Testosterone undecanoate replacement therapy in boys with pubertal delay or confirmed hypogonadism

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Keywords: pubertal delay, constitutional delay of puberty, hypogonadism.

1 INDEX

2 SUMMARY

Introduction: The absence of clinical signs of pubertal maturation, i.e. pubertal delay, is a relatively frequent reason for consultation in boys. In cases where it is necessary, the treatment to be established is the administration of testosterone with the aim of provoking the development of secondary sexual characteristics and optimizing growth.

Problem: Currently, the most commonly used treatment is empirical, with IM depot testosterone at increasing doses over a period of 2 to 3 years. The most commonly used formulation, testosterone enanthate, comes in ampoules of 250 mg in 1 ml of vehicle. The usual administration regimen begins with approximately 50-60 mg IM every 4 weeks (1/4 of the ampoule), increasing the dose by approximately 50-60 mg (1/4 ampoule) every 6 months until the full dose of 250 mg (1 ampoule) is reached every 4 weeks. The pharmacokinetic profile has not been described to see if it mimics the physiological progressive increase in testosterone levels at normal puberty. The pharmacokinetic profile of testosterone enanthate in adults shows supraphysiological serum testosterone concentrations the first week after the medication is administered, with a progressive drop to subphysiological levels in the fourth week.

In adults, testosterone undecanoate is used as a first-line medication. Its presentation in ampoules with 1000 mg in 4 ml, of IM administration every 12 weeks, is considered equivalent to testosterone enanthate 250 mg every 4 weeks. In adults, the circulating levels of testosterone obtained with the formulation of testosterone undecanoate show a profile within physiological ranges, more stable between doses than that observed with testosterone enanthate. Testosterone undecanoate via IM has not been tested in adolescents.

Hypothesis: The hypothesis of this work is that the initial administration of 1 ml (~250 mg) of testosterone undecanoate (1000 mg/4 ml) via IM every 12 weeks for 6 months, with a progressive increase of 1 ml (~250 mg) every 6 months until reaching 4 ml (1000 mg) per dose is safe and effective in inducing normal progression of secondary sex characteristics and growth spurt in boys with pubertal delay.

Objective: The general objective of this study is to evaluate whether a quarterly administration regimen of IM testosterone undecanoate, in boys with anorchia, hypogonadism or pubertal delay induces the progression in the development of secondary sexual characteristics, the speed of growth and the acquisition of bone mass in accordance with physiological pubertal development, and maintain blood testosterone levels similar to those seen during spontaneous pubertal maturation.

The primary specific objectives are to determine, in boys with pubertal delay:

a. If a treatment regimen of testosterone undecanoate (1000 mg/4 ml) via IM, with an initial dose of 1 ml (~250 mg) every 12 weeks (stage 1: weeks 1-24), 2 ml (~500 mg) every 12 weeks (stage 2: weeks 25-48), 3 ml (~750 mg) every 12 weeks (stage 3: weeks 49-72) and 4 ml (100 0 mg) every 12 weeks (stage 4: weeks 73-96), is accompanied by progression of secondary sexual characteristics and height velocity commensurate with those of normal pubertal development.

b. The safety of the administration of increasing doses of IM testosterone undecanoate, through the evaluation of biochemical parameters (see below) and bone age progression.

The secondary specific objectives are:

a. To assess whether blood testosterone levels are maintained at levels within established physiological limits, for 12 weeks after administering the dose of testosterone undecanoate via IM.

b. To explore the changes occurring in the pituitary-gonadal axis and bone mass of boys with pubertal delay who are treated with increasing doses of IM testosterone undecanoate.

Design and methods: An intervention study will be carried out, with prospective longitudinal follow-up of a cohort of boys with anorchia, hypogonadism or pubertal delay.

Testosterone undecanoate (1000 mg/4 ml) will be administered at a dose of 1 ml every 12 weeks for 6 months, 2 ml every 12 weeks for 6 months, 3 ml every 12 weeks for 6 months and 4 ml every 12 weeks for 6 months.

Progression commensurate with normal pubertal development shall be considered to exist if the clinical evaluation detects a Tanner stage G2 or G3 and VP2 or VP3 at the end of the first 6 months, G3 or G4 and VP3

or VP4 at the end of the first year, G3 to G5 and VP3 to VP5 after 18 months, and G4 or G5 and VP4 to VP6 at the end of 2 years. The proportion of patients with the hypothesized progression will be calculated. The therapeutic scheme shall be considered satisfactory if this proportion is ≥80%.

For growth, progression in line with normal pubertal maturation shall be considered if the clinical evaluation detects a height velocity of 6 to 10 cm/year at the end of the first year and 8 to 14 cm/year at the end of the second year. The proportion of patients with the hypothesized progression will be calculated. The therapeutic scheme shall be considered satisfactory if this proportion is ≥80%.

The number and type of adverse events at each visit will be reported. Changes in hormone levels of the pituitary-gonadal axis and bone mass will be analyzed.

Sample size: For this study, estimating that 80% of patients will have a development of secondary sexual characteristics within the range expected for each stage after the administration of 1, 2, 3 or 4 ml of testosterone undecanoate via IM every 12 weeks, it will be necessary to analyze 27 individuals to provide a conclusion with an accuracy of 15%. Accepting a maximum loss of 20% during follow-up, 34 patients should be recruited into the study.

Statistical analysis: A descriptive statistical report of the quantitative variables will be carried out, using mean and standard deviation or median and interquartile range according to whether they follow a normal distribution, evaluated by the Shapiro-Wilk test. The proportions will be reported by further calculating the 95% confidence interval.

3 INTRODUCTION

3.1 ETHICAL JUSTIFICATION OF THE RESEARCH.

The absence of clinical signs of pubertal maturation, i.e. pubertal delay, is a relatively frequent reason for consultation in boys. In cases where it is necessary, the treatment to be established is the administration of testosterone with the aim of provoking the development of secondary sexual characteristics and optimizing growth. Currently, the most commonly used treatment is empirical, with depot testosterone via IM at increasing doses over a period of 2 to 3 years. The most commonly used formulation, testosterone enanthate, comes in ampoules of 250 mg in 1 ml of vehicle. The usual off-label administration regimen begins with approximately 50-60 mg IM every 4 weeks (1/4 of the ampoule), increasing the dose by approximately 50-60 mg (1/4 ampoule) every 6 months until the full dose of 250 mg (1 ampoule) is reached every 4 weeks. The pharmacokinetic profile has not been described to see if it mimics the physiological progressive increase in testosterone levels occurring during spontaneous puberty. The pharmacokinetic profile of testosterone enanthate in adults shows supraphysiological serum testosterone concentrations the first week after medication is administered, with a progressive drop to subphysiological levels in the fourth week. In adults, testosterone enanthate has fallen into disuse, with testosterone undecanoate being used as a first-line medication. Its presentation in ampoules with 1000 mg in 4 ml, of IM administration every 12 weeks, is considered equivalent to testosterone enanthate 250 mg every 4 weeks. Testosterone undecanoate IM has not been tested in adolescents. In adults, the circulating levels of testosterone obtained with the formulation of testosterone undecanoate show a profile within physiological ranges, more stable between doses than that observed with testosterone enanthate. On the other hand, the presentation of testosterone undecanoate in ampoules of 4 ml facilitates the administration IM of 1/4 of ampoule compared to the formulation of testosterone enanthate that occurs in ampoules of 1 ml. Thus, it can be hypothesized that the use of testosterone undecanoate in its presentation in ampoules (1000 mg in 4 ml), properly dosed, would present the following benefits in the replacement therapy of adolescents with absence of adequate pubertal maturation, as compared to empirical treatment with testosterone enanthate currently used: 1) the concentration of circulating testosterone would show a more stable profile, within the physiological range; 2) IM administration would be more spaced: 1 time every 12 weeks instead of 1 time every 4 weeks; 3) dose fractionation would be easier and more accurate. Therefore, this project proposes to evaluate a regimen of administration of IM testosterone undecanoate in boys with pubertal delay.

3.2 THEORETICAL FRAMEWORK

3.2.1 Delayed puberty in the male

3.2.1.1 Definition, causes and epidemiology

Delayed puberty is defined as the absence of enlargement of the testicles in the male (≥ 4 ml, corresponding to Tanner stage G2) at an age that exceeds by 2 to 2.5 standard deviations that of the population average. In practice,

this means the existence of testicles < 4 ml at the age of 14 years (1, 2). It is accompanied by a lack of development of secondary sexual characteristics. Delayed puberty has a prevalence of approximately 2%, being a more frequent reason for consultation in men than in women (3, 4).

For practical purposes, the causes of pubertal delay can be divided into two large groups: (A) constitutional delay of growth and puberty (CDGP, also known as simple pubertal delay), a condition that is not strictly pathological but a simple delay in the maturation of the hypothalamic-pituitary-gonadal (HPG) axis and (B) pathological hypogonadism. The latter can be divided into two main categories: (B.1) primary hypogonadism (usually called hypergonadotrophic), and (B.2) secondary or central hypogonadism (hypogonadotrophic) (1, 2, 5). In turn, secondary or central hypogonadism can be: (B.2.a) functional, that is, due to a general disease that affects pituitary function and that reverses when the underlying disease is treated, or (B.2.b) persistent, either congenital or acquired (6, 7).

3.2.1.2 Diagnosis

The reason for consultation, along with an anamnesis and a physical examination that includes the careful evaluation of stature, weight and pubertal staging, is usually indicative. In the male, the biggest concern is usually short stature and small size of the genitals compared to their peers who have already started pubertal development. Puberty occurs at a crucial time of social and educational development. Delayed onset of puberty may have a greater impact on cognitive, psychological, and sociocultural changes as young people progressively take on adult roles. Males with pubertal delay manifest a related decreased quality of life, with increased anxiety and depressive symptoms. Patients complain about the bullying they suffer from their peers and tend to isolate themselves (8, 9).

The history provided by the pediatrician is of great help: nutritional and physical activity habits, previous illness and use of medications, as well as the evolution of height and weight in periodic check-ups. Some signs and symptoms or personal history should be sought as warning signs to guide or rule out the most frequent etiological diagnoses (Table 1).

In the physical examination, it is of the utmost importance to verify the standing and sitting height, as well as the arm span and genital development (correctly using the orchidometer in men to verify testicular volume less than 4 ml) and pubic hair, according to Tanner staging (10).

A history of cryptorchidism, micropenis and microorchidism at birth have a high predictive value for congenital hypogonadism. In boys with non-palpable testicles, the history of congenital anorchia, testicular regression syndrome or bilateral orchiectomy leads to a lack of endogenous testosterone production.

A meticulous analysis of the height and weight curves, as well as an X-ray to assess bone age are very useful in the first instance. If there is no relevant history, height and weight were always in the low percentiles (that is, the patient was always among the shortest among his peers) and bone age is 1 or 2 years behind, the diagnosis of CDGP is highly likely. If, on the other hand, the patient always grew within his mid-parental target height and

signs of body disproportion (eunuchoidism) begin to be noticed, with a bone age according to the chronological one, the diagnosis of hypogonadism is more likely. On the other hand, if the weight or the speed of growth fell abruptly, the cause of pubertal delay could be a condition of recent development, either general or endocrine, for example, Cushing syndrome or hypothyroidism (Table 1).

Table 1. "Red flags" in boys with pubertal delay.

Signs and symptoms	**Probable cause**
Abdominal pain, constipation, diarrhea, hematochezia Low weight for height, paleness, bloating	Inflammatory bowel disease, celiac disease
Excessive exercise, food restriction	Anorexia nervosa
Cold intolerance, fatigue, constipation Dry skin and hair, bradycardia, goiter	Hypothyroidism
Weight loss, heat intolerance, insomnia Low weight for height, exophthalmos, sweaty skin, tremor, hypertension, goiter	Hyperthyroidism
Weight gain, slowing growth Rounded facies, winey stretch marks, high blood pressure	Cushing syndrome
History of cranial radiation therapy	Acquired central hypogonadism
Headache, visual disturbances, seizures Visual field abnormalities, abnormal neurological examination	Acquired central hypogonadism (central nervous system tumor)
Bilateral cryptorchidism, micropenis, microorchidism Anosmia, hyposmia	Kallmann syndrome (isolated congenital central hypogonadism)
Bilateral cryptorchidism, micropenis, microorchidism without anosmia	Congenital central hypogonadism (isolated or multiple pituitary insufficiency)
Neonatal hypoglycemia and jaundice, postnatal growth deficiency	Multiple pituitary insufficiency
Visual disturbances, intellectual disability, seizures Midline defects (cleft lip, cleft palate, congenital heart disease), dysmorphic features (hypertelorism)	Congenital polymalformative syndrome (Septo-optic dysplasia, CHARGE syndrome, etc.)
Family history of delayed puberty	CDGP, congenital central hypogonadism
Family history of chronic diseases	Evaluate individually

Given the high prevalence of CDGP in boys without clinical guiding signs or symptoms, a "wait-and -see" strategy may be sufficient in patients up to 13 years of age, with growth and development control at 4 to 6 months, without the need for other diagnostic tests (6). If there are clinical data or informative history (e.g. of anorchia or hypogonadism), or the patient manifests psycho-social concern, it may be necessary to move forward with studies for diagnosis and to start testosterone therapy.

Among the initial endocrinological tests, the gonadal axis profile includes FSH, LH, testosterone, AMH, and inhibin B (2, 6, 11, 12). While primary hypogonadism as a cause of pubertal delay is rare, low values of testosterone, AMH and inhibin B, along with elevated gonadotropin values confirm the diagnosis. When LH and testosterone are in prepubertal values (13), FSH, inhibin B and AMH (as markers of Sertoli cells) may be helpful. If FSH and AMH or inhibin B are low, the diagnosis of central hypogonadism is likely (11, 12, 14). When the baseline laboratory is not informative, it may be necessary to use a dynamic test (14).

Other hormone measurements that may be useful are: prolactin to rule out hyperprolactinemia, a thyroid profile to rule out hypothyroidism, and plasma cortisol, to rule out adrenal insufficiency, or urinary free cortisol in case of suspected Cushing syndrome. All these evaluations allow to rule out a functional or reversible hypogonadism (6, 15).

3.2.2 Treatment of hypogonadism or pubertal delay in males

Medical behavior varies according to the etiological diagnosis. In functional hypogonadism the objective is to treat the underlying acute or chronic disease, generally not requiring replacement treatment with testosterone.

3.2.2.1 Testosterone Treatment Goals

In patients with CDGP or with persistent hypogonadism (including cases of anorchia), the main therapeutic objective of testosterone treatment is to achieve an adequate range of circulating testosterone to induce secondary sexual characteristics, and to optimize the height velocity, alleviate psychosocial discomfort and avoid the negative effect of steroid deficiency on the progression of bone mass.

The effects of sex steroids on target organs can be schematically classified into androgenic and anabolic. Androgenic effects include the development of secondary sexual characteristics and reproductive organs and the growth pattern of pilosebaceous follicles. Anabolic effects include increased muscle and skeletal mass which is associated with accelerating bone metabolism and growth.

Expectant management is acceptable in patients under 14 years of age in whom CDGP is suspected and who do not manifest psychic discomfort. However, testosterone therapy should not be delayed to the point of compromising final bone size and mass. In addition, treatment is usually necessary in patients between 12 and 14 years old who manifest psycho-social discomfort.

When the diagnosis of anorchia or hypogonadism has already been confirmed before pubertal age, the early onset of management of the situation has a double therapeutic and preventive objective. In these cases, treatment may be started around the age of 12 years in boys if the bone age is at least 11 years and the prognosis of adult height is consistent with the target size (2, 9, 15, 16). When the differential diagnosis with CDGP could not be established, the time needed to perform diagnostic tests coincides with the period of expectant observation to see if the first pubertal changes begin to be glimpsed. In cases of later diagnosis, testosterone treatment should be started as soon as possible (2, 9, 15, 16).

3.2.2.2 Treatment scheme

In adolescents with a confirmed diagnosis of anorchia or hypogonadism, or with CDGP, the most frequently used hormonal treatment is testosterone enanthate, cypionate or propionate esters. These compounds are prepared with

oil-based vehicles for slow-release IM injection (1, 9, 17, 18). Oral (19), transdermal (20) and subdermal (21) testosterone and oral oxandrolone have only been explored in research studies for some specific clinical situations (9, 18).

Testosterone replacement therapy in adolescents has not been approved by any regulatory body in the world. Empirically, it is usually started at low doses, with progressive increases in dose, with the aim of imitating the changes that occur throughout physiological puberty until the full development of the genitals and adult size is achieved. In patients with a confirmed diagnosis of primary (hypergonadotrophic) or central (hypogonadotrophic) hypogonadism, treatment is lifelong.

Among the side effects of testosterone treatment in adults, erythrocytosis, inhibition of gonadotropins with persistence of prepubertal testicular volume, persistent erections, gynecomastia, acne, nervousness, irritability and pain and swelling at the injection site are described (18, 22).

In Argentina, the only available formulation contains testosterone enanthate 250 mg in ampoules of 1 ml of oily solution. The half-life is approximately 4.5 days, and elimination occurs by hepatic metabolism and subsequent excretion of metabolites through the kidneys (90%) and bile (10%). In adults, a 250 mg (1 ml) IM ampoule is usually given every 2–4 weeks. On the other hand, testosterone undecanoate is also available in Argentina, and is marketed in 4 ml ampoules containing 1000 mg to be administered to adults via IM every 10-14 weeks (23). The difference in dose interval between testosterone enanthate and undecanoate is mainly due to a different pharmacokinetic profile, associated with a slower release of the undecanoate deposition site, which allows plasma concentrations to be achieved at physiological levels over longer periods (24).

3.3 PROBLEM IDENTIFICATION

All commercially available formulations of testosterone were designed for adults. Therefore, most treatment protocols used in adolescents are empirical and "*off-label*." As previously mentioned, testosterone enanthate is the only testosterone IM preparation commonly used in adolescents (9, 25), even though there are no pharmacokinetic studies in children or adolescents with this drug. In young adults with hypogonadism, a single injection of 250 mg of testosterone enanthate induced a rapid increase in serum testosterone, reaching even supraphysiological levels (700–1800 ng/dl or ~24–62 nmol/l) by day 7, decreasing to 260–700 ng/dl (~9–24 nmol/l) by day 14, and to infra-physiological levels (approximately 100–400 ng/dl or ~3.5–14 nmol/l) in most cases by day 21 (26). Interindividual variations in pharmacokinetics have been seen, depending on the patient's genomic *background* (27, 28). In adolescents, 0.25 ml of the ampoule (containing approximately 60 mg of testosterone enanthate) is applied per month at the beginning, progressively increasing by 0.25 ml every approximately 6 months until reaching the dose of 1 ml used in adults or (250 mg) (1, 2, 9, 22, 29).

Testosterone undecanoate exists as an oral formulation with a very low risk of hepatotoxicity; however, it is not widely available, has a short half-life, needs multiple daily doses and is not reliably absorbed, with variable effects,

so it has not had great acceptance for use in adolescents (9). On the other hand, the IM form of testosterone undecanoate has shown a more physiological pharmacokinetic profile than enanthate or cypionate in adults (30), with less frequent supraphysiologic levels in the first week and physiological levels for at least 10-12 weeks (24, 31). In clinical practice, the administration of 1000 mg of IM testosterone undecanoate every 10-12 weeks is equivalent to the use of 250 mg of IM testosterone enanthate every 3-4 weeks. Despite the advantages and acceptance shown by IM testosterone undecanoate in adults, its use in adolescents has not been tested until now (9).

3.4 HYPOTHESIS

The hypothesis of this work is that the initial IM administration of 1 ml (~250 mg) of testosterone undecanoate (1000 mg/4 ml) every 12 weeks for 6 months, with a progressive increase of 1 ml (~250 mg) every 6 months up to 4 ml (1000 mg) per dose is safe and effective in inducing normal progression of genital maturation and growth rate in boys with any cause of delayed puberty.

3.5 OBJECTIVE OF THE STUDY

The general objective of this study is to evaluate whether a quarterly administration regimen of IM testosterone undecanoate, in boys with anorchia, hypogonadism or pubertal delay induces the progression in the development of secondary sexual characteristics, the speed of growth and the acquisition of bone mass in accordance with physiological pubertal development, and maintain blood testosterone levels similar to those seen during spontaneous pubertal maturation.

The primary specific objectives are to determine, in boys with pubertal delay:

a. If a treatment regimen of testosterone undecanoate (1000 mg/4 ml) via IM, with an initial dose of 1 ml (~250 mg) every 12 weeks (stage 1: weeks 1-24), 2 ml (~500 mg) every 12 weeks (stage 2: weeks 25-48), 3 ml (~750 mg) every 12 weeks (stage 3: weeks 49-72) and 4 ml (100 0 mg) every 12 weeks (stage 4: weeks 73-96), is accompanied by progression of secondary sexual characteristics and height velocity commensurate with those of normal pubertal development.
b. The safety of the administration of increasing doses of IM testosterone undecanoate, through the evaluation of biochemical parameters (see below) and bone age progression.

The secondary specific objectives are:

c. To assess whether blood testosterone levels are maintained at levels within established physiological limits, for 12 weeks after administering the dose of testosterone undecanoate via IM.
d. To explore the changes occurring in the pituitary-gonadal axis and bone mass of boys with pubertal delay who are treated with increasing doses of IM testosterone undecanoate.

3.6 RELEVANCE

The verification of the efficacy and safety of the administration of IM testosterone undecanoate every 12 weeks to boys with anorchia, hypogonadism or CDGP will provide a standardized treatment regimen for conditions managed empirically so far, without proven pharmacokinetic bases.

4 SUBJECTS AND METHODS

4.1 DESIGN

An intervention study, with prospective longitudinal follow-up of a cohort of boys with anorchia, hypogonadism or CDGP, will be carried out in public tertiary care hospitals in the city of Buenos Aires.

4.2 POPULATION

The target population includes boys diagnosed with anorchia, hypogonadism, or CDGP requiring testosterone treatment.

All eligible patients who present consecutively until the sample size is completed will be invited to participate.

Inclusion criteria:

- Males between 13 and 18 years old without signs of pubertal development, or between 12 and 18 years old with a confirmed diagnosis of anorchia or hypogonadism.
- Normal male configuration of the external genitalia for Tanner G1 stage, with or without palpable testicles, as defined in 4.5.
- As appropriate, assent/consent of the patient and their parents or guardians.

Exclusion criteria

- Bone age < 11 years.
- Allergy to the medication of the study.

Termination criteria

- Testicular volume ≥ 10 ml at any follow-up visit.
- Acceleration of bone age of more than 1 year every 6 months of treatment: Δ bone age >12 months between visit 1 and visit 8, between visit 8 and visit 14, or between visit 14 and visit 20.

- Need for dose adjustment in 3 opportunities, as defined in 4.4.3.1.
- Erythrocytosis, defined as hematocrit > 54%.00
- Increased blood pressure above normal (>p90) for age and height.
- Priapism.
- Allergic reaction to the drug under study or to vehicle components.
- Serious adverse drug reaction or other adverse event that justifies it at the discretion of the principal investigator.

4.3 STUDY PROCEDURES

All study procedures will be recorded in medical records.

4.3.1 Enrolment

The Investigator or professionals participating in the study will inform the patient and their parents or guardians about the study and invite them to participate. The information will be described in the Information and Consent/Assent Form, answering the questions made by the patient or their parents/guardians. Only those patients who consent, with the consent or assent of their parents or guardians, and sign the corresponding forms, will be included in the study.

4.3.2 Selection visit

Visit 1: Informed assent and consent of the patient and his or her parents are obtained as appropriate, testicular volume and position of the testicles (as described in 4.3.5) shall be recorded after palpation and comparison with the Prader orchidometer, as well as genital and pubic hair development according to the Marshall and Tanner stages (10) and the existence of micropenis or any other anatomical or clinical sign evident on examination.

Once the inclusion and exclusion criteria have been verified, the following studies will be carried out: hand and wrist radiography for bone age calculation, bone mineral densitometry of the lumbar spine, blood count and laboratory routine (Table 2) and determination of AMH, estradiol, estrone, FSH, inhibin B, LH, SHBG and testosterone in blood (10 ml blood sample). The patient will be seen with the results within 3 months (Visit 2).

4.3.3 Inclusion visit

Visit 2: The results of bone age, blood count and laboratory routine and determinations of AMH, estradiol, estrone, FSH, inhibin B, LH, SHBG and testosterone in blood will be recorded. A physical examination will be performed to verify Tanner G1 genital development.

Patients who present any of the exclusion or termination criteria will be considered as selection failures and will not continue in the study.

A blood sample (2 ml) will be drawn for testosterone determination between 8 AM and 1 PM, and treatment with testosterone undecanoate (ampoule 1000 mg) will be started immediately by applying 1 ml (~250 mg) IM in the gluteal region.

4.3.4 Initial dose and gradual increase to full dose (Table 2)

4.3.4.1 Stage 1 (dose 1 ml), Visits 3 to 8, pharmacokinetics and clinical controls

Visit 3, period 1, week 1: 7 (± 2) days after visit 2, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone and SHBG.

Visit 4, period 1, week 6: 42 (± 4) days after visit 2, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone and SHBG.

Visit 5, period 1, week 12: 84 (± 4) days after visit 2, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone, FSH, LH, SHBG, AMH and inhibin B, and safety checks (blood count, chemistry and hepatic enzymes). A clinical examination will be performed, recording standing and sitting stature, weight, blood pressure, genital examination according to Tanner stages, and all relevant clinical data. Immediately afterwards, the dose of 1 ml (~250 mg) of testosterone undecanoate (ampoule 1000 mg) IM will be given in the gluteal region.

Visit 6, period 2, week 1: 7 (± 2) days after visit 5, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone and SHBG.

Visit 7, period 2, week 6: 42 (± 4) days after visit 5, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone, FSH, LH and SHBG.

Visit 8, period 2, week 12: 84 (± 4) days after visit 5, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone, FSH, LH, SHBG, AMH and inhibin B, and safety checks (blood count, chemistry and hepatic enzymes). X-ray of the left hand and wrist will be performed to determine bone age. A clinical examination will be performed, recording standing and sitting height, weight, blood pressure, genital examination according to Tanner stages, and all relevant clinical data. Immediately afterwards, the dose of 2 ml (~500 mg) of testosterone undecanoate (ampoule 1000 mg) IM will be given in the gluteal region.

4.3.4.2 Stage 2 (dose 2 ml), Visits 9 to 14, pharmacokinetics and clinical controls

Visit 9, period 3, week 1: 7 (± 2) days after visit 8, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone and SHBG.

Visit 10, period 3, week 6: 42 (± 4) days after visit 8, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone, FSH, LH and SHBG.

Visit 11, period 3, week 12: 84 (± 4) days after visit 8, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone, FSH, LH, SHBG, AMH and inhibin B, and safety checks (blood count, chemistry and hepatic enzymes). A clinical examination will be performed, recording standing and sitting height, weight, blood pressure, genital examination according to Tanner stages, and all relevant clinical data. Immediately afterwards, the dose of 2 ml (~500 mg) of testosterone undecanoate (ampoule 1000 mg) IM will be given in the gluteal region.

Visit 12, period 4, week 1: 7 (± 2) days after visit 11, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone and SHBG.

Visit 13, period 4, week 6: 42 (± 4) days after visit 11, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone, FSH, LH and SHBG.

Visit 14, period 4, week 12: 84 (± 4) days after visit 11, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone, FSH, LH, SHBG, AMH and inhibin B, and safety checks (blood count, chemistry and hepatic enzymes). X-ray of the left hand and wrist will be performed to determine bone age and lumbar spine densitometry. A clinical examination will be performed, recording standing and sitting height, weight, blood pressure, genital examination according to Tanner stages, and all relevant clinical data. Immediately afterwards, the dose of 3 ml (~750 mg) of testosterone undecanoate (ampoule 1000 mg) IM will be given in the gluteal region.

Table 2. Visits after testosterone undecanoate is administered.

Stage	**Visit**	Period	Week	**Laboratory**	**Other**
1	**3**	1	1	Testosterone, E2, E1, SHBG	
	4		6	Testosterone, E2, E1, SHBG	
	5		12	Blood count, hepatic enzymes, chemistry Testosterone, E2, E1, SHBG, LH, FSH, AMH, inhibin B	Standing and sitting height, weight, BP Genital examination 1 ml Undecanoate Testosterone
	6	2	1	Testosterone, E2, E1, SHBG	
	7		6	Testosterone, E2, E1, SHBG	
	8		12	Blood count, hepatic enzymes, chemistry Testosterone, E2, E1, SHBG, LH, FSH, AMH, inhibin B	Standing and sitting height, weight, BP Genital examination Bone age 2 ml Undecanoate Testosterone
2	**9**	3	1	Testosterone, E2, E1, SHBG	
	10		6	Testosterone, E2, E1, SHBG	
	11		12	Blood count, hepatic enzymes, chemistry	Standing and sitting height, weight, BP Genital examination

				Testosterone, E2, E1, SHBG, LH, FSH, AMH, inhibin B	2 ml Undecanoate Testosterone
	12	4	1	Testosterone, E2, E1, SHBG	
	13		6	Testosterone, E2, E1, SHBG	
	14		12	Blood count, hepatic enzymes, chemistry Testosterone, E2, E1, SHBG, LH, FSH, AMH, inhibin B	Standing and sitting height, weight, BP Genital examination Bone age, lumbar spine densitometry 3 ml Undecanoate Testosterone
3	**15**	5	1	Testosterone, E2, E1, SHBG	
	16		6	Testosterone, E2, E1, SHBG	
	17		12	Blood count, hepatic enzymes, chemistry Testosterone, E2, E1, SHBG, LH, FSH, AMH, inhibin B	Standing and sitting height, weight, BP Genital examination 3 ml Undecanoate Testosterone
	18	6	1	Testosterone, E2, E1, SHBG	
	19		6	Testosterone, E2, E1, SHBG	
	20		12	Blood count, hepatic enzymes, chemistry Testosterone, E2, E1, SHBG, LH, FSH, AMH, inhibin B	Standing and sitting height, weight, BP Genital examination Bone age 4 ml Undecanoate Testosterone
4	**21**	7	1	Testosterone, E2, E1, SHBG	
	22		6	Testosterone, E2, E1, SHBG	
	23		12	Blood count, hepatic enzymes, chemistry Testosterone, E2, E1, SHBG, LH, FSH, AMH, inhibin B	Standing and sitting height, weight, BP Genital examination 4 ml Undecanoate Testosterone
	24	8	1	Testosterone, E2, E1, SHBG	
	25		6	Testosterone, E2, E1, SHBG	
	26		12	Blood count, hepatic enzymes, chemistry Testosterone, E2, E1, SHBG, LH, FSH, AMH, inhibin B	Standing and sitting height, weight, BP Genital examination Bone age, lumbar spine densitometry

E2: estradiol; E1: estrone; BP: blood pressure.

4.3.4.3 Stage 3 (dose 3 ml), Visits 15 to 20, pharmacokinetics and clinical controls

Visit 15, period 5, week 1: 7 (± 2) days after visit 14, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone and SHBG.

Visit 16, period 5, week 6: 42 (± 4) days after the visit 14, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone, FSH, LH and SHBG.

Visit 17, period 5, week 12: 84 (± 4) days after visit 14, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone, FSH, LH, SHBG, AMH and inhibin B, and safety checks (blood count, chemistry and hepatic enzymes). A clinical examination will be performed, recording standing and sitting height, weight, blood pressure, genital examination according to Tanner stages, and all relevant clinical data. Immediately afterwards, the dose of 3 ml (~750 mg) of testosterone undecanoate (ampoule 1000 mg) IM will be given in the gluteal region.

Visit 18, period 6, week 1: 7 (± 2) days after visit 17, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone and SHBG.

Visit 19, period 6, week 6: 42 (± 4) days after visit 17, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone, FSH, LH and SHBG.

Visit 20, period 6, week 12: 84 (± 4) days after the visit 17, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone, FSH, LH, SHBG, AMH and inhibin B, and safety checks (blood count, chemistry and hepatic enzymes). X-ray of the left hand and wrist will be performed to determine bone age. A clinical examination will be performed, recording standing and sitting height, weight, blood pressure, genital examination according to Tanner stages, and all relevant clinical data. Immediately afterwards, the dose of 4 ml (~1000 mg) of testosterone undecanoate (ampoule 1000 mg) IM will be given in the gluteal region..

4.3.4.4 Stage 4 (dose 4 ml), Visits 21 to 26, pharmacokinetics and clinical controls

Visit 21, period 7, week 1: 7 (± 2) days after the visit 20, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone and SHBG.

Visit 22, period 7, week 6: 42 (± 4) days after the visit 20, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone, FSH, LH and SHBG.

Visit 23, period 7, week 12: 84 (± 4) days after the visit 20, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone, FSH, LH, SHBG, AMH and inhibin B, and safety checks (blood count, chemistry and hepatic enzymes). Immediately afterwards, the second dose of 4 ml (~1000 mg) of testosterone undecanoate (ampoule 1000 mg) IM will be given in the gluteal region..

Visit 24, period 8, week 1: 7 (± 2) days after the visit 23, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone and SHBG.

Visit 25, period 8, week 6: 42 (± 4) days after the visit 23, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone, FSH, LH and SHBG.

Visit 26, period 8, week 12: 84 (± 4) days after the visit 23, a blood sample (5 ml) will be drawn for measurement of testosterone, estradiol, estrone, FSH, LH, SHBG, AMH and inhibin B, and safety checks (blood count, chemistry and hepatic enzymes). X-ray of the left hand and wrist will be performed to determine bone age and lumbar spine densitometry. A clinical examination will be performed, recording standing and sitting height, weight, blood pressure, genital examination according to Tanner stages, and all relevant clinical data. The patient will be informed that he has reached the end of the study and that he will continue his usual clinical follow-up, during which he will be informed of the results of the studies carried out at this last visit.

At all visits, the physical examination and bone age assessment will be performed by a member of the research team who is unaware of the dose of testosterone undecanoate the patient is receiving.

4.3.5 Processing of blood samples

Blood samples will be processed for hormonal study as described below (4. 3.5.1 and 4.3.5.2). After the hormonal determinations have been made, the blood samples will be kept until the end of the study in a freezer at -80°C, under the responsibility of the Principal Investigator at CEDIE. After the stipulated deadlines, the samples will be destroyed, following the procedures of CEDIE.

4.3.5.1 Hormone assays

All hormone determinations are performed in the Laboratory of the Division of Endocrinology of the Ricardo Gutiérrez Children's Hospital, with the following methodology.

***Testosterone, estrone and estradiol*:** Mass spectrometry. 1) HPLC conditions: Shimadzu Nexera SIL-30 UHPLC system, Phenomenex Kinetex C18 (150x0.3 mm, 2.6 mm) system. 2) Tandem mass spectrometry conditions: Sciex Triple QuadTM 6500+ spectrometer. 3) Data analysis: Software Analyst and Multiquant (32).

***LH and FSH**:* Electro-chemiluminescence immunoassays (ECLIA, Roche Diagnostics GmbH, Mannheim, Germany) as described (33). The limits of quantification of both are 0.10 IU/L, according to the second NIBSC IS 80/552 for LH and the second WHO IRP 78/549 for FSH. The intra- and inter-assay coefficients of variation are 1.1% and 1.8% for LH, respectively, for an average LH concentration of 2.80 IU/L and 1.4% and 1.5% for an average concentration of 16.90 IU/L. The intra- and inter-assay coefficients of variation are 1.0% and 4.2% for FSH, respectively, for an average FSH concentration of 14.80 IU/L and 1.1% and 4.1% for a mean FSH concentration of 23.40 IU/L. When LH or FSH levels are undetectable, they are assigned the limit *of quantification* value (functional sensitivity = 0.05 IU/L).

AMH: Specific enzyme-immunoassay for human AMH (EIA AMH/MIS®, BeckmanCoulter), as described (13, 33). The intra- and inter-assay coefficients of variation are, respectively, 10.5% and 9.4% for a serum AMH concentration of 700 pmol/L, and 11.1% and 12.8% for a serum concentration of 7 pmol/L. When the serum concentration of AMH is undetectable, the value of the limit of quantification *(functional sensitivity=2.5 pmol/L)* is attributed.

Inhibin B: Specific enzyme-immunoassay for inhibin B (Inhibin B GEN II ELISA, A81303, BeckmanCoulter).

SHBG: chemiluminescence assay (Immulite, Siemens) with sensitivity of 5.0 nmol/l and intra-assay coefficients of variation <4.0% and inter-assay <8.2% (34).

The laboratory results will be processed and reported by a member of the research team who does not know the dose of testosterone undecanoate that the patient is receiving.

4.3.5.2 Routine laboratory:

The samples will be processed in the Central Laboratory of the Ricardo Gutiérrez Children's Hospital.

Blood count: includes red blood cell count, white blood cell count and relative formula, hematocrit, hemoglobin concentration.

Blood chemistry: Blood glucose, urea and creatinine in blood.

Hepatic tests: Total and Direct Bilirubin, GPT, GOT, Alkaline Phosphatase, Total Proteins, Albumin, Triglycerides, Total Cholesterol, HDL and LDL.

4.3.6 Imaging studies

Bone age: X-ray of the hand and left wrist. The interpretation of bone age will be performed by comparison with the atlas of Greulich and Pyle by a pediatric endocrinologist, member of the working team, but blinded (nowhich patient it is).

Bone mineral density (BMD):The BMD of the lumbar spine will be measured by a dual-energy X-ray absorptiometry (DXA) scan, on a Lunar Prodigy (GE Healthcare) equipment.

4.4 PREDICTOR VARIABLES AND OUTCOME MEASURES

4.4.1 Primary outcome variables

4.4.1.1 Progression in the development of secondary sexual characteristics

Tanner genital (G) and pubic hair (VP) stages will be reported at the visits as indicated in Table 2.

Progression of pubertal stages will be considered as in line with normal maturation if the clinical evaluation detects Tanner G2 or G3 and VP2 or VP3 at the end of stage 1 (visit 8), G3 or G4 and VP3 or VP4 at the end of stage 2 (visit 14), G3 to G5 and VP3 to VP5 at the end of stage 3 (visit 20), and G4 or G5 and VP4 to VP6 at the end of stage 4 (visit 26). The proportion of patients with progression as hypothesized will be calculated. The therapeutic regimen shall be considered satisfactory if this proportion is ≥80%.

4.4.1.2 Progression in height velocity

The height velocity, calculated as Δ stature (cm)/Δ time (months), shall be reported in the visits as indicated in Table 2.

Progression will be considered as in line with normal growth if the clinical evaluation detects a height velocity of 6 to 10 cm/year at the end of stage 2 (visit 14) and 8 to 14 cm/year at the end of stage 4 (visit 26). The proportion of patients with progression as hypothesized will be calculated. The therapeutic regimen shall be considered satisfactory if this proportion is ≥80%.

4.4.2 Safety variables

The number and type of adverse events at each visit will be reported.

The Δ bone age (months)/Δ chronological age (months) will be reported in the visits as indicated in Table 2. Bone age will be considered to be accelerated if an increase of more than 12 months is found for every 6-month period of treatment, between visit 1 and visit 8, between visit 8 and visit 14, or between visit 14 and visit 20.

4.4.3 Secondary outcome variables

4.4.3.1 Testosterone concentration in serum

The values of serum testosterone concentration will be reported as indicated in Table 2. Values will be considered to be within the target range if they are between 20 and 80 ng/dl in visits 4 and 7, between 100 and 180 ng/dl in visits 10 and 13, between 200 and 300 ng/dl in visits 16 and 19 and between 350 and 900 ng/dl in visits 22 and 25.

The mean and standard deviation or median and range of serum testosterone in the patient cohort will be calculated for each visit. The proportion of patients with values in range, below and above it will be estimated for each visit.

In the event that serum testosterone concentrations are lower than expected, the treatment regimen will not be modified since it does not jeopardize the patient's safety. The only difference with the expected outcome in this case would be that it would take more than two years to achieve full maturation of pubertal signs, which would be no different from what can be observed in normal development (up to 4 or 5 years (10)).

In case serum testosterone concentrations are >50% of the maximum established at 6 weeks of administration, the dose of testosterone undecanoate will be adjusted as follows:

a. If it occurs at visit 4, the dose will be 0.5 ml at visit 5, 1 ml at visits 8 and 11, 2 ml at visits 14 and 17 and 3 ml at visits 20 and 23. Further dose adjustment shall be made if the serum testosterone concentration exceeds by >50% the maximum concentration established for the dose .
b. If it occurs at visit 7, the dose will be 1 ml at visits 8 and 11, 2 ml at visits 14 and 17 and 3 ml at visits 20 and 23. Further dose adjustment shall be made if the serum testosterone concentration exceeds by >50% the maximum concentration established for the dose.

c. If it occurs at visit 10, the dose will be 1 ml at visit 11, 2 ml at visits 14 and 17 and 3 ml at visits 20 and 23. Further dose adjustment shall be made if the serum testosterone concentration exceeds by >50% the maximum concentration established for the doses.

d. If it occurs at visit 13, the dose will be 2 ml at visits 14 and 17 and 3 ml at visits 20 and 23. Further dose adjustment shall be made if the serum testosterone concentration exceeds by >50% the maximum concentration established for the doses.

e. If it occurs at visit 16, the dose will be 2 ml at visit 17 and 3 ml at visits 20 and 23. Further dose adjustment shall be made if the serum testosterone concentration exceeds by >50% the maximum concentration established for the doses.

f. If it occurs at visit 19, the dose will be 3 ml at visits 20 and 23. Further dose adjustment shall be made if the serum testosterone concentration exceeds by >50% the maximum concentration established for the doses.

g. If it occurs at visit 22, the dose will be 3 ml at visit 23.

h. Up to 2 dose adjustments will be accepted; in case of need for a third adjustment, the patient will be withdrawn from the study, sending the report to his attending physician so that he continues the follow-up and treatment in accordance with his usual practice. The proportion of patients requiring dose adjustment for each dose administered (1, 2, 3 or 4 ml) and the proportion of patients who had to be withdrawn from the study shall be reported. An adjustment at less than 30% of doses or a withdrawal from the study in less than 10% of patients will be considered acceptable.

4.4.3.2 Progression in the acquisition of bone mass

The bone mineral density of the lumbar spine L1-L4 in g/cm^2 and z-scores will be reported as indicated in Table 2. The mean and standard deviation or median and range of z-score in the patient cohort for each visit will be calculated. The proportion of patients with Z-score values <-2 for each visit will be estimated.

4.4.3.3 Hormone levels

Changes in the values of LH, FSH, AMH, inhibin B and estradiol will be reported as indicated in Table 2. The mean and standard deviation or median and range of serum hormone concentration in the cohort of patients will be calculated for each visit. The analysis will be stratified according to the diagnosis of anorchia, primary hypogonadism, central hypogonadism and CDGP.

4.5 DEFINITIONS

Signs of pubertal development: absence of signs of pubertal development shall be considered when:

a. Both testicles of volume < 4 ml (measurement by orchidometer); or
b. Volume of a testicle < 6 ml, stable for at least 6 months, in males with monorchia or with the atrophic contralateral testicle (≤ 1 ml) or not palpable in the scrotum; or
c. Tanner stage G1 genital development, in males without palpable testicles.

Normal male configuration of the external genitalia for Tanner G1 stage: Penis of any length (patients with micropenis or those with penile development due to previous treatment with androgens), with urethral orifice at the tip (no history of hypospadias), scrotum with normal central raphe (not bifid), with or without palpable testicles.

Anorchia: males without palpable testicles, with undetectable serum values of AMH or inhibin B before initiation of testosterone treatment.

Primary hypogonadism: Serum values of testicular hormones below Tanner G1 stage reference values: AMH <257 pmol/L (13) or inhibin B < 100 ng/mL (35) and FSH >1.2 IU/L (14, 33) prior to initiation of testosterone therapy.

Central hypogonadism: LH <5.8 IU/L in GnRH test (14) before initiation of testosterone treatment.

Erythrocytosis: Hematocrit > 54%.

Above normal blood pressure: Systolic or diastolic blood pressure > 90th percentile for sex, age and height (36).

4.6 CALCULATION OF SAMPLE SIZE AND STUDY DURATION

Estimating that 80% of patients will have developed secondary sexual characteristics (G and VP Tanner stages) within the expected range after the administration of 1, 2, 3 and 4 ml of testosterone undecanoate via IM every 12 weeks, it will be necessary to analyze 27 individuals to reach a conclusion with an accuracy of 15%. Accepting a maximum loss of 20% during follow-up, 34 patients should be recruited into the study.

4.7 STATISTICAL ANALYSIS

A descriptive statistical report of the quantitative variables will be made, using mean and standard deviation or median and interquartile range according to whether they follow a normal distribution, evaluated by the Shapiro-Wilk test. The proportions will be reported by further calculating the 95% confidence interval.

Statistical analyses will be performed using GraphPad Prism version 7.03 for Windows (GraphPad Software, San Diego, CA, USA) and STATA 13 (StataCorp LLC, College Station, TX, USA).

4.8 ETHICAL CONSIDERATIONS

All the procedures of this study will be carried out in strict compliance with the ethical standards for human research, listed in the Declaration of Helsinki of the World Medical Association, Law 3301/09 (Protection of Rights of Subjects in Health Research) of the Autonomous City of Buenos Aires and its decree 58/2011, and the regulatory provision 6677/2010 of ANMAT (Administración Nacional de Medicamentos, Alimientos y Tecnología Médica, Argentina) referring to clinical pharmacology studies . The confidentiality of each individual will be ensured using an identification code for each participant, as well as compliance with the Personal Data Protection Law n° 25.326 (Argentina). Only those patients who have consented to participate and their parents or guardians have given their written consent will be included in the study. The study protocol was evaluated by the IRB of the participating hospitals.

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