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Testicular Dysfunction in 47,XXY Boys: When It All Begins. A Semilongitudinal Study

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Abstract

Objective: Klinefelter syndrome is the most common chromosomal disorder in males and the most common cause of hypergonadotropic hypogonadism. We describe the natural history of testicular dysfunction in patients with Klinefelter syndrome through the integration of clinical, hormonal, and quantitative ultrasound data in a life-course perspective.

Design: Prospective semilongitudinal study.

Methods: We included 155 subjects with 47,XXY karyotype (age range: 7 months-55 years) naïve to testosterone replacement therapy. Subjects were divided according to pubertal stage and age group (transition age and adults). Serial clinical, hormonal, and testicular ultrasound (US) assessments were performed.

Results: Testicular development progresses until Tanner stage 4, with subsequent regression, whereas Sertoli and germ cell impairment is not hormonally detected before Tanner stages 3-4, as reflected by normal inhibin B values until stage 4 and the fall in the inhibin B/follicle-stimulating hormone ratio thereafter. The testosterone/luteinizing hormone ratio peaks during Tanner stages 2-3 and declines from Tanner stage 4 onward, preceding the development of overt hypogonadism. US echotexture progressively worsens until transition age, reflecting ongoing gonadal compromise, whereas quantitative US echotexture measures and the presence of both hypoechoic lesions and microlithiasis independently and significantly predict a lower circulating testosterone level.

Conclusions: The findings from this large prospective study contribute to our understanding of the natural history of testicular dysfunction in Klinefelter syndrome, underlining the importance of quantitative testicular US in infancy and childhood, as well as during pubertal development and transition age, for the optimal care of Klinefelter syndrome patients.

Key Words: Klinefelter syndrome, puberty, testicular dysfunction, hypogonadism, bilateral testicular volume, testicular ultrasound

Klinefelter syndrome (KS) was first described by Harry Klinefelter in 1942 (1) as a condition characterized by gynecomastia, small and firm testes, hyalinization of the seminiferous tubules, and azoospermia. It represents the most common sex chromosome aneuploidy in males, with an unadjusted occurrence of the classic genotype among prenatal diagnoses of approximately 1 every 520 male children (2-4), caused by the presence of one supernumerary X chromosome, usually acquired through nondisjunction events during maternal or paternal gametogenesis. The classic form (47,XXY) characterizes 80% to 90% of all KS patients, while other less common genotypes are also included under the KS terminology, although presenting with 46,XY/47,XXY mosaicism or structural X chromosome abnormalities (5, 6).

While the diagnosis rate has risen in recent decades (7), the true prevalence of KS is still thought to be underestimated,

probably due to its considerable phenotypic variation and lack of awareness among general practitioners (8-11). Its clinical expression varies significantly from patient to patient, and it may present with several subtle age-related clinical signs. However, in recent years, noninvasive prenatal testing during pregnancy has led to an increase in early diagnosis (12), thus enabling timely treatment and helping prevent possible complications in childhood, adolescence, and adulthood, such as neurodevelopmental dysfunctions, learning difficulties, and behavioral problems, as well as complications of hypogonadism (13).

The key clinical feature of KS is represented by testicular impairment with small testes, hypergonadotropic hypogonadism, and, consequently, primary infertility (4). The underlying mechanism responsible for the testicular degeneration is still not completely understood, although the influence

of the additional X chromosome seems clear. The most plausible hypotheses include a primary effect of the extra X chromosome on germ cell development and function or an adverse influence on the supporting somatic Sertoli and/or Leydig cells, with consequent alteration of testicular endocrine function, possibly due to lack of X-inactive specific transcript transcription and/or PAR1 gene overexpression (14, 15). The testes of KS subjects evolve toward seminiferous tubule hyalinization and fibrosis, leading to spermatogenetic failure. Recent studies, by our group and others, also demonstrated impaired testicular vascularization in KS patients (16), which affects peripheral testosterone (T) release (17).

Foci of residual function in seminal tubules are present in a proportion of subjects, and for this reason some have advocated early (micro)testicular sperm extraction to retrieve spermatozoa (or spermatids) immediately after pubertal completion or in the transition age period (18). However, there are insufficient data to establish the temporal dynamic of testicular failure in KS, with histological reports indicating a very early impairment of the spermatogenetic niche (19-22). According to seminal study by Aksglaede et al, testicular deterioration is already apparent during fetal life, progresses during infancy and childhood with a gradual degeneration of germ cells, and peaks from mid-puberty to adulthood, by which time the testes display appreciable seminiferous tubule hyalinization, associated with Leydig and Sertoli cell hyperplasia (19). Further studies confirmed the early disorder of either Leydig (23, 24) or Sertoli cells (25, 26). Nonetheless, recent evidence suggests that endocrine testicular function in KS children is normal, with no hormonal evidence of testicular dysfunction between mini-puberty and late prepuberty (27). In this regard, the serial evaluation of hormonal dynamics, ultrasound (US) testicular assessments via the measurement of testicular volume, and the appraisal of testicular echotexture and echogenicity can provide significant information on gonadal function (28). To our knowledge, no study has comprehensively investigated these features in KS so far.

The purpose of this large, semilongitudinal study is to describe the clinical, endocrine, and testicular US morphofunctional patterns in KS from infancy through pubertal development and the transition age, into adulthood, and to explore whether these features could reliably and precisely reflect the natural history of testicular degeneration in these patients.

Materials and Methods

Study Population

The study population included 155 KS patients with a classic karyotype (47,XXY), ages 7 months to 55 years, consecutively selected among those who performed a medical examination in the pediatric (Pediatric Department) or adolescent and adult (Department of Experimental Medicine, Section of Endocrinology and Andrology) endocrine outpatient clinics at Policlinico Umberto I, Hospital of Rome from January 2012 to July 2021. Apart from age, the sole inclusion criterion was 47,XXY KS diagnosed by peripheral leukocyte karyotyping on at least 30 metaphases. For subjects diagnosed prenatally, karyotyping was repeated after birth for confirmation. The exclusion criteria were (1) presence of mosaic karyotypes, other known genetic conditions, or chromosomal abnormalities; (2) any endocrine and metabolic-active drugs acting on the hypothalamic-pituitary-gonadal (HPG)

axis or potentially interfering with gonadal function tests (including T therapy); (3) previous pituitary gland surgery or radiotherapy. Infants younger than 7 months of age were also excluded, to avoid any potential influence of mini-puberty on gonadal function (29).

Ethical Approval

This study was approved by the Ethics Committee of Policlinico Umberto I (Rif. CE 6478, Prot. no. 1038) and was conducted in accordance with the Declaration of Helsinki. Each patient old enough to provide their assent or their parents (if the patient was under the age of consent), signed a complete written informed consent before the study.

Study design

We designed a prospective semilongitudinal study in which patients were enrolled at first visit and were followed thereafter until either (1) their last available observation, (2) they required T therapy commencement (according to exclusion criteria), or (3) they were lost at follow-up. As such, all patients were naïve to T replacement therapy.

Patients had been diagnosed before entering the study and were clinically evaluated at regular outpatient visits. For children diagnosed prenatally, at least one evaluation was performed in the prepubertal stage and one evaluation every 6 months from the onset of puberty. Medical history was recorded, and anthropometric measures were assessed at each visit. A general and genital physical examination was performed, with pubertal stage evaluated according to Tanner original description for external genitalia appearance (ie, scrotum, penis, pubic hair) (30, 31). Blood tests for hormonal assays and testicular US were performed at each evaluation. Transition age was defined as the phase between the end of puberty and young adulthood: therefore, we included in the transition age group subjects who had achieved Tanner stage V and completed linear growth (32, 33); subjects ages 26 years and older were included in the adult group.

Hormonal Evaluation

Pituitary and gonadal function were studied by measurement of the following hormones: follicle stimulating hormone (FSH), luteinizing hormone (LH), total T, 17β-estradiol (E2), sex hormone-binding globulin (SHBG), and inhibin B (INHB). Blood samples were obtained via antecubital venous puncture in the early morning (7:30-9:30 AM) after overnight fasting. Samples were centrifuged, and the serum was immediately frozen at −20°C. FSH, LH, T, E2, and SHBG levels were measured in duplicate using the chemiluminescent microparticle immunoassay (Architect System, Abbott Laboratories, IL, USA, catalog no. 7K75-25, RRID:AB_2813910; catalog no. 2P40-25, RRID:AB_2813909; catalog no. 2P13, RRID: AB_2895254; catalog no. 7K72-25, RRID:AB_2813911; and catalog no. 8K26, RRID:AB_2895255, respectively) with limits of detection of 0.07 mIU/mL, 0.05 mIU/mL, 0.1 nmol/L, 10 pg/mL, and 0.28 nmol/L, respectively. The intra- and interassay coefficients of variation were as follows: 3.8% and 5.5% at 4.1 mIU/mL (LH), 3.6% and 5.4% at 3.2 mIU/mL (FSH), 2.1% and 3.6% at 10.08 nmol/L (T), 5% and 7% at 190 and 600 pg/mL (E2), and 5.65% and 9.54% at 8.8 nmol/L (SHBG), respectively. Serum concentrations of INHB were measured by enzyme-linked immunosorbent assay (GEN II, Beckman Coulter Laboratories, USA,

catalog no. A81303, RRID:AB_2827405) (limit of detection 4.0 pg/mL, intra- and interassay coefficients of variation 3.3% and 7.2% at 122 pg/mL). The laboratory reference ranges at our laboratory are as follows: LH <1 mIU/mL (prepubertal) and 1.8-8.16 mIU/mL (adults); FSH <1 mIU/mL (prepubertal) and 1.38-9.58 mIU/mL (adults); T < 3.2 nmol/L (prepubertal) and 10.4-38.2 nmol/L (adults); E2 25-107 pg/mL; SHBG 11.2-78.1 nmol/L; INHB 80-380 pg/mL.

We used Vermeulen's formula to calculate the free T concentrations (cfT) from T and SHBG levels using a fixed albumin level of 4.3 g/dL (34). We calculated the T/LH ratio (T/LH), a marker of Leydig cell function (35, 36), and the inhibin B/FSH ratio (INHB/FSH), a marker of Sertoli cell function (37) and of the presence or absence of meiotic and post meiotic germ cells that contribute to INHB production (38).

Ultrasound Evaluation

Testicular US was performed using a Philips IU22 unit (Philips, Bothell, WA, USA) with a 7-15 MHz wideband linear transducer. The standardized protocol included axial and transverse examinations (39). All US examinations were performed by 2 experienced operators. Right and left testicular volumes were calculated using Lambert's formula height × width × length [cm] × 0.71, given its superiority in determining testicular volume in prepubertal testes (40), and expressed in mL. According to the European Academy of Andrology (EAA) US study on healthy, adult, fertile men, the mean reference range was considered between 11.8 and 24.4 mL when using the Ellipsoid formula (height × width × length [cm] × 0.52) and between 16.0 and 33.0 mL when using the Lambert formula (41). The total testicular volume (TTV) was then calculated (right + left volume). Images and loops were stored for subsequent analysis in a local image archiving system (eFilm Merge, Milwaukee, WI, USA) for a blind evaluation by a third operator, to increase the standardization of image interpretation. Testicular parenchymal echogenicity and testicular echotexture were evaluated by texture analysis, a member of the texture-based radiomics feature family, using the free software ImageJ (version 1.51j8, National Institutes of Health, Bethesda, MD, USA). This is a quantitative set of metrics computed to quantify the textural patterns of images and converts radiological images into a mineable matrix of automatically extracted data from characterization algorithms (42). Therefore, a quantitative evaluation of the images is allowed (43). The gray level co-occurrence matrix (GLCM) (44) was created from a 60×60 (or 50×50 in some prepubertal children) pixel square representative of the entire testis, selected as region of interest, obtained from both testes, and transformed into a grey-scale 8-bit image, employing the free ImageJ plug-in "Texture Analyzer" (version 0.4, available at: https://imagej.nih.gov/ij/plugins/texture.html, developed by Julio E. Cabrera) with the size of the step in pixels set to 1 and the direction set to 0°. Through this process, we obtained the second-order features homogeneity (or inverse difference moment), which assesses image homogeneousness and tightness of distribution of the matrix values (higher values describe an image containing larger homogeneous patches), and entropy, which measures disorder and randomness of all pixel intensity and is indicative of the complexity within the image (higher values indicate an inhomogeneous distribution of pixels).

Echogenicity was assessed by image gray-level histogram quantification. This function calculates and displays a histogram of the distribution of gray values in the active region of interest and provides a more detailed investigation of the testis because it evaluates the number of pixels, the brightness (intensity), and the gray scale of the user-defined area, with lower values for tissue hypoechogenicity. Testicular microlithiasis were graded as absent or present starting from at least 5 microcalcifications per US scan. The presence or absence of hypoechoic solid testicular lesions was also evaluated. Finally, the presence or absence of varicocele was recorded.

Statistical Analysis

Data are expressed as mean and SD or median and 25% to 75% interquartile range, as appropriate. Data distribution was visually inspected by analysis of the histograms and normality plots.

To account for the semilongitudinal nature of the data, we employed a general linear mixed model approach, using a first-order autoregressive structure with heterogenous variances as covariance structure (to account for increasing and diverging variances in outcome variables with increasing age groups), and restricted maximum likelihood method, to explore repeated-measures differences in outcome variables, with hormones, TTV, and US features acting as dependent variables, stage (prepubertal, Tanner stage I through V, transition age, and adulthood) acting as a fixed effect, and patient ID acting as a random effect. We further conducted post hoc pairwise comparisons of main effects, reporting Šidák-adjusted significance values for multiple comparisons, to determine the stages at which significant longitudinal changes occurred. Categorical variables were examined by chi-square or Fisher's exact tests. Partial correlations with bootstrapping on 2000 samples were conducted to explore the relationship between hormonal parameters and quantitative echogenicity and echotexture parameters, controlling for pubertal stage and age group; two-tailed significance levels and 95% bias-corrected accelerated confidence intervals are reported. A binomial logistic regression was used to ascertain the influence of hormones on testicular US features. The P-values were two-sided for all statistical tests, and $P < .05$ was considered statistically significant.

All statistical analyses were performed with SPSS Statistics version 27.0 (IBM SPSS Statistics Inc., Chicago, IL, USA) and Prism (version 9.0, GraphPad Software, LLC).

Results

Between January 2012 and July 2021, 180 consecutive patients ages 7 months to 55 years were screened to enter the study. None of the younger patients showed any HPG axis activation. Twenty-two patients with mosaicism at the peripheral blood karyotype and 3 subjects with translocation were excluded. The study therefore included 155 subjects with classic karyotype (47,XXY). The main reasons for referral were prenatal diagnosis (67.4%), followed by delayed puberty (22.1%), infertility evaluation (6.6%), and general andrological screening (3.9%). Cryptorchidism was reported in 13.2% of patients and was bilateral in half of them. Twelve boys underwent surgical orchidopexy between 0 and 24 months, while the remainder had spontaneous testicular descent by the age of 1 year. None of the patients had micropenis or hypospadias. Gynecomastia was found in 14.3% and was

bilateral in 84% of these. Anthropometric measurements and hormone and US features are shown in Tables 1 and 2.

Hormone Assessment According to Pubertal Status

There were statistically significant longitudinal differences in hormone values assessed across different pubertal stages and age groups evaluated via general linear mixed model analyses. All reported effects are significant at $P < .001$ for the overall model. Hormonal values through the various growth stages are shown in Figures 1 and 2 and in Table 1.

Prepuberty and puberty

Post hoc analyses confirmed statistically significant differences in hormone values in KS children at Tanner stage 1 and across pubertal development: all values were lower during prepuberty, except for SHBG and the INHB/FSH ratio, which were highest in these subjects (Table 1). There was a slight increase in FSH from Tanner stage 2 to 3 and a sharp increase from stage 3 to 4, settling to pathologically high values in stage 5. LH first started rising in stage 2, when it was significantly higher than at stage 1; thereafter it gradually increased up to stage 5, when a significant difference was observed and reached pathological levels. T progressively increased during puberty until Tanner stage 5. INHB values were stable and within the normal range until stage 3 and then sharply declined in stage 4, remaining extremely low at stage 5. SHBG gradually decreased through pubertal stages, and a significant variation was observed between stages 1 and 2. The cfT showed gradual significant increases at the transition between Tanner stages 2, 3, and 4. E2 levels increased from mostly undetectable at stage 1 to normal range pubertal values at stage 5, but the only significant rise was from stage 3 into stage 4. The mean T/LH ratio rose from prepuberty to Tanner stage 3, then dropped significantly at stage 5. The INHB/FSH ratio showed a slight decline from stage 2 to 3 but fell dramatically in stage 4. The T/E2 ratio fell significantly from stage 1 to stage 2. All the comparisons were repeated after excluding the patients with a history of cryptorchidism, and findings were consistent with the entire cohort (data not shown).

Transition age and adulthood

During transition age a few significant differences were observed with regard to late pubertal development (Tanner stage 5). Both FSH and LH levels plateaued, and likewise T and cfT levels reached their maximum concentrations, although not significantly different from the end of puberty. INHB levels showed a slight decrease, and a minor increase was observed in SHBG levels. The T/LH ratio significantly declined, indicating ongoing worsening of Leydig cell function, whereas the INHB/FSH level did not significantly so. The T/E2 ratio significantly increased, because of slight modifications in both T and E2. No differences were observed with regard to E2 levels. During adulthood both T and cfT levels declined significantly from plateauing during transition age, and the T/LH ratio further worsened. On the contrary, no significant differences could be detected with regard to other HPG axis hormones or function indexes in adult life.

US Assessment by Pubertal Status

As each testis was considered separately for GLCM analysis, the average values for each parameter were used for the GLMM statistical analysis. All reported effects are significant at $P < .001$ for the overall model. Ultrasonographic parameters and characteristics are presented in Table 2 and Figure 3.

Prepuberty and puberty

There was a significant increase in TTV from Tanner stage 1 onward, reaching maximum median levels in stage 4 and then dropping in stage 5. Testicular echogenicity linearly increased during pubertal development, with no significant differences from one stage into the next. The prepubertal testes also appeared more homogeneous, as demonstrated by higher homogeneity values in prepuberty, which decreased throughout stages 2 to 5, alongside increasing entropy levels. All the comparisons were repeated after excluding the patients with a history of cryptorchidism, and findings were consistent with the entire cohort (data not shown).

Testicular microlithiasis was found in 3 prepubertal patients age 8 months, 2.9 years, and 6.6 years, with the youngest patient presenting a starry sky appearance. During puberty, microlithiasis was found in 4 patients at their first ever US assessment (stage 3, 13.5 years; stage 4, 13.0 and 17.3 years; stage 5, 17.5 years) and emerged in 3 patients during a follow-up US assessment performed at Tanner stage 2 (13.1 years), stage 3 (13.5 years), and stage 4 (14.1 years). Hypoechoic intratesticular lesions were found in stage 4 (n = 1) and stage 5 (n = 5). Three out of 6 patients had a previous US scan, where the lesions were not visible.

Transition age and adulthood

During transition age the TTV was lower than at the end of puberty development, although this difference was not statistically significant. There were no significant differences in echogenicity, homogeneity, or entropy entering transition age. Presence of microlithiasis was found in 10 patients: in 4 of these it appeared during follow-up US evaluations at 18.8 years, 19.3 years, 23.5 years, and 24.1 years (Fig. 4). Overall, 16 patients in transition age had hypoechoic intratesticular lesions, including in the count those patients who developed lesions in late pubertal stages. Hypoechoic lesions appeared de novo in 3 out of 9 patients for whom we had a previous US follow-up (Fig. 5).

During adulthood the median TTV further significantly fell and was accompanied by a worsening in testicular echotexture, whereas echogenicity remained unchanged and the prevalence of microlithiasis and hypoechoic lesions also increased.

Relationship Between Testicular US Parameters and Endocrine Function

We explored the relationship between HPG axis hormones (LH, FSH, T, E2, cfT) and quantitative US parameters. Through partial correlations on the whole cohort, no significant predictors of testicular echogenicity could be identified and, similarly, homogeneity was not associated with endocrine function ($P > .05$ for all), whereas testicular entropy showed a significant inverse correlation with both T [r = −0.21 (−0.32, −0.10), $P < .001$] and cfT [r = −0.18 (−0,29, −0,08), $P = .003$]. Similar results were obtained after the exclusion of prepubertal subjects (data not shown).

To test whether hypoechoic lesions might be representative of a worse testicular endocrine function we conducted a multivariate analysis according to presence of testicular hypoechoic

Table 1. Anthropometric measurement and hormonal assessment of patients with Klinefelter syndrome

	Prepuberty	Puberty				Transition age	Adulthood		
	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5			P Stage 5 vs transition age	P Transition age vs adulthood
n	70	22	19	24	28	32	41		
Clinical parameters									
Age, years	5.5 (3.0;8.3)	11.3 (10.2;12.2)	12.2 (11.2;13.1)	13.7 (12.6;14.5)	15.9 (15.0;17.1)	20.7 (18.6;23.5)	38 (33;44)	/	/
Height, cm	118 (96.7;135.0)	148.5 (145.7;156.8)	157.5 (149.6;166.1)	168.4 (161.1;174.2)	176.4 (173.2;180.5)	178.2 (174.5;180.0)	184 (177.6;186.5)	<.001	<.001
Weight, kg	22 (15.3;30.7)	42 (36.7;50.5)	47.5 (37.5;54.3)	53.5 (46;60.5)	60 (53.9;69.3)	68 (59.5;80.0)	83.2 (78.5;92.2)	<.001	<.001
Hormonal evaluations									
FSH, mIU/mL	0.52 (0.27;0.76)	1.42 (0.75;2.08)	2.14[a] (1.00;13.28)	16.78[a] (8.30;27.83)	29.45[a] (20.58;36.82)	26.65 (20.20;38.08)	28.92 (20.54;35.98)	.974	.907
LH, mIU/mL	0.07 (0.04;0.13)	0.69[a] (0.37;1.15)	2.28 (1.58;3.15)	5.45 (2.93;9.52)	12.04[a] (7.71;16.85)	15.66 (11.43;22.49)	16.04 (11.96;20.05)	.541	.843
T, nmol/L	0.20 (0.10;0.30)	1.20[a] (0.53;2.70)	8.40[a] (4.25;12.85)	12.60 (9.30;16.45)	15.60[a] (12.40;19.30)	16.50 (11.40;19.45)	9.90 (5.10;15.40)	.906	<.001
Inhibin B, pg/mL	125.0 (96.5;166.5)	137.5[a] (81.0;238.8)	176.0 (44.65;256.0)	26.1[a] (8.0;69.9)	10.20 (4.0;23.5)	8.35 (4.0;14.75)	4.0 (4.0;4.0)	.627	.089
Estradiol, pg/mL	10.0 (10.0;12.0)	13.0 (10.0;29.5)	12.0 (10.0;26.0)	19.0[a] (15.0;27.5)	30.0 (24.0;36.7)	36.5 (29.0;44.0)	25.0 (19.5;33.0)	.738	.965
SHBG, nmol/L	135.2 (103.7;159.6)	78.4[a] (59.1;104.1)	59.3 (40.4;73.6)	42.1 (35.6;50.2)	36.0 (30.1;47.8)	39.4 (30.2;51.5)	41.8 (32.3;57.5)	1.000	.108
cfT, pmol/L	1 (1;3)	12 (5;27)	131[a] (56;212)	212[a] (148;320)	327[a] (240;391)	330 (211;397)	163 (57;289)	1.000	<.001
T/LH ratio	2.86 (1.50;6.00)	2.94[a] (1.58;3.79)	3.55 (2.38;4.83)	2.08 (1.33;3.93)	1.43 (0.95;2.11)	1.03 (0.56;1.91)	0.54 (0.32;0.96)	.041	.020
INHB/FSH ratio	259.6 (155.1;476.8)	127.9 (54.9;229.5)	126.0[a] (4.03;217.4)	1.35[a] (0.33;6.60)	0.34 (0.11;0.92)	0.30 (0.15;0.96)	0.14 (0.11;0.18)	.564	.508
T/E2 ratio	95.5 (47.6;125.0)	11.2[a] (3.91;30.5)	1.83 (0.92;4.97)	1.67 (1.11;2.07)	2.18 (1.48;2.53)	2.37 (1.52;3.73)	2.53 (2.01;5.14)	.036	.373

Values are expressed as median and interquartile range (IQR) or number and percentage, as appropriate. Comparisons between stage 5 and transition age, between transition age and adulthood, and among stages of puberty were performed with general linear mixed model analyses with outcome variables acting as dependent variables, stage acting as a fixed effect, and patient ID acting as a random effect. We report Šidák-adjusted significance values for multiple comparisons from post hoc pairwise comparisons of main effects to determine the stage at which significant changes occurred. Significant P-values are presented in bold.

Abbreviations: cfT, free T concentrations; E2, 17β-estradiol; FSH, follicle-stimulating hormone; INHB, inhibin B; LH, luteinizing hormone; SHBG, sex hormone-binding globulin; T, testosterone.

[a]Indicates significant differences with the previous stage of pubertal development (P < .05).

Table 2. Ultrasonographic features of patients with Klinefelter syndrome

	Prepuberty	Puberty				Transition age	Adulthood		
	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5		*P*		*P*
n	70	22	19	24	28	32	Stage 5 vs transition age	41	Transition age vs adulthood
Ultrasound features									
Total testicular volume, mL	1.90 (1.43;2.50)	4.40[a] (3.33;6.03)	6.30 (5.45;7.43)	8.70 (5.40;10.60)	7.05 (4.70;9.25)	6.05 (4.73;7.65)	.295	4.45 (3.23;6.35)	**.040**
GCLM									
Echotexture									
Homogeneity	0.34 (0.33;0.37)	0.33 (0.30;0.33)	0.31[a] (0.30;0.33)	0.32 (0.30;0.35)	0.30 (0.28;0.31)	0.31 (0.29;0.32)	.135	0.29 (0.27;0.30)	**.008**
Entropy	6.24 (6.09;6.39)	6.16 (6.03;6.27)	6.18 (6.03;0.24)	6.19 (5.93;6.26)	6.31 (6.21;6.38)	6.31 (6.20;6.58)	.580	6.40 (6.26;6.58)	**.018**
Echogenicity									
Gray-levels	40.9 (34.2;46.2)	41.7 (38.1;46.5)	37.7 (32.6;51.4)	43.4 (36.4;59.6)	43.4 (36.8;50.1)	41.0 (35.7;54.0)	.747	44.1 (38.7;59.6)	.508
Microlithiasis, n (%)	3 (1.8)	1 (3.8)	2 (11.1)	3 (9.4)	1 (2.6)	10 (18.5)	**.012**	15 (38.5)	**.032**
Hypoechoic lesions, n (%)	0 (0)	0 (0)	0 (0)	1 (3.1)	5 (12.8)[a]	16 (29.6)	**<.001**	33 (84.6)	**<.001**
Varicocele, n (%)	1 (0.6)	0 (0)	0 (0)	4 (12.5)	1 (2.6)	6 (11.1)	.097	7 (17.−9)	.348

Values are expressed as median and Interquartile range (IQR) or number and percentage, as appropriate. Comparisons between stage 5 and transition age, between transition age and adulthood, and among stages of puberty were performed with GCLM analyses with outcome variables acting as dependent variables, stage acting as a fixed effect, and patient ID acting as a random effect. We report Šidák-adjusted significance values for multiple comparisons from post hoc pairwise comparisons of main effects to determine the stage at which significant changes occurred. Significant *P*-values are presented in bold.
Abbreviation: GCLM, general linear mixed model.
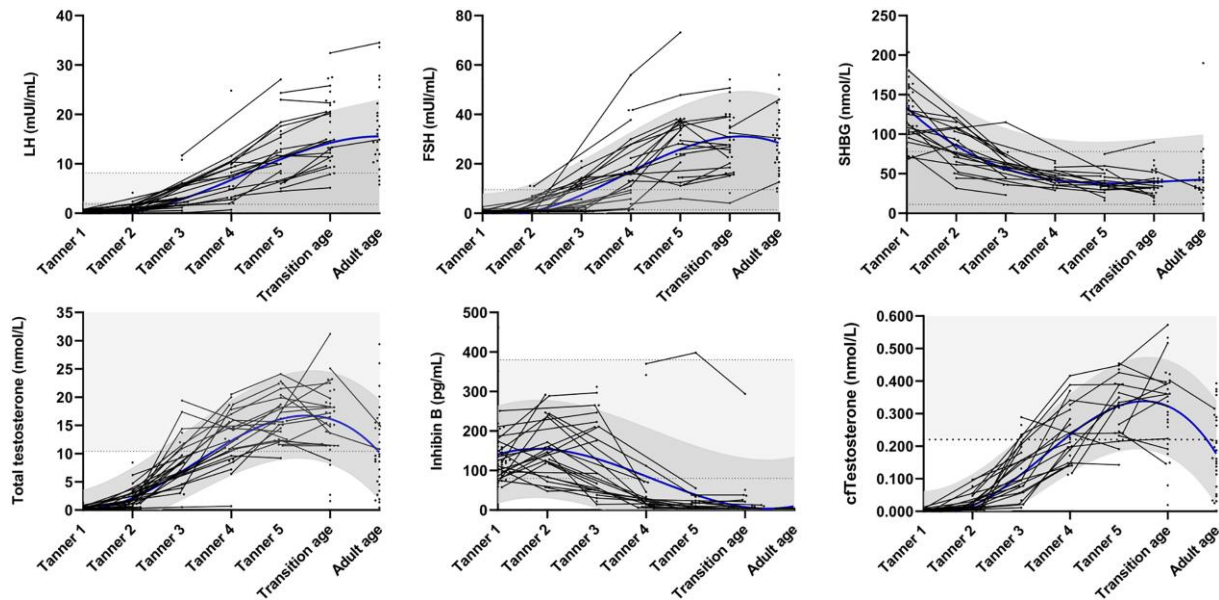[a]Indicates significant differences with the previous stage of pubertal development (*P* < .05).

Figure 1. Serum concentration of hormone values (FSH, LH, SHBG, INHB, T, cfT) in the different pubertal stages, transition age, and adulthood of patients with Klinefelter syndrome. Symbols mark individual samples. Lines connect consecutive samples in a single individual. Abbreviations: cfT, free testosterone concentrations; FSH, follicle-stimulating hormone; INHB, inhibin B; LH, luteinizing hormone; SHBG, sex hormone binding globulin; T, total testosterone.

lesions, after controlling for pubertal stage and age group (and excluding prepubertal subjects). Subjects with testicular hypoechoic lesions, indeed, showed higher LH levels (13.08 vs 9.42 mUI/mL, *P* = .003) and lower T (8.95 vs 13.03 nmol/L, *P* = .007) and cfT levels (0.12 vs 0.24 nmol/L, *P* < .001), indicating a worse steroidogenic function. Similar results were
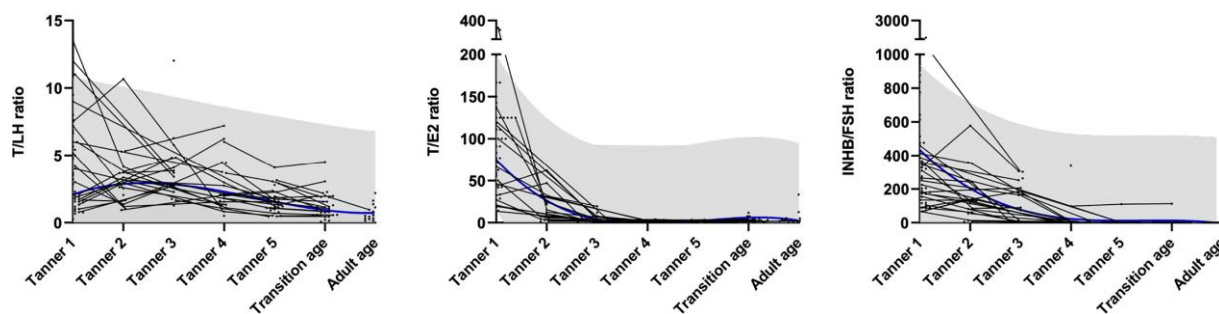
Figure 2. INHB/FSH, T/LH, T/E2 ratios in the different pubertal stages, transition age, and adulthood of patients with Klinefelter syndrome. Symbols mark individual samples. Lines connect consecutive samples in a single individual. Abbreviations: E2, 17β-estradiol; FSH, follicle-stimulating hormone; INHB, inhibin B; LH, luteinizing hormone; T, total testosterone.
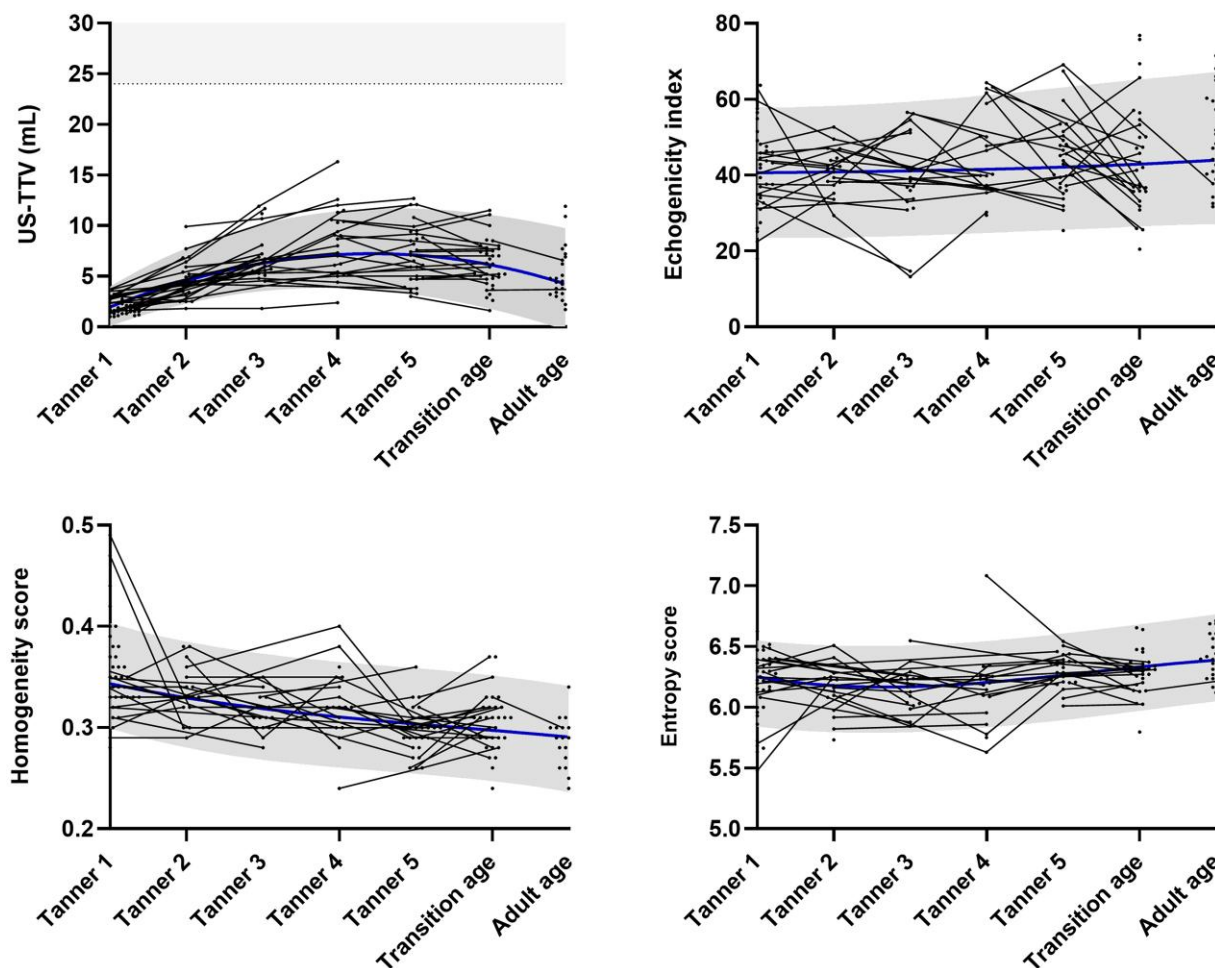

Figure 3. Total testicular volumes (mL) and gray level co-occurrence matrix analysis parameters in the different pubertal stages, transition age, and adulthood of patients with Klinefelter syndrome. Symbols mark individual evaluation. Lines connect consecutive evaluations in a single individual. Abbreviations: TTV, total testicular volume; US, ultrasound.

obtained for the presence of microlithiasis with regards to cfT concentrations (0.20 vs 0.22 nmol/L, $P = .030$). To further validate these findings, we conducted a linear regression analysis with T and cfT as the dependent variables, and entropy, presence of hypoechoic lesions, and microlithiasis as independent variables, adjusting for pubertal stage and age group (after the exclusion of prepubertal children). Hypoechoic lesions (for both T and cfT) and entropy and microlithiasis (only for cfT) were independently and significantly associated with lower T concentrations (Table 3).

Discussion

In this study we describe the clinical, hormonal, and US characteristics of a cohort of KS patients from 7 months to 55 years of age. We aimed to detail the changes occurring in a life-course prospective, spanning from infancy through puberty and transition age to adulthood, and to assess the role of US in revealing these changes. Our main findings are as follows: (1) Sertoli and germ cell impairment is not hormonally detected before Tanner stages 3 to 4, as reflected by normal INHB values until stage 4 and the fall in the INHB/FSH ratio
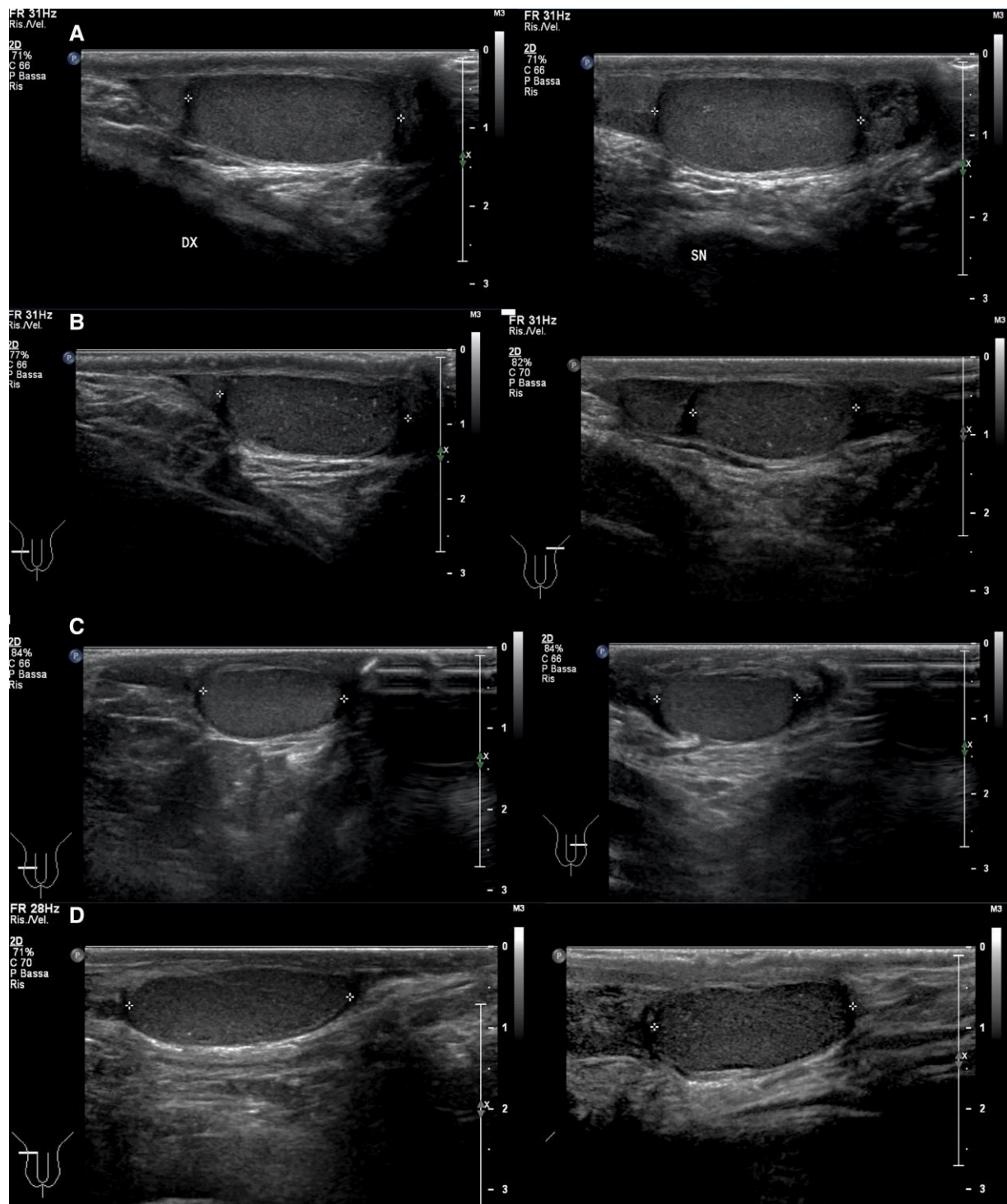
Figure 4. Panels A and B: Appearance of testicular microlithiasis in a Klinefelter syndrome patient of 23.5 years in the transition age (Panel B), compared with the same patient at Tanner stage 4 (15.9 years) (Panel A). Panels C and D: An additional case of testicular microlithiasis discovered during ultrasound follow-up assessment. During Tanner stage 2 (11.9 years, Panel C), the testes of this patient were homogeneous and slightly hypoechoic, while at Tanner stage 4 (14.1 years, Panel D), they appeared inhomogeneous with a few hyperechoic spots (<5 per scan).

between stages 3 and 4; (2) the T/LH ratio peaks during Tanner stages 2 to 3 and declines from Tanner stage 4 onward; (3) testicular volume progresses until Tanner stage 4, with subsequent regression; (4) US echotexture progressively worsens until transition age, reflecting ongoing gonadal compromise; (5) quantitative US echotexture measures and the presence of both hypoechoic lesions and microlithiasis predict a worse testicular endocrine function.

The uniqueness of the present study is its prospective evaluation of a large caseload of young nonmosaic KS patients in different stages of life from a single referral center using a standardized protocol. Patient selection from both pediatric and transition age outpatient clinics enabled us to conduct a comprehensive follow-up of these patients, combining clinical, laboratory, and US data with a semilongitudinal approach. The available literature on KS during childhood and
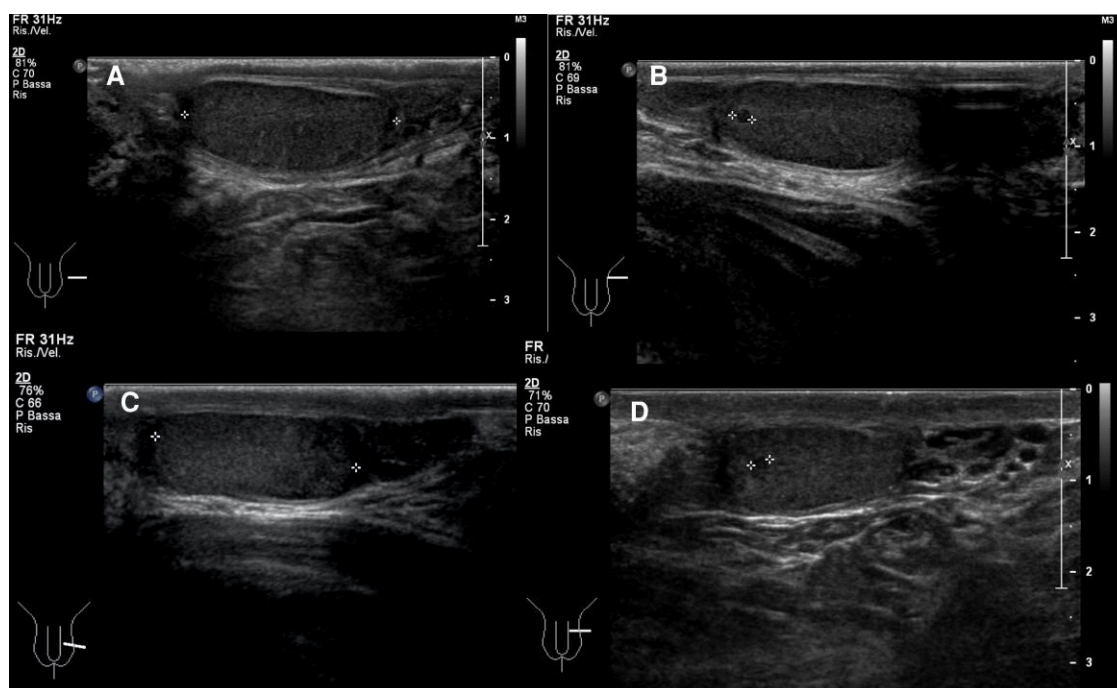
Figure 5. Appearance of hypoechoic lesions in 2 Klinefelter syndrome patients assessed during the transition age. Panel A shows an 11.2-year-old patient in Tanner stage 3; in Panel B the same patient, now 15.2 years, has developed a hypoechoic lesion (Tanner stage 5). Panel C shows the right testis of a 10-year-old Klinefelter syndrome boy in Tanner stage 2; in Panel D, a small hypoechoic lesion has appeared in the upper pole of the same testis, scanned at the end of pubertal development (Tanner stage 5, 15.2 years).

adolescence is summarized in Table 4. To the best of our knowledge, this is the first study to comprehensively evaluate testicular function and US characteristics in a large population of nonmosaic KS from infancy to adulthood with an important number of patients in puberty and transition age. Most of the other studies on KS have focused on late pubertal and adult gonadal function. Those assessing pubertal development (19, 45-57) are hampered by the small caseload, and Tanner stage is not always considered. Above all, only 2 studies have explored US features, both of which focused on sperm retrieval and were based on a small cohort (58) or did not include prepubertal and pubertal patients (59).

As expected, some features of KS were invariably present in all late puberty and transition age patients, namely small testes, high gonadotropin levels, low-to-undetectable INHB levels, and a low T/LH ratio. Cryptorchidism was found in 13.2% and gynecomastia in 14.3% of patients, relatively lower rates than reported in the literature (19, 46, 51, 60). The deterioration of seminiferous tubules accelerates dramatically during puberty and is accompanied by a rise in FSH and a sharp decline in INHB levels (56). In a previous study, our group showed the absence of hormonal signs of testicular failure in the period from mini-puberty to prepuberty, as indicated by higher levels of anti-mullerian hormone and INHB during early childhood (6 months to 8 years), and no differences between KS and 46,XY children in mid-childhood (8 to 12 years) (27).

As known, serum INHB and anti-mullerian hormone reflect Sertoli cell function during prepuberty. Both these markers become mainly germ-cell dependent during mid-puberty (61). However, in KS patients, changes in FSH and INHB may not be indicative of reduced spermatogenesis, since this process is globally disrupted in the testes of KS patients. As suggested by Gies et al (57), the tubular hyalinization process

starts at the onset of puberty and is followed, rather than preceded, by a decline in serum INHB. This decline is accompanied by a simultaneous rise in FSH and serum T. FSH levels rise from Tanner stage 2, somewhat more markedly than LH levels. A hypergonadotropic state with compensated hypoandrogenism develops in virtually all adult patients, and our data, however, show how impaired testosterone secretion is typically not apparent in KS boys during puberty. We speculate that these findings might imply the absence of testicular vascular abnormalities hindering peripheral testosterone release in early puberty (17). Plasma T/LH ratio is considered a sensitive marker of Leydig cell function: a reduced ratio can reflect Leydig cell dysfunction, even where plasma T levels are normal. In our cohort we observed a lower T/LH ratio in the last pubertal stages with further reductions in transition age and adulthood, alongside an increase in serum FSH.

The testicular US features of children and adolescents with KS have so far been described only superficially (27) with no clear distinction between the transition age and adulthood. Garolla et al (59) evaluated 111 KS patients (range 16-51 years) undergoing testicular sperm extraction. They assessed testicular volume using the ellipsoid formula and reported the presence of varicocele. Nahata et al (58) reported that 5 of 15 KS subjects (33.3%) ages 15 to 23 years had testicular microlithiasis. Physiologically, the stage of germ cell development and tubular maturation determines the testicular echotexture. The low echogenicity typical of prepubertal testes rises progressively to reach a medium echogenicity by postpuberty. This increase is primarily due to the growth of the seminiferous tubules, which increase in diameter and develop a lumen. As seminiferous tubules comprise 70% to 80% of the adult testicular mass (40), testicular volume is believed to reflect spermatogenesis. In KS subjects, testicular histopathology typically reveals hypotrophy with fibrotic, hyalinized

Table 3. Linear model of predictors of LH, total, and calculated free testosterone in KS subjects after bootstrapping

	b	SE	*P*
Total testosterone, nmol/L			
Constant	8.303 (0.640, 18.195)	4.502	.071
Pubertal stage	−0.491 (−0.720, −0.282)	0.109	**<.001**
Entropy	−0.634 (−2.227, 0.567)	0.716	.384
Presence of hypoechoic lesions	−0.492 (−0.884, −0.067)	0.201	**.017**
Presence of microlithiasis	0.459 (−0.432, 1.585)	0.551	.427
LH, mIU/mL			
Constant	1.584 (−28.411, 29.921)	14.634	.903
Pubertal stage	2.834 (2.321, 4.400)	0.276	**<0.001**
Entropy	−0.839 (−5.593, 4.158)	2.440	0.712
Presence of hypoechoic lesions	6.415 (3.107, 10.234)	1.805	**<.001**
Presence of microlithiasis	−2.440 (−5.927, 0.818)	1.698	.155
Calculated free testosterone, pmol/L			
Constant	0.604 (0.056, 1.148)	0.287	.039
Pubertal stage	0.057 (0.040, 0.074)	0.008	**<.001**
Entropy	−0.100 (−0.193, −0.009)	0.047	.038
Presence of hypoechoic lesions	−0.115 (−0.185, −0.056)	0.033	.001
Presence of microlithiasis	−0.061 (−0.132, −0.010)	0.032	.041

95% bias corrected accelerated confidence intervals from 2000 bootstrapped samples are reported in parentheses. *P* < .001 for the overall models. Significant *P*-values are presented in bold.
Abbreviations: LH, luteinizing hormone; KS, Klinefelter syndrome.

seminiferous tubules, and Leydig cell hyperplasia. These features are reflected in a reduced TTV and very irregular and inhomogeneous US features. Comparing our results to data from the literature, we found some discrepancies regarding the TTV (19, 46). This could be imputable to the use of US compared to Prader orchidometer, the latter of which may overestimate small testicles (62). However, using gold-standard US methodology, we show how the TTV increases during puberty (from stage 2 until stage 4), while testicular degeneration becomes apparent at the end of pubertal development (stage 5) and into adulthood, with a significant drop in TTV compared to transition age patients, and a lower T, cfT, and T/LH ratio. Furthermore, the absence of differences in FSH and LH values between adult and transition age patients possibly reflects an HPG axis already stretched to its maximum.

With GLCM analysis, we observed that testicular echotexture worsened as puberty progressed, as demonstrated by reduced homogeneity values and increased entropy values throughout pubertal development. Interestingly, testicular US appearance further significantly worsened in adulthood, with decreasing homogeneity and increasing entropy, alongside an increased prevalence of microlithiasis and hypoechoic lesions, which were significant independent predictors of worse testicular endocrine function after adjusting for pubertal stage and age group, highlighting their importance in the longitudinal follow-up of patients for the development of hypogonadism. Of note, the quantitative echotexture parameter entropy was also significantly, inversely and independently associated with cfT after controlling for pubertal stage and age group, possibly reflecting the development of small foci of Leydig cell hyperplasia, a frequent finding in patients with KS (19, 63). Leydig cell micronodules, histologically defined as more than 15 Leydig cells in a cross-section, have previously been associated with a low T/LH ratio, reflecting an endocrine dysfunction (64, 65). High LH causes Leydig cell hyperstimulation, which usually presents as small multifocal hypoechoic testicular micronodules at US with increased vascularization. Holm et al (66) observed in infertile men that patients with bilateral micronodules had higher LH values and a lower T/LH ratio and that the frequency of micronodules was clearly correlated with the degree of spermatogenetic impairment. They hypothesized that whatever caused the alterations in the spermatogenic compartment might also induce an increase in Leydig cell cluster size and concluded that the presence of micronodules indicated impaired testicular function at both the endocrine and spermatogenic level. As already stated, a lower T/LH ratio was typical of late puberty, which is in line with the appearance of Leydig cell hyperplasia (hypoechoic lesions) during later pubertal stages and transition age in our cohort.

The main strength of this study resides in the comprehensive data collection in a large sample across a broad age range and in its prospective design, as the literature on this topic mainly comprises retrospective studies. In addition, the combination of US with clinical and hormonal data allowed an all-round evaluation of testicular development in young KS patients, especially concerning very young children and during transition age, two partly neglected contexts. However, our study did have some limitations. Specifically, we could not combine our results with testicular histology, as there were no clinical indications for testicular biopsy in our KS infants or children and the hormonal assessment was not performed with high performance liquid chromatography-tandem mass spectrometry, which is the current gold-standard methodology for steroid hormone determination. Moreover, we did not compare our results with a 46,XY control group; however, our considerations are mostly related to relative dynamic changes in our cohort of KS subjects rather than in absolute terms.

Conclusions

In conclusion, in this semilongitudinal study we confirmed, in a large cohort of 47,XXY KS patients, that spermatogenetic and Sertoli cell function deteriorates dramatically from pubertal stage 3 onward, as demonstrated by the fall in INHB levels. Furthermore, we found that T levels increase in the first pubertal stages and remain stable in the latter and in transition age, decreasing only in adulthood. Supraphysiologic LH levels are required to achieve adequate testosterone concentrations starting from Tanner stage 5. Concurrently, T/LH ratio drops in the final pubertal stages, as a hypergonadotropic state with compensated hypogonadism becomes established. Data from

Table 4. Summary of the existing literature on nonmosaic KS children and adolescent patients

Reference	Study	Study cohort (n)	Age (years)	Gynecomastia (%)	Cryptorchidism (%)	Testicular biopsy	US assessment	Volume evaluation	Tanner staging	TRT	Mosaic/other	Outcome
Ratcliff et al, 1982	Retrospective	12	16.6 ± 0.9	33.3	NR	No	No	Prader orchidometer	Yes	No	No	Clinical and biochemical characteristics
Christiansen et al, 2003	Prospective	22	8.9-29.5	NR	NR	No	No	Prader orchidometer	No	Yes	No	Clinical and biochemical characteristics
Wikstrom et al, 2004	Prospective	14	11.5 (median) 10.0-13.9	NR	0	Yes	No	Prader orchidometer	Yes	No	No	Sperm retrieval
Wirkstrom et al, 2006	Prospective	14	11.5 (median) 10-13.9	78.5	0	No	No	Prader orchidometer	Yes	No	No	Clinical and biochemical characteristics
Bastida et al, 2007	Retrospective (1986-2006)	29	0.8-22	20.7	51.7	No	No	Prader orchidometer	Yes	No	No	Clinical and biochemical characteristics
Aksglaede et al, 2011	Retrospective (1999-2010)	166	0.25-17 (n = 82) 18-57 (n = 68)	44	14	Yes	No	Prader orchidometer	No	Yes	No	Clinical and biochemical characteristics
Gies et al, 2012	Retrospective	7	10-16	NR	0	Yes	No	Prader orchidometer	Yes	No	No	Sperm retrieval
Van Saen et al, 2012*	Retrospective	7	13-16	NR	N/A	Yes	No	NR	No	No	No	Sperm retrieval
Tincani et al, 2012	Retrospective (1989-2011)	33	5.3-59.7	27.2	NR	No	No	Prader orchidometer	No	No	Yes	Clinical and biochemical characteristics
Pacenza et al, 2012	Retrospective (1982-2008)	98	0-17.9 (n = 44) >18 (n = 54)	25 10.9	36.3 0	No	No	Prader orchidometer	Yes	No	Yes	Clinical and biochemical characteristics
Rohayem et al, 2015	Retrospective (2008-2013)	135	13-19 (n = 50) 20-61 (n = 85)	NR	15.5	Yes	No	Prader orchidometer and US	Yes	Yes	No	Sperm retrieval
Nahata et al, 2016	Prospective (2013)	15	15-23	NR	NR	Yes	Yes	US	No	No	No	Sperm retrieval
Akcan et al, 2017	Retrospective	23	3.0 (median) 0.04-16.3	8.7	34.7	No	No	NR	No	No	No	Clinical and biochemical characteristics
Garolla et al, 2018	Retrospective (2005-2016)	111	29.9 ± 7.3 (16-51)	NR	NR	Yes	Yes	US	No	Yes	No	Sperm retrieval
Van Saen et al, 2018	Prospective	56	<18 (n = 29) > 18 (n = 27)	NR	N/A	Yes	No	NR	No	No	Yes	Sperm retrieval
Tanner et al, 2021	Retrospective (2004-2018)	72	10.2-17.2	NR	NR	No	No	Prader orchidometer	Yes	No	No	Clinical and biochemical characteristics

Abbreviations: KS, Klinefelter syndrome; N/A, not applicable; NR, not reported; TRT, testosterone replacement therapy; US, ultrasound.

the combined analysis of hormonal and US data suggest that quantitative testicular US parameters are responsive to the evolution of the gonadal status in these patients, and specifically we could show how the presence of hypoechoic testicular lesions, microlithiasis, and higher entropy are independent predictors of worse testicular endocrine function. We thus propose that quantitative US alongside texture analysis should be assessed in future studies as a clinical tool to monitor testicular parenchymal health and to support the clinicians in the management of KS patients, possibly with regard to the timing of T replacement therapy start or microsurgical testicular sperm extraction procedures.

Our findings open new questions on the role of testicular US in KS children and adolescents in order to provide them with optimal care and reduce their long-term morbidity. Future prospective studies combining hormone and quantitative US features (comprising GLCM analysis) with histological correlates are needed to further explore and validate this approach as potential predictor of the success of sperm retrieval.

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Author Contributions

All authors contributed to the conception and design of the review. M.T., F.S., C.T., F.C., and R.P. performed the outpatient visits, reviewed the literature, and extracted the respective data. C.P. and D.G. performed all US exams. M.T., F.S., and M.S. wrote the first draft. C.P., F.S., and F.C. performed the statistical analysis. C.P. performed the first revision and synthesis of the manuscript. M.S., A.L., and A.R. performed a second critical revision of data. C.P., A.M.I., D.G., A.R., and L.T. performed the last critical revision for important intellectual content and granted the final approval of the version to be published. All authors are accountable for the accuracy and integrity of the work, and they all reviewed and approved the final manuscript.

Disclosures

The authors have nothing to disclose.

Data Availability

The data underlying this article cannot be shared publicly due to the privacy of individuals that participated in the study. The data will be shared on reasonable request to the corresponding author.

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