.* Vol 2. Fifth ed. New York, NY: Informa Healthcare USA; 2007.

66. Ma G, Yao M, Liu Y, et al. Validation of a new pediatric air-displacement plethysmograph for assessing body composition in infants. *The American journal of clinical nutrition.* 2004;79(4):653-660.
67. Fields DA, Gilchrist JM, Catalano PM, Gianni ML, Roggero PM, Mosca F. Longitudinal body composition data in exclusively breast-fed infants: a multicenter study. *Obesity.* 2011;19(9):1887-1891.
68. Darrah J, Piper M, Watt MJ. Assessment of gross motor skills of at-risk infants: predictive validity of the Alberta Infant Motor Scale. *Developmental medicine and child neurology.* 1998;40(7):485-491.
69. Snyder P, Eason JM, Philibert D, Ridgway A, McCaughey T. Concurrent validity and reliability of the Alberta Infant Motor Scale in infants at dual risk for motor delays. *Physical & occupational therapy in pediatrics.* 2008;28(3):267-282.
70. Spittle AJ, Doyle LW, Boyd RN. A systematic review of the clinimetric properties of neuromotor assessments for preterm infants during the first year of life. *Developmental medicine and child neurology.* 2008;50(4):254-266.
71. Provost B, Heimerl S, McClain C, Kim NH, Lopez BR, Kodituwakku P. Concurrent validity of the Bayley Scales of Infant Development II Motor Scale and the Peabody Developmental Motor Scales-2 in children with developmental delays. *Pediatric physical therapy : the official publication of the Section on Pediatrics of the American Physical Therapy Association.* 2004;16(3):149-156.
72. Bayley N. *Bayley Scales of Infant and Toddler Development, 3rd Edition.* San Antonio, TX: Harcourt Assessment Inc; 2006.
73. Harrison P. OT. *Adaptive Behavior Assessment System—Second edition (ABAS-II)* San Antonio, TX: The Psychological Corporation; 2015.
74. Chace DH, DiPerna JC, Mitchell BL, Sgroi B, Hofman LF, Naylor EW. Electrospray tandem mass spectrometry for analysis of acylcarnitines in dried postmortem blood specimens collected at autopsy from infants with unexplained cause of death. *Clinical chemistry.* 2001;47(7):1166-1182.
75. Shen JJ, Matern D, Millington DS, et al. Acylcarnitines in fibroblasts of patients with long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency and other fatty acid oxidation disorders. *J Inherit Metab Dis.* 2000;23(1):27-44.
76. Yang Y, Cruickshank C, Armstrong M, Mahaffey S, Reisdorph R, Reisdorph N. New sample preparation approach for mass spectrometry-based profiling of plasma results in improved coverage of metabolome. *J Chromatogr A.* 2013;1300:217-226.
77. Cruickshank-Quinn C, Quinn KD, Powell R, et al. Multi-step preparation technique to recover multiple metabolite compound classes for in-depth and informative metabolomic analysis. *J Vis Exp.* 2014(89).
78. Davis S, Kowal K, Ross J. Prenatal Diagnosis of 47,XXY is Associated with Better Cognitive and Behavioral, But Not Physical Phenotypes in Childhood. 10th International Meeting of Pediatric Endocrinology; 2017; Washington D.C.
79. Chang S, Skakkebaek A, Trolle C, et al. Anthropometry in Klinefelter Syndrome - Multifactorial Influences Due to CAG Length, Testosterone Treatment and Possibly Intrauterine Hypogonadism. *The Journal of clinical endocrinology and metabolism.* 2015;100(3):E508-517.
80. Bojesen A, Hertz JM, Gravholt CH. Genotype and phenotype in Klinefelter syndrome - impact of androgen receptor polymorphism and skewed X inactivation. *International journal of andrology.* 2011;34(6 Pt 2):e642-648.
81. Zitzmann M. The role of the CAG repeat androgen receptor polymorphism in andrology. *Frontiers of hormone research.* 2009;37:52-61.
82. Zinn AR, Ramos P, Elder FF, Kowal K, Samango-Sprouse C, Ross JL. Androgen receptor CAGn repeat length influences phenotype of 47,XXY (Klinefelter) syndrome. *The Journal of clinical endocrinology and metabolism.* 2005;90(9):5041-5046.