BMJ Open

Effect of testosterone treatment during puberty in boys with Klinefelter syndrome (The TIPY Study): protocol for a nationwide randomised, double-blinded, placebo-controlled study

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To cite: Caspersen ID, Fritzbøger AFØ, Petersen JH, *et al*. Effect of testosterone treatment during puberty in boys with Klinefelter syndrome (The TIPY Study): protocol for a nationwide randomised, double-blinded, placebo-controlled study. *BMJ Open* 2025;**15**:e095628. doi:10.1136/bmjopen-2024-095628

► Prepublication history and additional supplemental material for this paper are available online. To view these files, please visit the journal online (https://doi.org/10.1136/bmjopen-2024-095628).

IDC and AFØF are joint first authors.

Received 25 October 2024
Accepted 20 February 2025

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ABSTRACT

Introduction Klinefelter syndrome (KS) is a genetic condition characterised by the presence of an extra X chromosome in males (47,XXY). KS is associated with various phenotypic characteristics in adult life, including infertility, hypogonadism and increased risk of type II diabetes, cardiovascular disease and osteoporosis. Additionally, individuals with KS often experience mental health challenges and functional impairments that significantly impact their quality of life. Currently, testosterone replacement therapy (TRT) in adolescence is considered the first-line treatment by some physicians for patients with KS and biochemical signs of hypogonadism. However, comprehensive evidence on its effectiveness in preventing typical phenotypic traits associated with KS remains limited, and, currently, no evidence-based recommendations for TRT in this population exist. We therefore aim to evaluate the effects of two years of TRT during puberty in boys with KS. The primary endpoint is to monitor changes in body fat percentage. Secondary endpoints include changes in pubertal development and virilisation, growth and body proportions, bone mineralisation, muscle strength, lipid and glucose metabolism, systemic inflammation, methylation, fertility and effects on the cognitive and psychopathological features of KS.

Methods and analysis The TIPY study is a multicentre, national, randomised, double-blind, placebo-controlled intervention study. Participants will be recruited from four tertiary paediatric endocrine units in Denmark that manage boys with KS. Participants will be randomised to treatment with transdermal placebo or transdermal testosterone (Androgel; Besins Healthcare, Paris, France) with dose titration every 3 months based on individual measurements of serum concentrations of testosterone. Dose titration will be conducted by a single physician to ensure free testosterone remains between +1 and +3 SD for age.

Thorough clinical and biochemical evaluation will be performed at baseline, after 12 months and 24 months. Additional visits for minor evaluations will occur every 3 months. Neuropsychological assessment will be conducted at baseline and after 24 months of treatment.

Ethics and dissemination The study will be conducted in accordance with the Helsinki Declaration. The study has been approved by the Danish National Medical Research Ethics Committee and the Danish Medicines Agency (Clinical trials information system number 2023-505854-16-00). Results will be submitted for publication in peer-reviewed journals.

Trial registration number NCT06294990.

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ Klinefelter syndrome (KS) is often diagnosed late or remains undiagnosed, leading to an inherent risk of ascertainment bias in this study.
⇒ Boys with milder cognitive challenges or stronger family support systems may be over-represented in the study cohort, creating an unbalanced sample.
⇒ A key strength of this study is that participants are recruited from the four tertiary paediatric endocrine units in Denmark that manage boys with KS, ensuring that potentially all boys diagnosed with KS in Denmark will be invited to participate.
⇒ To ensure consistency in assessments, all participants will undergo comprehensive neuropsychological and clinical examinations conducted by the same psychologist and clinicians.
⇒ Biochemical evaluation of reproductive hormones will be performed using liquid chromatography with tandem mass spectrometry in the Hormone Laboratory at the Department of Growth and Reproduction, Rigshospitalet, ensuring high accuracy and reliability.

BACKGROUND

Klinefelter syndrome (KS), characterised by the presence of an additional X chromosome (47,XXY), is the most frequent sex chromosome aberration affecting approximately 1 in 660 newborn boys.[1] KS encompasses a wide range of phenotypic characteristics including small testes, hypergonadotropic hypogonadism, gynaecomastia, infertility, learning disabilities and psychosocial challenges. Individuals with KS also face an increased risk of developing cardiovascular disease, metabolic syndrome, diabetes, osteoporosis and psychiatric disorders.[2–4]

The severity of symptoms varies significantly, with some individuals experiencing mild impairments while others face more severe physical, developmental, psychosocial, behavioural and learning challenges. Deficits in language, executive function and social cognition may be evident in early childhood, while other symptoms are not apparent until adulthood. Symptoms before puberty are often sparse and, if present, are mainly related to learning disabilities as well as psychosocial and behavioural challenges. Despite the profound impact on the health of most patients with KS, the condition is highly underdiagnosed or diagnosed late. Less than 10% of cases are identified before puberty, and only between 25% and 38% of the expected cases ever receive a diagnosis.[1 5] This is particularly important as timely intervention may prevent complications such as osteoporosis, cardiovascular disease and mental health impairment.

Mental health and functioning

Multiple developmental, cognitive and psychiatric issues characterise KS at a group level, all of which may significantly affect life achievements and life satisfaction. Full-scale intelligence quotient scores typically fall in the lower range of normal performance,[6–8] and intellectual disability is not common.[9] However, in measures of adaptive functioning, children with KS are less capable of integrating and regulating cognition and emotion to navigate everyday challenges, compared with unaffected peers.[7 10 11] Psychopathology related to attention (attention deficit/hyperactivity disorder), emotion and mood regulation (anxiety and depression) and social skills (autism spectrum disorders) is significantly more common among individuals with KS, compared with the general population.[7 9 12–18] During the course of a lifetime, KS considerably increases the risk of adverse psychosocial and socioeconomic factors such as lower education and income levels,[19] higher morbidity and mortality rates[20 21] and reduced quality of life.[15 22–26]

Testosterone deficiency

It is well-established that most adults with KS develop primary testicular failure, necessitating testosterone replacement therapy (TRT). However, studies examining testicular endocrine function in infants and children with KS are limited, and some controversy remains. Thus, some studies indicate that boys with KS may already present with lowered testosterone concentrations in infancy and in the years preceding puberty,[27–33] whereas other studies could not confirm this.[34–38] Clinical signs suggesting early testosterone deficiency in KS infants include increased incidence of cryptorchidism, smaller testis volume, reduced penile length at birth and impaired penile growth in childhood.[27 29 30 39 40]

Testosterone supplementation in minipuberty

One randomised trial has evaluated the effects of testosterone injections in KS for 3 months during infancy.[27] This pivotal study demonstrated significant increases in percentage fat mass in untreated boys, whereas fat-free mass, body length and growth velocity, as well as stretched penile length, increased significantly in infants receiving testosterone compared with untreated boys.[27]

Oxandrolone treatment in prepubertal KS

A single double-blinded, randomised, placebo-controlled study has evaluated the effect of treatment with low-dose oral androgen (oxandrolone) in prepubertal boys with KS for 24 months and showed significant improvements in cortical bone mass, body fat, triglycerides and high-density lipoprotein and in visual-motor integration, measures of anxiety, depression and social problems (including self-reported self-esteem) compared with placebo.[41–43] However, no significant changes in cognition, gross motor function or attention were observed[43]

Testosterone treatment in pubertal KS

Boys with KS generally enter puberty, defined as a Tanner stage of G2, spontaneously at the expected time.[35] However, around mid-puberty, defined as Tanner stage PH3, luteinising hormone (LH) and follicle stimulating hormone (FSH) increase to very high concentrations, followed by a levelling off of testosterone in Tanner stage PH4.[35 44] Testosterone concentrations in boys with KS are typically sufficient to support normal male genital development. However, signs of reduced virilisation are often evident in adolescence and adulthood, including a lack of voice deepening, sparse facial and body hair and decreased muscle mass. Gynaecomastia is a physiological phenomenon seen in 20–70% of otherwise healthy pubertal boys and occurs with a similar frequency (18–59%) in pubertal boys with KS. However, gynaecomastia may be more likely to persist in KS, particularly if hypogonadism is present and untreated.[45]

No randomised, placebo-controlled studies have evaluated TRT during puberty. However, few descriptive real-world evidence studies have evaluated the effects of testosterone on biological outcomes.[45–49] It makes biological sense to consider TRT during puberty in KS to prevent the further development of hypogonadal features, which are frequently observed in newly diagnosed young adults with KS, although there are no evidence-based recommendations. In recent years, the use of TRT during puberty has been questioned and is no longer recommended in some countries.[50 51]

AIM

The aim of this randomised, double-blind, placebo-controlled intervention study is to evaluate the effect of 2 years of testosterone treatment in boys with KS aged 10–14 at the time of inclusion.

Primary outcome

The primary objective is to evaluate the impact of 24 months of TRT on body fat mass as evaluated by whole-body dual-energy X-ray absorptiometry (DEXA) scans.

Secondary outcomes

The secondary objectives are to evaluate the effects of 2 years of TRT on:

- Pubertal development and virilisation.
- Growth and body proportions.
- Bone mineralisation.
- Secondary measurements of body composition.
- Muscle strength.
- Electrocardiogram (QTc) results.
- Lipid and glucose metabolism and systemic inflammation.
- Fertility.
- Neurocognitive development, adaptive behavioural functioning, psychopathology and quality of life.
- Epigenetics, that is, changes in DNA methylation.

METHODS AND ANALYSIS

For an overview, see the graphical abstract (figure 1).

Study population

The study 'Testosterone treatment in Puberty in Males with Klinefelter syndrome' (The TIPY-study) is a national, multicentre study including a total of 32 boys with KS aged 10–14 years. The study was registered with an estimated recruitment start date of 21 August 2023, and the first patient was included on 15 August 2024. The estimated completion date is 31 December 2029.

The main inclusion criteria are a diagnosis of 47,XXY, age between 10 and 14 years, serum concentration of LH above +2 SD and free testosterone (free T) (calculated using the Vermeulen equation[52]) below +2 SD measured at the Hormone Laboratory in the Department of Growth and Reproduction, Rigshospitalet. Additional inclusion and exclusion criteria are presented in table 1, and an example of the changes in LH and free T before and after initiation of TRT in a potential participant is illustrated in figure 2.

Recruitment and informed consent

Participants will be recruited from the four paediatric endocrinology centres following boys with KS in Denmark, located at Copenhagen University Hospital,

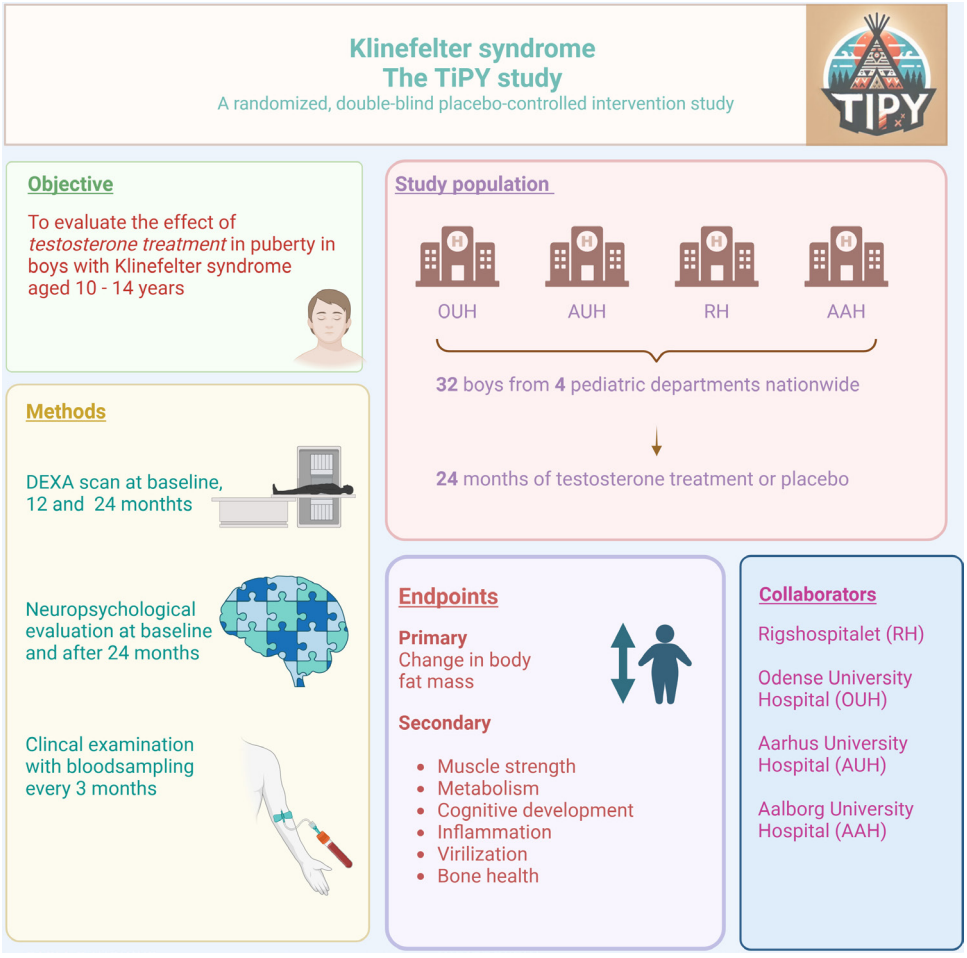

Figure 1 Graphical abstract illustrating The TIPY study.

Table 1 Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Boys with 47,XXY Klinefelter syndrome verified by conventional chromosome analysis or array CGH.	Previous or ongoing T treatment except for TRT because of micropenis during minipuberty
Age 10 to 14 years at inclusion	Contraindications to TRT
LH > +2 SD by ultrasensitive LH assay	Participation in any other clinical trial
Free T<+2 SD	
Signed consent from both parents	

.LH, luteinising hormone; T, testosterone; TRT, testosterone replacement therapy.

Rigshospitalet; Odense University Hospital; Aarhus University Hospital and Aalborg University Hospital.

As part of the standard care for boys with KS in Denmark, the boys are seen at regular visits every three to four months as they approach puberty. At each of these visits, the pubertal stage according to Tanner is evaluated, height and weight are measured and a blood sample is collected to measure serum concentrations of reproductive hormones, assessing biochemical signs of hypogonadism. Based on this standard evaluation, it is determined whether the inclusion criteria are met. When a boy meets the inclusion criteria, the boy and his parents will be invited to participate in the study. Participants and their parents will receive both oral and written information about the study, and, if the family agrees to participate, the parents will sign an informed consent form (online supplemental file 1). The boy and his parents will then be invited for visit 1 at the Department of Growth and Reproduction, Rigshospitalet. Participants may withdraw from the study at any time without any consequences for their future follow-up at the hospital.

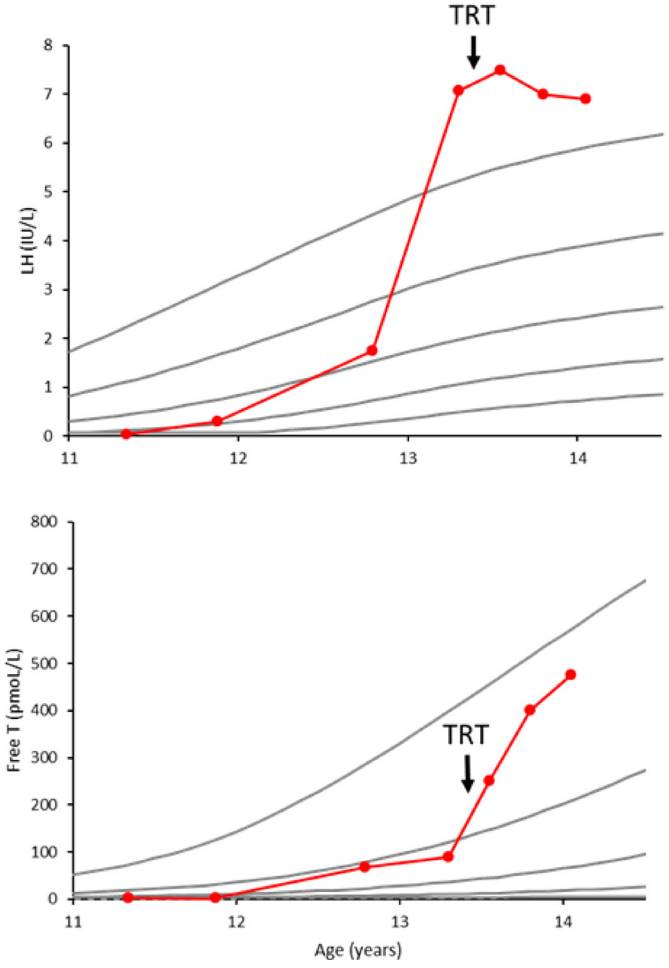

Figure 2 Serum concentrations of luteinising hormone (LH) and free testosterone (free T) according to chronological age in a potential boy with Klinefelter syndrome before and after initiation of testosterone replacement therapy (TRT).

Randomisation and blinding

At the first visit, participants will be randomised 1:1 to receive either TRT or placebo. The randomisation will be double-blinded. Subjects will be assigned a specific and unique identification number. Randomisation will be performed at the regional pharmacy in the Capital Region of Denmark.

Procedures for breakage of the randomisation code

The randomisation code will be broken for a participant if a serious adverse event is deemed to be related to the treatment. The randomisation code may be broken at any time during the study period.

Intervention

The active drug (Androgel 1%) and the placebo will be provided in identical containers, each labelled with a unique identification number. Each container has a pump mechanism that dispenses a fixed amount of gel (1.25 g). One pump of Androgel 1% delivers 12.5 mg of testosterone.

Study arm 1: testosterone (Androgel 1%)

Participants assigned to active treatment will receive treatment with Androgel applied transdermally in the morning for 2 years. During the first 3 months, the dose will be increased from 12.5 mg testosterone (one pump) every second day to a daily dose of 12.5 mg. Thereafter, dose adjustments will be made every 3 months based on the results of the blood samples taken at each visit. The goal is to maintain free T between +1 SD and +3 SD for age (see figure 2).

Study arm 2: placebo

Participants assigned to placebo will apply the placebo transdermally in the morning for 2 years. Identical dose

increments will be performed during the first 3 months to a dose of one pump per day. Thereafter, dose adjustments in participants receiving placebo will be identical pairwise to the adjustments performed in a participant receiving testosterone to preserve the double-blinded study design.

An unblinded investigator, who will not interact with the participants or their families, will evaluate safety issues and determine dose titrations.

Adverse events

Adverse events will be evaluated at each visit and at each phone call planned to be made 2–4 weeks after each visit.

Sample size and power calculation

Based on data from our previous study on 275 boys aged 8–14 years who underwent whole-body DEXA scan using the same Lunar Prodigy Advance equipment, the coefficient of variation (CV) for body fat percentage (%BF) was 13.1% (max CV for inter-rater variability).[53] The BF% for 10–12 year-olds was approximately 20%, resulting in an estimated SD of 2.6%. To detect a 5% difference in BF% (primary outcome) between the two groups over a 2-year period, using a two-sample t-test on paired differences, 13 patients are required in each group. The test is at the nominal 5% significance level with a power of 90%. With an expected dropout rate of approximately 20%, a total of 32 boys will be included to ensure that 13 participants in each arm will complete the study.

Analytical plan

The analysis will be a restricted linear mixed effects model which takes into account that baseline and follow-up measurements are on the same individual, while ensuring that the expected baseline levels in the two groups are the same due to the randomisation. Missing observations are handled in this (maximum likelihood estimation approach) under the usual assumption that missing data are missing at random. Comparison of the two groups at a particular time will be carried out by a t-test (or non-parametric test depending on the distribution of the outcome).

Ethics and dissemination

The study will be conducted in accordance with the Helsinki Declaration, and all necessary study approvals have been obtained from the Danish National Medical Research Ethics Committee and the Danish Medicines Agency (Clinical trials information system number 2023-505854-16-00). The study will be monitored and quality assured by the regional Unit for Good Clinical Practice (GCP) at each site. The clinical trial will be conducted in compliance with the protocol and with the declaration of GCP and FORORDNING (EU) Nr. 536/2014 April 16.

Positive, negative and inconclusive results from the study will be disseminated at national and international conferences and in peer-reviewed scientific international journals.

Public and patient involvement

Participants and their parents will be asked to provide feedback after each visit to help make the research days more participant-friendly. Additionally, the study team will create newsletters to keep families updated with information tailored for a non-specialist audience.

MEASUREMENTS

The data assessment schedule is shown in online supplemental file 2. Each participant's study period will span 2 years and include a total of nine assessments. Evaluations will take place every 3 months. The visits at baseline (visit 1), after 1 year (visit 5) and after 2 years (visit 9) will include fasting blood sampling and a thorough clinical examination. Between these evaluations, participants will attend routine visits every 3 months at the paediatric department where they are usually followed. For details about the timing of each examination, please see table 2. In addition, participating families will be contacted by phone 2–4 weeks after each visit to ensure the boy is taking the correct dose and is tolerating the treatment. Should the blood samples indicate the need for dose modifications, the family will be notified both orally and in writing by a study doctor or nurse and provided with instructions on how to adjust the dosage.

All other measurements are outlined in table 3.

The baseline and final evaluation will involve identical procedures including clinical examination, fasting blood sample, urine sample and neuropsychological assessment and will take place over the course of two consecutive days. The neuropsychological assessment will start on the first day and be completed on the second day. The 1-year evaluation will include a fasting blood sample and a clinical examination. No neuropsychological assessment will be conducted at that visit.

Results from individual analyses (blood and urine) will only be communicated to families if health issues requiring further medical evaluation are identified. In contrast, all neuropsychological assessments will be followed by a written summary of the child's performance along with recommendations for intervention, if relevant. If needed, the neuropsychologist responsible for the assessment will offer meetings involving the family and relevant professionals, such as schoolteachers or school psychologists. If symptoms of psychiatric disorders requiring acute or subacute intervention arise during assessment (specifically: severe depression or anxiety, psychotic episodes or thoughts related to death or suicide), the family will be directed to the nearest acute psychiatric admission centre or guided to seek help from the family's general practitioner or local municipal services.

Clinical visits

The participants will be examined every 3 months during the study period, and they will follow a standardised examination schedule as outlined in table 2. Clinical examination will be performed by trained medical doctors.

Table 2 Hormone analyses at the Department of Growth and Reproduction, Rigshospitalet

Hormone	LOD	Method/assay	Manufacturer
Follicle-stimulating hormone	0.3 IU/L	Chemiluminescence immunoassay	Atellica, Siemens Healthineers, Tarrytown, NY, USA
Luteinising hormone	0.07 IU/L	Chemiluminescence immunoassay	Atellica, Siemens Healthineers, Tarrytown, NY, USA
Sex-hormone binding globulin	0.33 nmol/L	Chemiluminescence immunoassay	Access2, Beckman Coulter, Brea, CA, USA
Anti-Müllerian hormone	0.14 pmol/L	Chemiluminescence immunoassay	Access2, Beckman Coulter, Brea, CA, USA
Inhibin B	3 ng/L	ELISA	Immunotech, Beckman Coulter, Brea, CA, USA
Insulin-like growth factor (IGF) I	10 µg/L	Chemiluminescence immunoassay	iSYS, Immunodiagnostic Systems, Boldon, Tyne and Wear, UK
IGF-binding protein (IGF-BP) III	80 µg/L	Chemiluminescence immunoassay	iSYS, Immunodiagnostic Systems, Boldon, Tyne and Wear, UK
IGF I	80 µg/L	LC/MS-MS	*In-house*
IGF II	6 µg/L	LC/MS-MS	*In-house*
IGF-BP I	2 µg/L	LC/MS-MS	*In-house*
IGF-BP II	16 µg/L	LC/MS-MS	*In-house*
IGF-BP III	90 µg/L	LC/MS-MS	*In-house*
IGF-BP IV	20 µg/L	LC/MS-MS	*In-house*
IGF-BP V	18 µg/L	LC/MS-MS	*In-house*
IGF-BP VI	50 µg/L	LC/MS-MS	*In-house*
Acid-labile subunit	300 µg/L	LC/MS-MS	*In-house*
Insulin-like 3	10 pg/mL	LC/MS-MS	*In-house*
Dehydroepiandrosterone sulphate	4.51 nmol/L	LC/MS-MS	*In-house*
Testosterone	0.031 nmol/L	LC/MS-MS	*In-house*
17α-Hydroxy-progesterone	0.033 nmol/L	LC/MS-MS	*In-house*
Dehydroepiandrosterone	4.4 nmol/L	LC/MS-MS	*In-house*
Progesterone	0.036 nmol/L	LC/MS-MS	*In-house*
Estrone	2.9 pmol/L	LC/MS-MS	*In-house*
Estradiol	4.0 pmol/L	LC/MS-MS	*In-house*
Estrone sulphate	0.025 nmol/L	LC/MS-MS	*In-house*
Aldosterone	0.038 nmol/L	LC/MS-MS	*In-house*
Cortisone	0.64 nmol/L	LC/MS-MS	*In-house*
Cortisol	5.57 nmol/L	LC/MS-MS	*In-house*
21-deoxycortisol	0.035 nmol/L	LC/MS-MS	*In-house*
Corticosterone	0.22 nmol/L	LC/MS-MS	*In-house*
11-Deoxycortisol	0.042 nmol/L	LC/MS-MS	*In-house*
Androstenedione	0.031 nmol/L	LC/MS-MS	*In-house*
11-Deoxycorticosterone	0.029 nmol/L	LC/MS-MS	*In-house*
17-Hydroxypregnenolone	0.19 nmol/L	LC/MS-MS	*In-house*

LOD, limit of detection; LC/MS-MS, liquid chromatography-tandem mass spectrometry.

Body fat percentage will be measured by whole-body DEXA scans using Lunar Prodigy (GE Healthcare, Madison, WI) and analysed using Encore software enhanced, V.16. For quality control, calibration is performed as recommended by the manufacturer using a quality assurance block daily and using a spine phantom

Table 3 Other biochemical analyses

Systemic effect	Measurement
Thyroid function	TSH, T3, T4, free T4, TPO, TRABs and TG-ab
Adrenal antibodies	Cyto11a1-Ab, Cyto17a1-Ab and Cyto21a2-Ab
Coeliac disease	Transglutaminase-IgA
Haematology	Haemoglobin, haematocrit, leukocytes and platelets
Bone health	25-OH-vitamin D, magnesium, calcium, phosphate, PTH, alkaline phosphatase, osteocalcin, PINP, CTX, RANKL, OPG, Klotho, sclerostin and TRACP 5b
Liver and kidney function	ALAT, ASAT, GGT, bilirubin, creatinine, albumin, GFR, NA, K and carbamide
Organ marker	PSA
Glucose metabolism	Fasting glucose, insulin and HbA1c
Inflammation	hsCRP+CRP, IL-6, TNF-α, IL-1RA and Mesoscale
Lipid status	Total cholesterol, HDL, LDL, triglyceride, free fatty acids and glycerol
Adipocytokines	Adiponectin and leptin

ALAT, Alanine Aminotransferase; ASAT, Aspartate Aminotransferase; CRP, C-reactive protein; CTX, C-terminal telopeptide of type I collagen; Cyp11a1, Cytochrome P450 Family 11 Subfamily A Member 1; cyp17a1, Cytochrome P450 Family 17 Subfamily A Member 1; Cyto17a1, Cytochrom P450 17A1-Ab; Cyto11a1-ab, Cytochrom P450 11A1-Ab; Cyto21a2-ab, Cytochrom P450 21A2-Ab; GFR, Glomerular Filtration Rate; GGT, Gamma-Glutamyl Transferase; HbA1c, Hemoglobin A1c; HDL, High-Density Lipoprotein; hsCRP, high-sensitivity C-reactive protein; IL-6, Interleukin-6; IL-1RA, Interleukin-1 Receptor Antagonist; K, Potassium; LDL, Low-Density Lipoprotein; Na, Sodium; OPG, Osteoprotegerin; PINP, Procollagen Type I N-Terminal Propeptide; PSA, Prostate-Specific Antigen; PTH, Parathyroid Hormone; RANKL, Receptor Activator of Nuclear Factor Kappa-B Ligand; T3, Triiodothyronine; T4, Thyroxine; TG-ab, Thyroglobulin Antibodies; TNF-a, Tumor Necrosis Factor-alpha; TPO, Thyroid Peroxidase; TRAB, Thyrotropin Receptor Antibodies; TRACP 5b, Tartrate-Resistant Acid Phosphatase 5b; TSH, Thyroid-Stimulating Hormone.

on a weekly basis. In addition, fat percentage will be evaluated by bioimpedance (Tanita MC 780S). *Skin fold* measurement at four sites, including the biceps, triceps, sub-scapular and iliac crest on the left side of the body will be performed using the Harpenden skin-fold calliper (Harpenden, British Indicators Ltd, London, UK).

Pubertal development and virilisation will be evaluated according to the Tanner genital stage G1 (prepubertal)–G5. The development of pubic hair will be graded from Tanner stages PH1 (no hair)–PH5[54] and testicular volume will be measured using an orchidometer.[55] Puberty SD score will be calculated by applying data on genital and pubic hair stage as well as testicular volume to a previously published puberty nomogram.[56] *Penile measurement will be* performed using a dial calliper to the nearest millimetre (Wiha, dialMax Calliper). *Voice frequency* will be measured using the app 'Voice Analyst' (Speechtools Ltd.).

Furthermore, *ultrasound of the testes* will be performed using a linear transducer (L14-5WE). The ultrasound examination will be performed with the boy in supine position and the length (L), width (W) and depth (D) will be measured of both testicles. The probe will be placed in the mid-sagittal testicular plane, perpendicular to the skin, and the examiner will then move the probe back and forth until the largest diameter is recorded (L). Afterwards, the probe will be rotated 90 degrees and W and D will be measured in the mid-transverse plane. The volume of the testes will then be calculated using the formula: L × W × D × 0.52. Echo-score (1–5) will be registered according to the study by Lenz *et al*.[57]

Breast ultrasound will be scored using a system for scoring maturation of glandular breast tissues based on description by García *et al*[58] with the addition of a second prepubertal stage proposed by Bruserud *et al*.[59] The ultrasound will be performed with the boy in supine position, with his arms resting at the side. The probe will be placed perpendicular to the skin and centred on the nipple to produce a sagittal standard section which will be used for all measurements. Based on this section, the depth and diameter of both breasts will be measured, and morphological staging on a scale from US B0 to US B5 will be used.[59]

Anthropometry will include *standing and sitting height* as measured to the nearest 0.1 cm using a wall-mounted stadiometer. *Parental heights* will be measured at the first visit or recorded if both parents are not present. *The weight* of participants and their parents will be measured using a digital electronic scale with a precision of 0.1 kg (SECA delta, model 707). *Body mass index* (weight/height^2 (kg/m^2)) will be calculated. *Waist, hip and head circumference* will be measured using measuring tape, and measurement of *arm span* will be performed using a laser measurer (Bosch GLM 40 Professional) and a measuring tape will be used to measure the arm span every 3 months at the local visits. *Bone age* will be assessed using X-ray of the left hand and evaluated by Bone Xpert V.3 (Hørsholm, Denmark).[60]

Bone mineralisation will be evaluated by whole-body DEXA scans and *bone health index* will be assessed using X-ray of the left hand and evaluated using the software BoneXpert V.3 (Hørsholm, Denmark).[60]

Table 4 Neuropsychological assessment battery

Domain	Test	Type	Outcome
General intellectual functioning	The Wechsler Intelligence Scale for Children – Fifth Edition (Wechsler)	Test	The General Ability Index
Attention	The test of variables of attention, V.9 (Leark *et al*)	Test	Response time variability and response sensitivity (*d'*)
Visuo-spatial abilities	The judgement of line orientation test (Benton, Varney and Hamsher)	Test	Total number of correct responses
	The mental rotation test (Vandenberg and Kuse)	Test	Total number of correct responses
Memory	The test of memory and learning, second edition (Tomal-2, Ritchie and Nierenberg)	Test	Scale scores of selected verbal subtests (word selective reminding; object recall) and non-verbal subtests (abstract visual memory; visual sequential memory)
	The Wechsler Intelligence Scale for Children – Fifth Edition (Wechsler)	Test	The Working Memory Index
Fine motor function and processing speed	The Beery-Buktenica developmental test of visual-motor integration (Beery, VMI)	Test	The Beery VMI score
	The Baseline Speed subtask of the Amsterdam Neuropsychological Tasks programme (de Sonneville).	Test	Mean response time
	The Wechsler Intelligence Scale for Children, Fifth Edition (Wechsler)	Test	Processing Speed Index
Language, communication and social cognition	The Children's Communication Checklist (Bishop)	Questionnaire	The index of general communication and index of social interaction
	The Social Responsiveness Scale-2 (Constantino)	Questionnaire	Total score
	Subtasks of the Amsterdam Neuropsychological Tasks programme (de Sonneville).	Test	The feature identification, face recognition and identification of facial emotions
Executive functioning	The Delis-Kaplan executive function system (Delis, Kaplan and Kramer)	Test	Primary speed and accuracy-measures of the trail-making test and the verbal fluency
	The BRIEF-2 rating scale, parent version (Gioia *et al*)	Questionnaire	Total score
Psychopathology	Behavior Assessment System for Children, Third Edition, parent and self-report version (Reynolds and Kamphaus).	Questionnaire	Clinical scales and index scores for attention deficit/hyperactivity disorder (ADHD) probability, autism spectrum disorder probability, and emotional behavioural disturbance probability
	Self-report version of The Multidimensional Anxiety Scale for Children – second edition (March and Parker)	Questionnaire	Total score as well as individual subscales
	The parent version of the ADHD-rating scale (DuPaul)	Questionnaire	Total scores of inattention, hyperactivity and behaviour scales
Adaptive behavioural functioning	The Adaptive Behavior Assessment System, Third Edition (Harrison and Oakland)	Questionnaire	The general adaptive composite

Continued

Table 4 Continued

Domain	Test	Type	Outcome
Quality of Life (self-perceptions, social stress, adaptability and fatigue)	The Beck Youth Self-Concept Inventory (Beck *et al*), the Self Concept subscale	Questionnaire	Total score of the Self Concept subscale
	Behaviour Assessment System for Children, Third Edition, self-report version	Questionnaire	Total scores of the Adaptive Skills subscales
	PedsQL Multidimensional Fatigue Scale, parent and self-report versions (Varni)	Questionnaire	Total score

BRIEF-2, Behavior Rating Inventory of Executive Function - 2nd Edition; PedsQL, Pediatric Quality of Life Inventory.

Muscle strength will be measured by grip strength (a proxy of maximum isometric upper limb strength) and standing jump length (a proxy of lower limb bilateral explosive physical performance) as previously described.[61] In brief, grip strength will be measured using a digital hand dynamometer (Baseline BIMS, digital hand dynamometer, functional model) with the dominant hand and the arm extended. The boys will be instructed to squeeze as hard as possible for at least 3 s. The best performance of three trials will be recorded (in kg). Standing jump length will be performed on a non-slippery mat where the boys will be instructed to stand on a line in an upright position and to bend their knees and jump forward as far as possible. The distance will be measured from the start line to the back of the heel nearest to the start line. The longest jump of four trials will be recorded (in cm).

Blood pressure will be measured three times using an automated device after 5 min of resting time.

Electrocardiography will be evaluated and analysed according to the national recommendation from The Association of Danish Pediatricians.[62]

Skeletal deformities associated with KS[30], and the presence of tremor (yes/no) will be registered. Examination for hypermobility will be performed using the Beighton scoring system.[63]

Blood samples will be drawn from an antecubital vein after local anaesthesia (EMLA), if needed. Blood will be centrifuged at 3500 RPM for 10 min and stored at −20°C or, if added to the biobank, at −80°C. Collection will happen approximately 4 hours after application of gel to ensure correct hormone measurements. The time of application of gel will be recorded at each visit. All reproductive hormones and growth factors will be measured at the hormone laboratory at the Department of Growth and Reproduction (table 2).

DNA will be analysed using a selected set of genetic polymorphisms in target genes with established or theoretical effects on hormone production and hormone receptor sensitivity. They will be analysed either by PCR genotyping or targeted sequencing (max. 200 selected genes). Single nucleotide polymorphism (SNP) arrays that exclusively target common variants and not any rare variants will be used to determine the influence of common genetic variation on the observed associations. Rare variants are defined as variants with a minor allele frequency of <1%. DNA methylation patterns will be analysed on DNA from white blood cells by applying Illumina methylation arrays.

RNA analysis of circulating small, non-coding RNA will be performed as a biomarker for the circulating concentrations of reproductive hormones[64] and for overweight.[65]

Urine sampling will be obtained at the first, fasting, morning voiding and evaluated for LH, FSH and bone markers.

Semen sampling at the end of the study period, all boys who are willing and able will be offered the opportunity to make a semen deposit in the semen bank at the Department of Growth and Reproduction. The semen volume, sperm concentration and percentage of motile sperm cells will be registered before cryopreservation. If preferred, the sample can be collected at home provided it is delivered to the semen bank within 1 hour and maintained at body temperature until delivery.

Neuropsychological evaluation will be performed by a neuropsychologist using a standardised battery of tests outlined in table 4.

The testing will be performed in Danish over 2 days with an estimated total duration of 4 hours and 30 min with allocated breaks. All families will be invited to an online feedback session and receive a written summary with recommendations for intervention if relevant.

DISCUSSION

This randomised, double-blind, placebo-controlled intervention study aims to evaluate the effect of 2 years of treatment with testosterone in boys with KS aged 10–14 at the time of inclusion. No evidence-based recommendations for TRT exist, and this study contributes to filling this significant research gap.

KS is associated with a wide range of adverse health and life circumstances, ultimately impacting quality of life and life expectancy. Puberty marks a sensitive period of particular interest in boys with KS, as the precursors of adolescent and adult health trajectories are rapidly shaped by significant hormonal changes. For these reasons, the present study encompasses endpoints related to body composition, bone health, growth, cognition and psychopathology. By adopting this comprehensive approach, we

expect to detect and evaluate the potential effects of TRT in an integrative manner.

The study is the first randomised, placebo-controlled, intervention study to date to assess the effects of TRT during puberty in boys with KS. To gain more certainty of TRT as a valid future treatment for KS, we need to know if the dosage matters. Although previous low-dose oxandrolone intervention studies have shown improvements on specific measures of body composition, visual-motor integration and psychopathology in preteen years,[41–43] the effects of testosterone during puberty may yield additional beneficial outcomes to pubertal development, muscle strength and cognitive functions such as attention.

In conclusion, this study may generate new perspectives for the treatment of boys and men with KS. Evidence-based guidance for parents and clinicians on how to address the paediatric and psychosocial needs of boys with KS during childhood and adolescence will enhance the chances of alleviating health issues related to quality of life.

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Acknowledgements We would like to thank the participants in advance, collaboration partners and study assistants.

Contributors The protocol was developed by IDC and LIA in collaboration with the co-investigators (AFØF, JHP, NB, A-MRC, AJS, KK and AJ) and an international advisory board (JLR, SD, GB and SvR). All authors provided feedback and approved the final version of the manuscript. LIA is principal investigator and sponsor. LIA is responsible for the overall content as guarantor.

Funding The study has received funding from the Novo Nordisk Foundation (grant number: NNF22OC0080944). LIA received funding from Rigshospitalet's Research Council and the Independent Research Fund Denmark (grant number: 10.46540/4262-00014B). The study drug and placebo are provided by Besins Healthcare. None of the above-mentioned has any influence on the development of the study design or results.

Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Provenance and peer review Not commissioned; externally peer reviewed.

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