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ORIGINAL ARTICLE *Andrology*

Delaying testicular sperm extraction in 47,XXY Klinefelter patients does not impair the sperm retrieval rate, and AMH levels are higher when TESE is positive

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STUDY QUESTION: Should testicular sperm extraction (TESE) in non-mosaic 47,XXY Klinefelter syndrome (KS) patients be performed soon after puberty or could it be delayed until adulthood?

SUMMARY ANSWER: The difference in sperm retrieval rate (SRR) in TESE was not significant between the 'Young' (15–22 years old) cohort and the 'Adult' (23–43 years old) cohort of non-mosaic KS patients recruited prospectively in parallel.

WHAT IS KNOWN ALREADY: Several studies have tried to define predictive factors for TESE outcome in non-mosaic KS patients, with very heterogeneous results. Some authors have found that age was a pejorative factor and recommended performing TESE soon after puberty. To date, no predictive factors have been unanimously recognized to guide clinicians in deciding to perform TESE in azoospermic KS patients.

STUDY DESIGN, SIZE, DURATION: Two cohorts (Young: 15–22 years old; Adult: 23–43 years old) were included prospectively in parallel. A total of 157 non-mosaic 47,XXY KS patients were included between 2010 and 2020 in the reproductive medicine department of the University Hospital of Lyon, France. However 31 patients gave up before TESE, four had cryptozoospermia and three did not have a valid hormone assessment; these were excluded from this study.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Data for 119 patients (61 Young and 58 Adult) were analyzed. All of these patients had clinical, hormonal and seminal evaluation before conventional TESE (c-TESE).

MAIN RESULTS AND THE ROLE OF CHANCE: The global SRR was 45.4%. SRRs were not significantly different between the two age groups: Young SRR=49.2%, Adult SRR = 41.4%; $P = 0.393$. Anti-Müllerian hormone (AMH) and inhibin B were significantly higher in the Young group (AMH: $P = 0.001$, Inhibin B: $P < 0.001$), and also higher in patients with a positive TESE than in those with a negative

TESE (AMH: $P = 0.001$, Inhibin B: $P = 0.036$). The other factors did not differ between age groups or according to TESE outcome. AMH had a better predictive value than inhibin B. SRRs were significantly higher in the upper quartile of AMH plasma levels than in the lower quartile (or in cases with AMH plasma level below the quantification limit): 67.7% versus 28.9% in the whole population ($P = 0.001$), 60% versus 20% in the Young group ($P = 0.025$) and 71.4% versus 33.3% in the Adult group ($P = 0.018$).

LIMITATIONS, REASONS FOR CAUTION: c-TESE was performed in the whole study; we cannot rule out the possibility of different results if microsurgical TESE had been performed. Because of the limited sensitivity of inhibin B and AMH assays, a large number of patients had values lower than the quantification limits, preventing the definition a threshold below which negative TESE can be predicted.

WIDER IMPLICATIONS OF THE FINDINGS: In contrast to some studies, age did not appear as a pejorative factor when comparing patients 15–22 and 23–44 years of age. Improved accuracy of inhibin B and AMH assays in the future might still allow discrimination of patients with persistent foci of spermatogenesis and guide clinician decision-making and patient information.

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Introduction

Klinefelter syndrome (KS) is the most frequent cause of genetic infertility and gonosomal anomaly. The prevalence is estimated at 1/500 to 1/700 newborn males, 11% of azoospermic patients and 1–2% of infertile men (Lanfranco *et al.*, 2004). Until the late 1990s, KS was considered to cause total and definitive sterility. Now, patients with KS may father their own children thanks to intracytoplasmic injection techniques using testicular sperm extracted from residual foci of preserved spermatogenesis (Palermo *et al.*, 1998; Reubinoff *et al.*, 1998; Corona *et al.*, 2017). The first testicular sperm extraction (TESE) technique described was conventional TESE (c-TESE), consisting of small biopsies of testicular parenchyma. Since 1999, a new technique has also been used by a growing number of surgeons: microsurgical TESE (m-TESE), using an operating microscope to direct the biopsy toward the dilated seminiferous tubes (Schlegel, 1999). To date, we lack studies showing that m-TESE is superior to c-TESE in KS patients. In 2017, a meta-analysis found a sperm retrieval rate (SRR) of 44% (95% CI (39; 48%)) in KS patients. There was no significant difference in the SRR between c-TESE and m-TESE (Corona *et al.*, 2017).

Some studies have suggested that age could be a prognostic factor and that younger patients have better chances of positive TESE (Okada *et al.*, 2005; Bakircioglu *et al.*, 2006, 2011; Kyono *et al.*, 2007; Ferhi *et al.*, 2009; Ramasamy *et al.*, 2009; Yarali *et al.*, 2009; Sabbaghian *et al.*, 2014; Chehrazi *et al.*, 2017; Garolla *et al.*, 2018; Ozer *et al.*, 2018; Vloeberghs *et al.*, 2018; Yücel *et al.*, 2021; Özkan *et al.*, 2022). Several authors have suggested that it might be advisable to perform TESE in younger patients and even adolescents (Damani *et al.*, 2001; Mehta *et al.*, 2013; Rives *et al.*, 2013).

To answer the question of the optimal timing for TESE, we designed a prospective study comparing the SRR between two groups of patients enrolled prospectively in parallel: a 'Young' group referred for fertility preservation, aged 15–22 years, and an 'Adult' group referred for infertility above 23 years of age. Preliminary results were published in 2015 (Plotton *et al.*, 2015). We found no significant difference in the SRR between the Young and Adult patients. We report here the results of the completed study. In addition to age, we studied testicular volume and hormonal data, including anti-Müllerian hormone (AMH), as potential predictive factors for sperm cell extraction. AMH is a dimeric glycoprotein belonging to the transforming growth factor-B superfamily and acting on tissue growth and differentiation. The most specific effect of AMH is to cause the involution of Müllerian ducts during male embryogenesis (Jost, 1947). Serum concentrations are high in boys until puberty. Then a decrease occurs when intratesticular testosterone increases and spermatogenesis develops, and concentrations remain low during adulthood (Lee *et al.*, 1996). Aksglaede *et al.* (2011) in a study of 95 KS patients, showed that AMH stayed within the normal range until puberty, then the pubertal decline was delayed, but in adulthood their AMH was below −2 SD in 85% of cases.

We previously showed that AMH plasma levels are lower in genetic non-obstructive azoospermia (NOA) than in acquired (chemotherapy-induced) NOA or obstructive azoospermia, suggesting a variation in AMH plasma levels according to Sertoli cell differentiation (Plotton *et al.*, 2012). Moreover, AMH plasma levels were recently found to be related to sperm extraction in NOA (Aboukhshaba *et al.*, 2021; Benderradji *et al.*, 2021).

Here, we report the data of our complete study, confirming our preliminary report (Plotton *et al.*, 2015), showing that SRR is not significantly higher soon after puberty compared with that in young adulthood for patients with non-mosaic 47,XXY karyotype KS. Thus, TESE could be delayed if necessary, for example, if the patient is not psychologically ready. In addition, we showed that, among the potential predictive factors studied, the AMH plasma level, as a marker of Sertoli cell health, is the most valuable to predict the TESE outcome in KS patients.

Materials and methods

The design of the Fertipreserve study was described in the preliminary report (Plotton *et al.*, 2015) (clinical trial NCT01918280). The protocol was approved by the institutional review board, in line with French legislation. Written informed consent was obtained from the patients (and parents for patients aged under 18) at least 1 week after the protocol was explained by one of the investigators (I.P. or H.L.).

The aim of the study was to compare SRR in a group of young KS patients referred for fertility preservation and with that of a group of

adult KS patients referred for infertility. The patients included in the preliminary report are also included in the present report.

Patients

Non-mosaic 47,XXY karyotype KS patients were recruited prospectively in parallel, between April 2010 and March 2020, into two groups, Young aged 15–22 years and Adult aged 23 years and over, in the reproductive medicine department of the University Hospital of Lyon, France. The cutoff age between the two groups at 23 years was chosen as being the lowest age for Assisted Reproduction Technology in our institution.

All had a clinical examination and two semen analyses at a 3-month interval with extensive research of spermatozoa after centrifugation in the reproductive medicine laboratory of our hospital. Blood samples were drawn while fasting in the morning and assayed in the hormonology laboratory of our hospital, and bilateral c-TESE was performed by a single experienced urological surgeon (B.C.). Patients with associated infertility factors were excluded. One patient, aged over 15 years but with prepubertal hormonal data, was excluded. Testosterone treatment, if any, was suspended for at least 6 months before inclusion: i.e. at least 9 months before TESE. Psychological support was systematically offered to the patients of the Young group and was on demand for the patients of the Adult group.

Clinical data

Clinical examination, including testicular volume measurement using Prader's orchidometer, was performed by one of the authors (I.P.).

Karyotype and genetic testing

Homogeneity was defined as a standard karyotype with 25 47,XXY cells on peripheral blood sample confirmed by FISH analysis in at least 100 cells. All patients were tested for Y-chromosome microdeletions by multiplex PCR.

Hormonal assays

FSH blood concentration was measured on immunologic chemiluminescence assay (Architect; Abbott, Chicago, IL, USA). Intra- and inter-assay coefficients of variation at 20 IU/l were 1.9% and 7.6%, respectively.

LH blood concentration was measured using an immunologic chemiluminescence assay (Architect; Abbott, Chicago, IL, USA). Intra- and inter-assay coefficients of variation at 20 mIU/l were 3.6% and 6.9%, respectively.

Total testosterone (TT) blood concentration was measured using in-house liquid chromatography-mass spectrometry after supported liquid extraction (Grandhaye *et al.*, 2021). Mean intra-assay coefficients of variation were 3.7 and 3.3% for TT concentrations of 0.080 and 0.274 nmol/l, respectively; inter-assay coefficients of variation were 4.71 and 5.98% for TT concentrations of 2.254 and 8.934 nmol/l, respectively.

Bioavailable testosterone blood concentration was measured by radio-immunologic assay after ammonium sulfate precipitation followed by extraction and chromatography. Normal ranges were determined in normal 20- to 40-year-old men (Déchaud *et al.*, 1989).

Inhibin B blood concentration was measured using the INHIBIN B Gen II ELISA kit (DSL-10684100). Normal range defined from the data of 377 normozoospermic men was from 92 to 316 ng/l (Barbotin *et al.*, 2015).The limit of quantification was 5 ng/l.

17β-estradiol blood concentration was measured using a radio-immunologic kit after extraction.

AMH blood concentration was measured by electrochemiluminescence assay (Roche® Cobas PRO e801). Intra- and inter-assay coefficients of variation were 1.8% and 2%, respectively, for AMH 7.6 pmol/l. The normal range defined from the data of 578 normozoospermic men was from 16.4 to 90.3 pmol/l (Benderradji *et al.*, 2022). The limit of quantification was 1.85 pmol/l.

Prolactin blood concentration was measured by electrochemiluminescence assay (Roche® Cobas PRO e801). Intra- and inter-assay coefficients of variation were 0.92% and 1.4%, respectively, for prolactin 37.6 pmol/l.

Semen analysis, c-TESE and cryopreservation were performed as previously described (Plotton *et al.*, 2015). TESE was considered positive when at least one sperm cell was observed. Cases with positive TESE are referred to as TESE+ and cases with negative TESE as TESE−. Sperm was frozen only if at least 10 sperm cells were observed. To maximize the rate of sperm cell conservation, we decided to test vitality by a mobility test with theophylline at the time of the ICSI.

Histopathological study

A sample of testicular tissue, in addition to the TESE sample, was fixed in AFA (Alcohol, Formaldehyde, Acetic acid) and stained with hematoxylin and eosin, periodic acid Schiff and Masson's trichrome for histopathological study. The aspects of the spermatogenic tubules varied between patients and within the same sample from complete atrophy, to Sertoli cell only, the presence of immature germ cells, and the presence of mature sperm cells. Tubules were classified according to Johnsen's score (Johnsen, 1970), a 10-point scoring system for quantifying spermatogenesis. A Johnsen score of 10 indicates maximum spermatogenesis activity, and a score of 1 indicates complete absence of germ cells. The higher score observed for each patient in either the right or the left testicular sample, referred to as maximal Johnsen's score, was compared to the TESE results, age group and clinical and hormonal parameters.

Statistics

We estimated that a 25% improvement in the percentage of TESE+ would count as substantial, supporting TESE soon after puberty rather than waiting until adulthood. Calculation of the number of patients showed that 54 azoospermic patients should be included in each age group to reach power greater than 80%.

Clinical and biological characteristics were compared between TESE− and TESE+ groups and Young (<23 years) and Adult (≥23 years) groups. The χ^2 test was used to compare frequencies between categorical variables. Since age distribution was bimodal according to recruitment (fertility preservation or infertility) and the distributions of several variables, especially AMH and inhibin B values, were non-normal on Shapiro–Wilk test, data were presented as median and interquartile range and non-parametric tests were used for comparison and correlation analysis. For statistical analysis, the limit of

quantification was set at 4.9 ng/l for inhibin B and 1.84 pmol/l for AMH. All statistical analyses were carried out with IBM® SPSS Statistics software. The significance threshold was set at $P < 0.05$.

Results

Patients

Patient inclusion is shown in the flowchart (Fig. 1A and B). Out of 157 pubertal patients with non-mosaic 47,XXY karyotype recruited, 119 were azoospermic and completed the protocol up to and including TESE. Of the 31 patients who abandoned the process before TESE, 24 (77.4%) belonged to the Young group and seven (22.6%) belonged to the Adult group. This included three men who used sperm donation to as infertility management. Nine patients (7.6%) (three in the Young group and six in the Adult group) had a history of treated cryptorchidism with both testes in scrotal situation at inclusion. In the Young cohort, diagnosis was made prenatally because of maternal age in 23 patients (37.7%), during childhood in 11 patients (18%) and at puberty in 27 patients (44.3%). In the Adult cohort, diagnosis was made in adulthood due to infertility in 36 patients (62%), and due to other symptoms for four patients, while 13 were diagnosed at puberty, four during childhood and one prenatally because of maternal age.

Hormonal plasma levels and testicular volume did not differ between patients who abandoned the process and those who went on

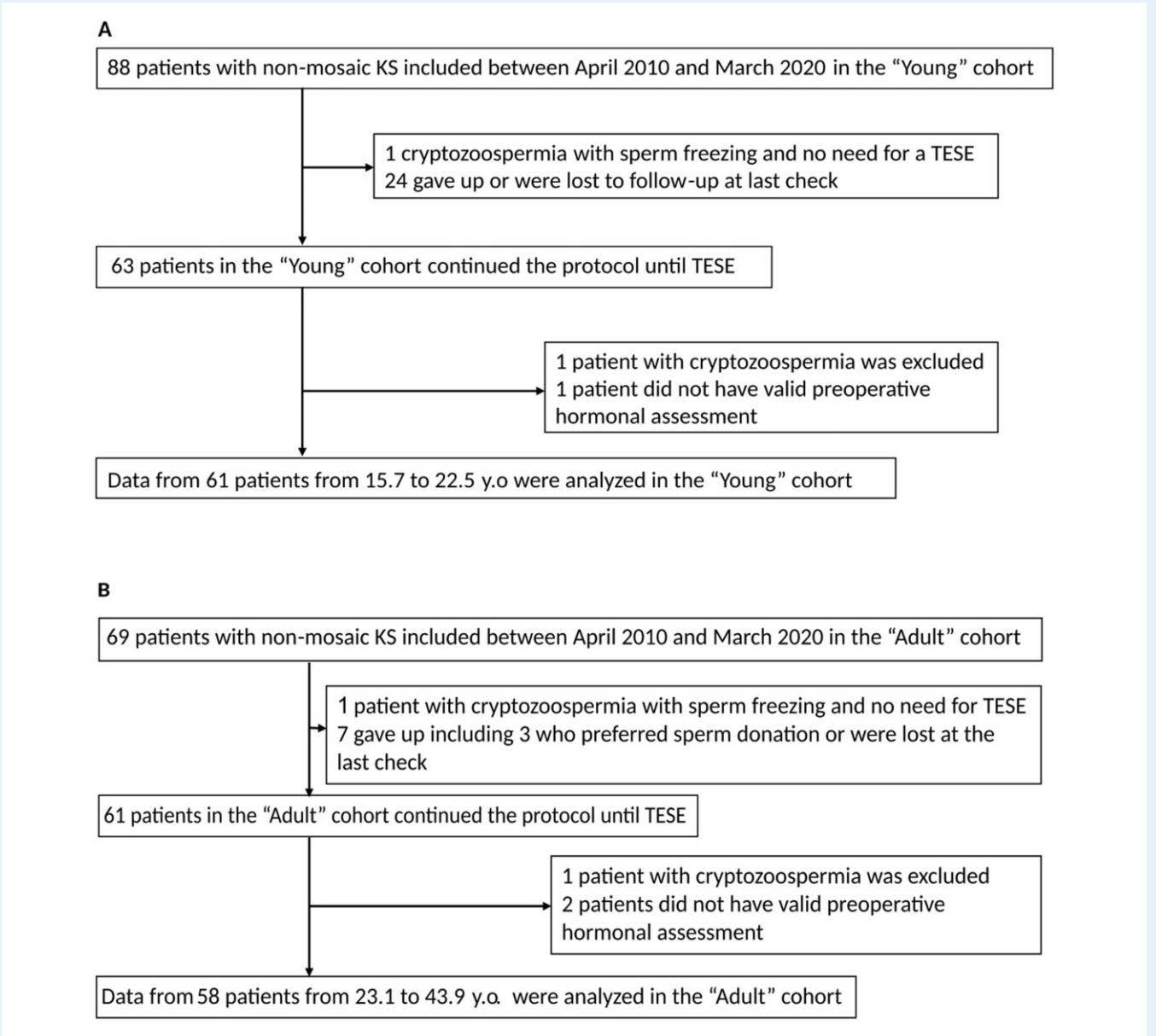

Figure 1. Flowchart. (**A**) Flowchart of the Young cohort. (**B**) Flowchart of the Adult cohort.

Table I Patient characteristics in the 'Young' and 'Adult' cohorts.

	Young	Adult	Young vs adult
	Median [IQR] range	Median [IQR] range	(Mann–Whitney)
Characteristic (unit) [normal range]			*P*-value
Age (years)	17.9 [16.8–19.7] 15.7–22.5	31.6 [28.7–34.4] 23.1–43.9	**<0.001**
AMH (pmol/l) [16.4–90.3]	12.7 [3.1–28.1] 1.84–153.6	1.84 [1.84–7] 1.84–46.0	**0.001**
Inhibin B (ng/l) [92–316]	7.0 [4.9–19.5] 4.9–124.0	4.9 [4.9–5.0] 4.9–30	**<0.001**
Testicular volume (R + L) (ml)	8.5 [6.3–10] 4–18	8.0 [6–10] 3–12	0.626
FSH (IU/l) [1.1–7.2]	34.1 [26.8–46.6] 8.1–114.2	28 [20.9–42.4] 8.7–88.6	0.086
LH (IU/l) [1.3–5.8]	17 [10.8–21.3] 5.6–79	16.6 [12.3–20] 7.2–40.0	0.960
Total testosterone (nmol/l) [10.4–26]	11.3 [8.0–14.8] 3.8–27.4	9.6 [6.9–13.6] 2.3–24	0.132
SHBG (nmol/l) [17–45]	29.5 [19.5–38] 9–74	27.0 [16–55.6] 5–64	0.451
Bioavailable testosterone (nmol/l) [2.25–10.70]	1.84 [1.31–2.7] 0.8–5.52	2.01 [1.29–2.54] 0.15–4.8	0.968
17β-estradiol (pmol/l) [66–139]	47.0 [33–66] 16.9–113	42.8 [27–65] 16.9–143	0.381
Prolactin (ng/ml) [4–15.2]	8.7 [6.3–11.4] 3.6–44.8	9.2 [7.4–10.7] 2.4–38.6	0.855

AMH, anti-Müllerian hormone; SHBG, sex hormone-binding globulin; R + L, right+left; IQR, interquartile range. Bold indicates statistically significant values.

to TESE (data not shown). Patient characteristics in both groups are shown in Table I.

TESE outcome

The global SRR was 45.4% (54/119). TESE was positive for 30/61 patients in the Young group (49.2%) and for 24/58 patients in the Adult group (41.4%); the difference was not significant ($P = 0.393$). Figure 2 shows the number of TESE+ and TESE− patients according to age. The median [IQR] age was 21.1 [17.9–30.1] years in the 54 TESE+ patients and 24.3 [17.8–32.2] years in the 65 TESE− patients; the difference was not significant ($P = 0.468$) (Table II).

In the Young group, the SRR was 47.8% (11/23) in the patients diagnosed prenatally, 63.6% (7/11) in those diagnosed during childhood and 44.4% (12/27) in those diagnosed at puberty.

In the Adult group, the patient diagnosed prenatally was TESE−, while the SRR was 50% (2/4) in patients diagnosed during childhood, 38.5% (5/13) in those diagnosed at puberty, and 42.5% (17/40) in those diagnosed during adulthood.

Potential predictive factors

Differences between TESE+ and TESE− patients were studied for the following potential predictive factors: testicular volume (R + L), FSH, LH, TT, sex hormone-binding globulin, bioavailable testosterone, 17β-estradiol, prolactin, inhibin B and AMH plasma levels. As shown in Table II, the differences between TESE+ and TESE− patients were significant only for AMH and inhibin B. AMH and inhibin B levels were positively correlated with each other ($\rho = 0.717$; $P < 0.001$).

As shown in Table III and in Fig. 3A and B, plasma levels of inhibin B and AMH were slightly higher in TESE+ than in TESE− patients and in the Young group than in the Adult group. The difference between TESE+ and TESE− patients in the proportion of inhibin B plasma concentrations below the limit of quantification was not significant, in the whole population ($P = 0.053$) or in the Young ($P = 0.054$) or the Adult cohort ($P = 0.629$); in contrast, the difference for AMH was significant for the whole population ($P = 0.014$), almost significant in the Young cohort ($P = 0.053$), but not in the Adult cohort ($P = 0.198$).

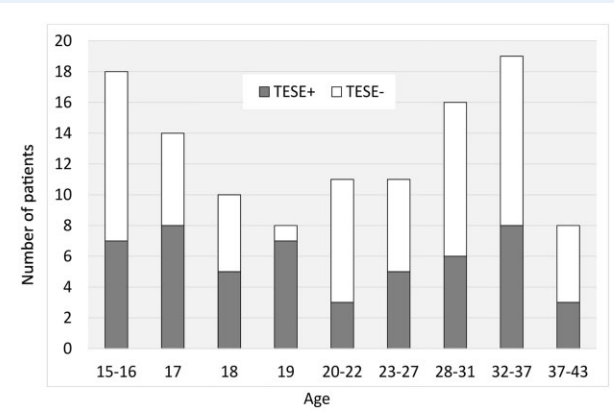

Figure 2. Testicular sperm extraction (TESE) outcome according to age. Histogram representing the number of patients with successful (TESE+) or negative (TESE−) sperm retrieval at different ages.

Both AMH and inhibin B plasma levels correlated with age ($\rho = -0.539$; $P < 0.001$ for AMH, and $\rho = -0.389$; $P < 0.001$ for inhibin B). AMH and inhibin B plasma levels were significantly higher in the Young group ($P = 0.001$ for AMH, $P < 0.001$ for inhibin B). None of the other parameters correlated with age (Supplementary Table SI). The age distribution of plasma AMH concentration according to TESE outcome is represented in Supplementary Fig. S1.

The usefulness of AMH and inhibin B plasma levels in predicting TESE outcome was investigated by receiving operator characteristic

Table II Population characteristics according to TESE outcome.

n (%)	Total 119 (100)	TESE+ 54 (45.4)	TESE− 65 (54.6)	TESE+ vs TESE− (Mann–Whitney)	ROC	Correlation with age
Potential prognostic factor [normal range]	**Median [IQR] range**	**Median [IQR] range**	**Median [IQR] range**	***P*-value**	**AUC [95% CI] *P*-value**	**ρ (Spearman) *P*-value**
Age (years)	22.4 [17.8–31.5] 15.7–43.9	21.1 [17.9–31.0] 15.7–43.2	24.3 [17.8–32.2] 15.8–43.9	0.468	0.539 [0.435–0.643] 0.468	–
AMH (pmol/l) [16.4–90.3]	6.1 [<1.85–16.0] <1.85–153.6	11.5 [3.0–19.3] <1.85–153.6	2.8 [<1.85–9.0] <1.85–98.1	**0.001**	**0.676** **[0.578–0.775]** **0.001**	**−0.539** **<0.001**
Inhibin B (ng/l) [92–316]	<5 [<5–10.0] <5–124.0	<5 [<5–14] <5–124.0	<5 <5–6.5 <5–62.0	**0.036**	0.598 [0.495–0.701] 0.066	**−0.389** **<0.001**
Testicular volume (R + L) (ml)	8.0 [6.0–10.0] 3.0–18.0	8.0 [6.0–10.0] 4.0–18.0	8.0 [6.0–10.0] 3.0–14.0	0.639	0.511 [0.406–0.616] 0.831	−0.120 0.205
FSH (IU/l) [1.1-7.2]	31.6 [23.2–44.8] 8.1–114.2	34.2 [22.6–45.0] 8.1–93.4	31.3 [24.1–44.9] 12.2–114.2	0.977	0.502 [0.396–0.607] 0.977	−0.107 0.247
LH (IU/l) [1.3–5.8]	16.9 [11.3–21.0] 5.6–79.0	16.6 [10.7–21.1] 6.5–79.0	17 [13.3–21.0] 5.6–52.4	0.479	0.538 [0.431–0.644] 0.479	0.132 0.151
Total testosterone (nmol/l) [10.4–26]	9.9 [7.1–14.0] 2.3–27.4	9.7 [7.0–14.8] 2.4–27.4	10.0 [7.2–14.0] 2.3–25.6	0.731	0.515 [0.413–0.624] 0.731	−0.141 0.126
SHBG (nmol/l) [17–45]	28.5 [18.0–38.3] 5.0–74.0	28.0 [17.5–39.0] 6.0–74.0	29.0 [19.5–37.5] 5.0–64.0	0.877	0.513 [0.408–0.619] 0.802	−0.047 0.613
Bioavailable testosterone (nmol/l) [2.25–10.70]	1.90 [1.31–2.60] 0.15–5.52	1.95 [1.26–2.53] 0.25–4.07	1.90 [1.40–2.71] 0.15–5.52	0.575	0.530 [0.425–0.635] 0.575	−0.063 0.497
17β-estradiol (pmol/l) [66–139]	46.0 [30.3–65.0] 16.9–143.0	46.0 [30.5–62.7] 16.9–143.0	46.0 [29.5–66.0] 16.9–113.0	0.848	0.515 [0.410–0.620] 0.783	−0.113 0.226
Prolactin (ng/ml) [4–15.2]	9.0 [7.0–11.2] 2.4–44.8	9.2 [7.1–10.9] 4.0–44.8	9.0 [6.6–12.1] 2.4–38.6	0.703	0.529 [0.425–0.633] 0.582	0.027 0.774

TESE+, successful TESE; TESE−, negative TESE; AMH, anti-Müllerian hormone; SHBG, sex hormone-binding globulin; R + L, right+left; ROC, receiver operating characteristics; IQR, interquartile range; TESE, testicular sperm extraction. Bold indicates statistically significant values.

(ROC) analysis (Table II and Fig. 4). The AUC was significantly different from 0.5 for AMH but did not reach significance for inhibin B. Figure 5 shows the ROC curve of AMH plasma levels in each age group. The AUC in the Young cohort was 0.683 (95% CI (0.546–0.820); $P = 0.009$) and in the Adult cohort, it was 0.642 (95% CI (0.491–0.792); $P = 0.066$). Sensitivity, specificity, predictive values and likelihood ratios were calculated for the limits between the quartiles of AMH plasma levels, in the whole group and in the Young and Adult cohorts (Supplementary Table SII). Tables IV and V show the SRRs according to AMH (Table IV) and inhibin B (Table V) plasma level quartiles in the whole population and in the Young and Adult groups. Differences between quartiles were significant for AMH but not for inhibin B.

In the sub-group of the nine patients with history of treated cryptorchidism, none of the three in the Young cohort and four of the six in the Adult cohort had positive TESE. The median AMH plasma level was 3 pmol/l (range 2.3–76.9 pmol/l) in the Young cohort and 4.9 pmol/l (range 1–46 pmol/l) in the Adult cohort.

Table III AMH and inhibin B plasma levels according to age group and TESE outcome.

	Young			Adult		
	TESE+ Median [IQR] range	TESE− Median [IQR] range	*P*-value	TESE+ Median [IQR] range	TESE− Median [IQR] range	*P*-value
AMH (pmol/l) [16.4–90.3]	17 [8.3–34.1] 1.84–153.6	8.5 [1.9–19.0] 1.84–98.1	**$P = 0.013$**	4.4 [<1.85–11.7] <1.85–46	<1.85 [<1.85–5] <1.85–34.3	**$P = 0.049$**
Proportion of AMH <1.85 pmol/l	1/30 (3.3%)	7/31 (22.6%)	$P = 0.053$*	10/24 (41.6%)	20/34 (58.8%)	$P = 0.198$
InhB (ng/l) [92–316]	10 [<5–24.3] <5–124	<5 [<5–13] <5–62	$P = 0.067$	<5 [<5–5.8] <5–30	<5 [<5–5] <5–17	$P = 0.602$
Proportion of InhB <5ng/l	11/30 (36.7%)	19/31 (61.3%)	$P = 0.054$	17/24 (70.8%)	26/34 (76.5%)	$P = 0.629$

TESE+, successful TESE; TESE−, negative TESE; AMH, anti-Müllerian Hormone, InhB, inhibin B; IQR, interquartile range; TESE, testicular sperm extraction.
Bold indicates statistically significant values.
*Because of the low number of patients, we used Fisher exact test.

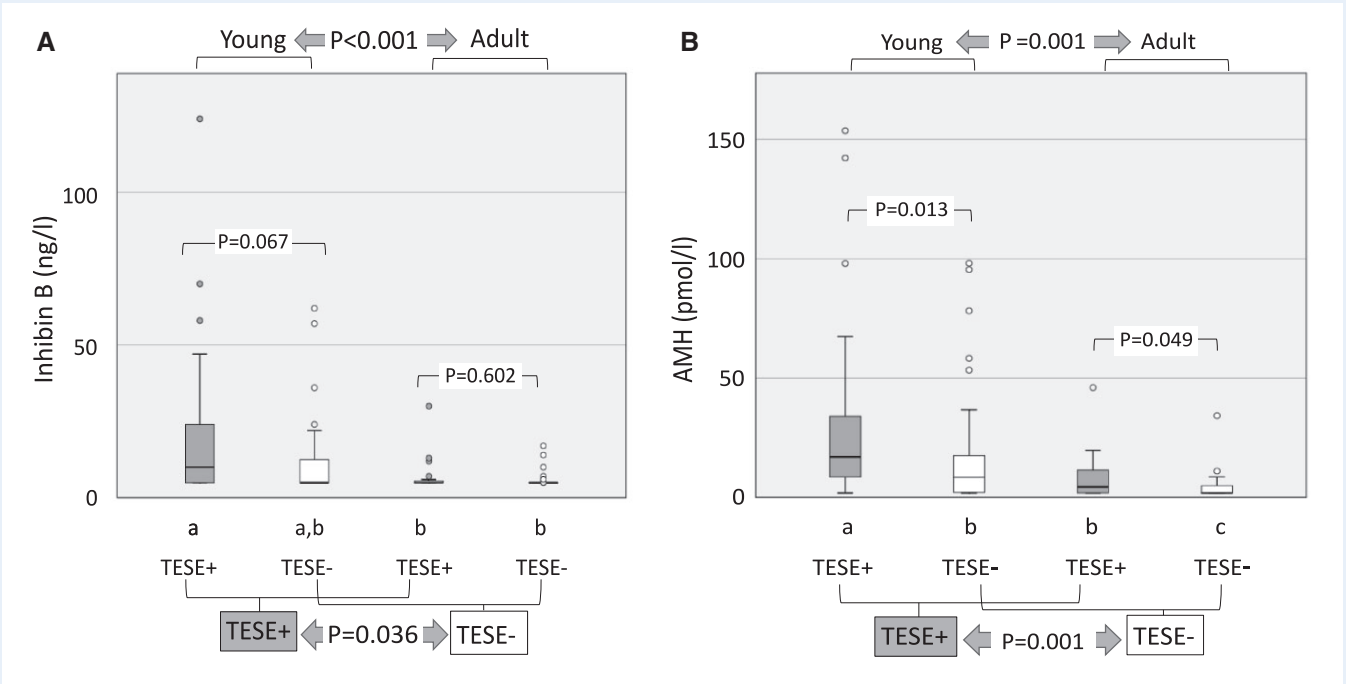

Figure 3. Box plot of inhibin B and AMH plasma levels according to age group and testicular sperm extraction (TESE) outcome. (**A**) Inhibin B plasma levels: Comparisons between patients with successful TESE (TESE+, in gray) and negative TESE (TESE−, in white) and between Young and Adult groups were performed by Mann–Whitney *U* test. Comparisons between the four subgroups (Young-TESE+, Young-TESE−, Adult-TESE+ and Adult-TESE−) by Kruskal–Wallis test were significant ($P<0.001$). Pairwise comparisons of the four subgroups by the Mann–Whitney *U* test are summarized with letters; conditions labeled with the same letter did not significantly differ (Young-TESE+ versus Young-TESE−: $P = 0.067$; Adult-TESE+ versus Adult-TESE−: $P = 0.602$). (**B**) AMH plasma levels: Comparisons between patients with successful TESE (TESE+, in gray) and negative TESE (TESE−, in white) and between Young and Adult groups were performed by Mann–Whitney *U* test. Comparisons between the four subgroups (Young-TESE+, Young-TESE−, Adult-TESE+ and Adult-TESE−) by Kruskal–Wallis test were significant ($P<0.001$). Pairwise comparisons of the four subgroups by the Mann–Whitney *U* test are summarized with letters; conditions labeled with the same letter did not significantly differ (Young-TESE+ versus Young-TESE−: $P = 0.013$; Adult-TESE+ versus Adult-TESE−: $P = 0.049$).

Histopathology

The maximal Johnsen's score according to TESE outcome was significantly higher in TESE+ than in TESE− patients ($P < 0.001$). The difference according to age group was not significant ($P = 0.322$) (Fig. 6). A significant correlation was obtained with AMH ($\rho = 0.381$; $P < 0.001$) and inhibin B ($\rho = 0.268$; $P = 0.004$) plasma levels.

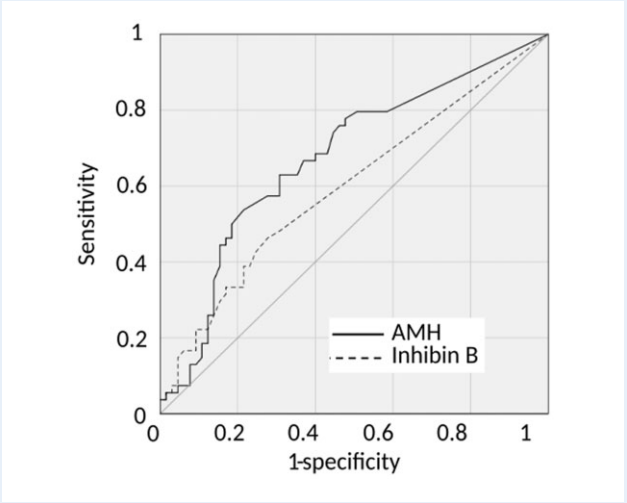

Figure 4. ROC curve of AMH (plain) and inhibin B (dotted) plasma levels to predict positive TESE in non-mosaic 47,XXY KS. The area under the curve [95% CI] was 0.676 [0.578–0.775] ($P = 0.001$) for AMH and 0.598 [0.495–0.701] ($P = 0.066$) for inhibin B plasma levels. ROC, receiver operator characteristic; TESE, testicular sperm extraction.

Previous testosterone therapy

There were 32 patients (14 in the Young group and 18 in the Adult group) who had been under testosterone treatment, withdrawn for at least 6 months before inclusion and 9 months before TESE. The SRRs were 50% (16/32) for the previously treated cases and 43.7% (38/87) for patients who had never received testosterone ($P = 0.539$). The duration of testosterone treatment was 25.5 [10–90] months in TESE+ patients and 34.0 [10–109] months in TESE− patients ($P = 0.546$). AMH plasma levels (median (IQR)) were similar whether patients had received prior testosterone treatment (6.07 (<1.85–16.0) pmol/l) or not (5.73 (<1.85–16.0) pmol/l; $P = 0.944$).

Discussion

Since the late 1990s, TESE-ICSI has enabled paternity in non-mosaic 47,XXY KS patients (Palermo *et al.*, 1998; Reubinoff *et al.*, 1998). The SRR was 44% in the meta-analysis by Corona *et al.* (2017) including 1248 patients. Factors influencing TESE outcome have been investigated with very heterogeneous results, identifying age, hormonal levels, TESE procedure, previous testosterone treatment, treatment designed to increase testosterone secretion (hCG, aromatase inhibitors, antiestrogens), and/or hCG-stimulated testosterone levels (Westlander *et al.*, 2001; Madgar, 2002; Vernaeve *et al.*, 2004; Okada *et al.*, 2005; Bakircioglu *et al.*, 2006, 2011; Koga *et al.*, 2007; Kyono *et al.*, 2007; Ferhi *et al.*, 2009; Ramasamy *et al.*, 2009; Yarali *et al.*, 2009; Selice *et al.*, 2010; Greco *et al.*, 2013; Madureira *et al.*, 2014; Sabbaghian *et al.*, 2014; Plotton *et al.*, 2015; Rohayem *et al.*, 2015; Franik *et al.*, 2016; Majzoub *et al.*, 2016; Vicdan *et al.*, 2016; Chehrazi *et al.*, 2017; Garolla *et al.*, 2018; Ozer *et al.*, 2018; Vloeberghs *et al.*, 2018; Guo *et al.*, 2020; Huang *et al.*,

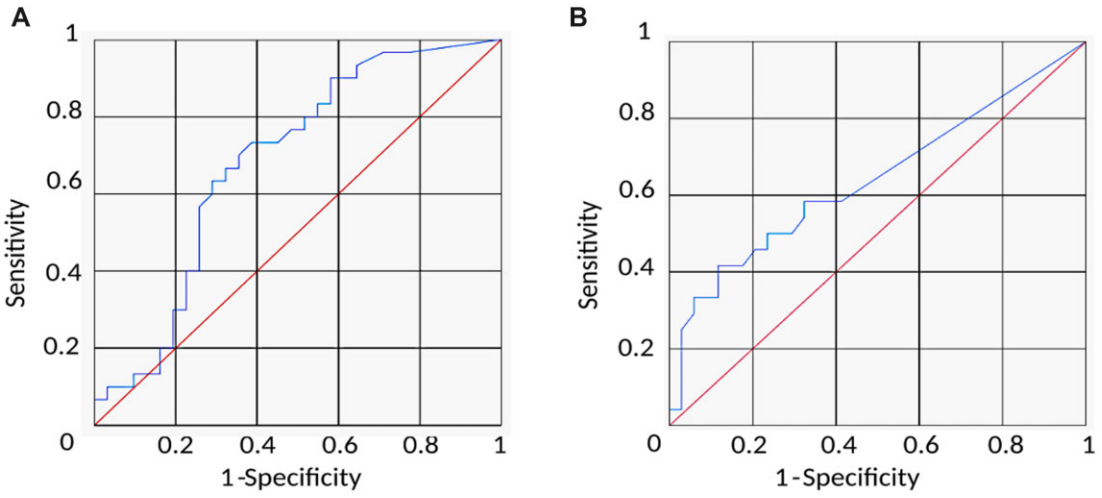

Figure 5. ROC curve of AMH plasma levels to predict positive TESE in non-mosaic 47,XXY KS in the Young cohort (A) and in the Adult cohort (B). The area under the curve [95% CI] was 0.683; 95% CI [0.546–0.820]; $P = 0.009$ for the young cohort and was 0.642; 95% CI [0.491–0.792]; $P = 0.066$ for the adult cohort. ROC, receiver operator characteristic; TESE, testicular sperm extraction.

Table IV Sperm retrieval rate according to AMH plasma levels in non-mosaic 47,XXY karyotype KS.

Whole cohort		
Subgroups	**AMH (pmol/l)**	**SRR(%) (N TESE+/N patients)**
<quantification limit	AMH <1.85	28.9 (11/38)
2nd quartile*	1.85 ≤ AMH <5.8	33.3 (7/21)
3rd quartile	5.8 ≤ AMH <16	51.7 (15/29)
4th quartile	AMH ≥16	67.7 (21/31)
Total		45.4 (54/119)
P-value		**0.007**
Young group		
Subgroups	**AMH (pmol/l)**	**SRR(%) (N TESE+/N patients)**
1st quartile	AMH <3.1	20.0 (3/15)
2nd quartile	3.1 ≤ AMH <12.7	46.7 (7/15)
3rd quartile	12.7 ≤ AMH <28.1	68.8 (11/16)
4th quartile	AMH ≥28.1	60.0 (9/15)
Total		49.2 (30/61)
P-value		**0.040**
Adult group		
Subgroups	**AMH (pmol/l)**	**SRR(%) (N TESE+/N Patients)**
<quantification limit	AMH <1.85	33.3 (10/30)
3rd quartile	1.85 ≤ AMH <7	28.6 (4/14)
4th quartile	AMH ≥7	71.4 (10/14)
Total		41.4 (24/58)
P-value		**0.031**

SRR, sperm retrieval rate; AMH, anti-Müllerian hormone; TESE+, successful TESE; *P*-values on χ^2 test; N, number; TESE, testicular sperm extraction; KS, Klinefelter syndrome.
Bold indicates statistically significant values.
*The number of patients in this subgroup was lower than expected because of data below the limit of quantification.

Table V Sperm retrieval rate according to inhibin B plasma levels in non-mosaic 47,XXY karyotype KS.

Whole cohort		
Subgroups	**Inhibin B (ng/l)**	**SRR(%) (N TESE+/N patients)**
<quantification limit	Inhibin B < 5	38.4 (28/73)
3rd quartile*	5 ≤ InhB <10	57.1 (8/14)
4th quartile	InhB ≥10	56.3 (18/32)
Total		45.5 (54/119)
P-value		0.153
Young group		
Subgroups	**Inhibin B (ng/l)**	**SRR(%) (N TESE+/N patients)**
<quantification limit	Inhibin B < 5	36.7 (11/30)
3rd quartile	5 ≤ InhB <19.5	62.5 (10/16)
4th quartile	InhB ≥19.5	60.0 (9/15)
Total		49.2 (30/61)
P-value		0.156
Adult group		
Subgroups	**Inhibin B (ng/l)**	**SRR(%) (N TESE+/N patients)**
<quantification limit	Inhibin B < 5	39.5 (17/43)
4th Quartile	InhB ≥5.0	46.7 (7/15)
Total		41.4 (24/58)
P-value		0.629

SRR, sperm retrieval rate; InhB, inhibin B; TESE+, successful TESE; TESE, testicular sperm extraction; KS, Klinefelter syndrome.
P-values on χ^2 test. Bold indicates statistically significant values.
*The number of patients in this subgroup was lower than expected because of data below the limit of quantification.

2020; Pozzi *et al.*, 2020; Yücel *et al.*, 2021; Özkan *et al.*, 2022). To date, none of these factors have been shown to be predictive (Corona *et al.*, 2017; Majzoub et al., 2022).

In the literature, in 13 of the 30 cohorts of KS patients recruited for infertility, aging was associated with lower SRR (Okada *et al.*, 2005; Bakircioglu *et al.*, 2006, 2011; Kyono *et al.*, 2007; Ferhi *et al.*, 2009; Ramasamy *et al.*, 2009; Yarali *et al.*, 2009; Sabbaghian *et al.*, 2014; Chehrazi *et al.*, 2017; Garolla *et al.*, 2018; Ozer *et al.*, 2018; Vloeberghs *et al.*, 2018; Yücel *et al.*, 2021; Özkan *et al.*, 2022), with a threshold at about 30–35 years (Okada *et al.*, 2005; Bakircioglu *et al.*, 2006; Kyono *et al.*, 2007; Ferhi *et al.*, 2009; Ramasamy *et al.*, 2009; Ozer *et al.*, 2018; Yücel *et al.*, 2021). Thus, a progressive decrease in focal spermatogenesis from adolescent to older KS patients was suggested, raising the possibility that SRR could be more successful when TESE is performed soon after puberty. To investigate this, we prospectively compared two groups of non-mosaic 47,XXY KS patients enrolled in parallel. The global SRR of 45.4% was similar to previous reports in large cohorts (Bakircioglu *et al.*, 2006; Rohayem *et al.*, 2015; Vicdan *et al.*, 2016; Guo *et al.*, 2020) and meta-analyses (Corona *et al.*, 2017; Majzoub *et al.*, 2022).

The two age cohorts were comparable in terms of testicular volume and most hormone levels, but the modality of diagnosis differed. However, the SRRs were similar regardless of diagnosis modality, which therefore probably did not bias our results. In addition, the proportion of patients who abandoned the protocol was higher in the Young cohort. This could be explained by the fact that young patients are less interested in fertility issues. In addition, few hospitals practice TESE in young patients, unlike in adult patients, and therefore many of the young patients came from places far from our center, increasing the complexity of care. The number of patients with history of cryptorchidism in our cohort was lower than previously reported in KS patients (Lanfranco *et al.*, 2004) because we did not include patients with persistent cryptorchidism (untreated or after unsuccessful orchidopexy).

The difference in SRR between young patients aged 15.7–22 years (49.2%) and patients aged between 23 and 43.9 years (41.4%) was not statistically significant. This confirms, in a larger population, our preliminary report (Plotton *et al.*, 2015). Rohayem *et al.* (2015) also reported, in a retrospective study of KS with a larger age range, an SRR of 38% in 50 teenagers (13–19 years) and 31% in 85 adults (20 to >60 years) and the difference was not significant; though it should be noted that SRR was low (10%) in the youngest group (13–14 years). In the present study, younger patients were asked to wait until the age of 15 to be recruited. Low SRRs were also found in studies including younger

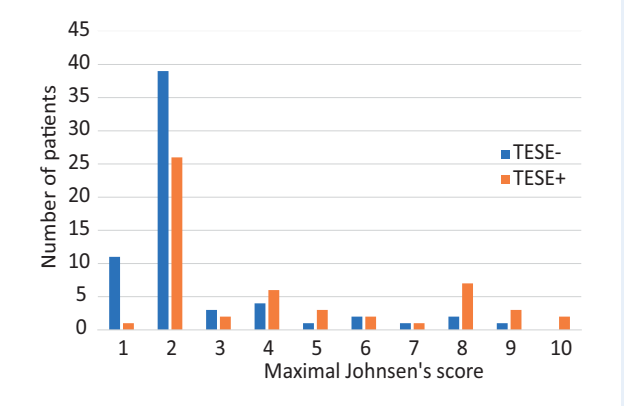

Figure 6. Maximal Johnsen's score according to TESE outcome. Histogram representing the number of patients for each maximal Johnsen's score in TESE+ (orange) and TESE− (blue) patients. TESE, testicular sperm extraction. Score 1 = no seminiferous epithelium; 2 = Sertoli cell only; 3 = spermatogonia only; 4 = no spermatozoa or spermatids, few spermatocytes; 5 = no spermatozoa or spermatids, many spermatocytes; 6 = no spermatozoa, no late spermatids, few early spermatids; 7 = no spermatozoa, no late spermatids, many early spermatids; 8 = less than five spermatozoa per tubule, few late spermatids; 9 = slightly impaired spermatogenesis, many late spermatids, disorganized epithelium; 10 = full spermatogenesis.

patients (Wikström *et al.*, 2007; Gies *et al.*, 2012; Van Saen *et al.*, 2012, 2018; Rives *et al.*, 2013; Heckmann *et al.*, 2018). This could be explained by the fact that focal spermatogenesis was not complete in these very young patients. Some of these studies were designed with a view to cryopreserving spermatogonia (Gies *et al.*, 2012; Van Saen *et al.*, 2012; Rives *et al.*, 2013; Heckmann *et al.*, 2018), which may decrease the amount of testicular tissue available for further TESE and therefore is not recommended (Zitzmann *et al.*, 2021). Since we did not find a significant difference in SRR between the Young and the Adult groups of non-mosaic 47,XXY KS patients, our data further support the recommendation by the European Academy of Andrology to perform TESE between 20 and 30 years of age (Zitzmann *et al.*, 2021). This allows younger patients to be psychologically mature enough and avoids the possible degradation of SRR in older KS patients.

c-TESE was used in our study. Several studies have compared c-TESE and m-TESE outcomes in NOA patients (Punjani *et al.*, 2021). In 2015, a meta-analysis of 15 comparative studies with almost 2000 patients showed that m-TESE had a 1.5-fold greater likelihood of successful sperm retrieval than c-TESE (Bernie *et al.*, 2015). On the contrary, Corona *et al.*'s meta-analysis including over 21 000 patients with various etiologies for NOA (Corona *et al.*, 2019) and a meta-analysis including only KS patients (Corona *et al.*, 2017), found no difference between the two techniques. Table VI gives an update of the SRRs reported in the literature in m-TESE versus c-TESE; the difference does not reach significance. Thus, the superiority of m-TESE over c-TESE, if any, is not definitively demonstrated.

Table VI Literature review of TESE outcomes in KS patients according to surgical technique (conventional versus microsurgical TESE).

References with c-TESE only	c-TESE n	c-TESE+ n (SRR)	References with m-TESE only	m-TESE n	m-TESE+ n (SRR)
(Vernaeve *et al.*, 2004)	50	24 (48%)	(Schiff *et al.*, 2005)	29	29 (69%)
(Kyono *et al.*, 2007)	17	6 (53.3%)	(Bakircioglu *et al.*, 2006)	74	42 (56.7%)
(Ferhi *et al.*, 2009)	27	8 (29.6%)	(Koga *et al.*, 2007)	26	13 (50%)
(Madureira *et al.*, 2014)	65	25 (38.5%)	(Ramasamy *et al.*, 2009)	68	45 (66%)
(Majzoub *et al.*, 2016)	43	6 (14%)	(Yarali *et al.*, 2009)	22	22 (56%)
(Franik *et al.*, 2016)	9	3 (33.3%)	(Selice *et al.*, 2010)	26	9 (37.5%)
(Garolla *et al.*, 2018)	111	38 (34.2%)	(Bakircioglu *et al.*, 2011)	106	50 (47%)
Current data	119	54 (45.4%)	(Sabbaghian *et al.*, 2014)	134	38 (28.4%)
			(Rohayem *et al.*, 2015) *young*	50	19 (38%)
			(Rohayem *et al.*, 2015) *adult*	85	26 (30.6%)
			(Chehrazi *et al.*, 2017)	134	38 (28.4%)
			(Ozer *et al.*, 2018)	110	22 (20%)
			(Vloeberghs *et al.*, 2018)	138	48 (34.8%)
			(Huang *et al.*, 2020)	66	24 (34.6%)
			(Guo *et al.*, 2020)	184	80 (43.5%)
			(Özkan *et al.*, 2022)	67	35 (52.2%)
Total	**441**	**164 (37.19%)**	**Total**	**1074**	**380 (35.38%)**[§]

c-TESE, conventional TESE; m-TESE, microsurgical TESE; SRR, sperm retrieval rate; TESE, testicular sperm extraction; KS, Klinefelter syndrome.
Bold indicates statistically significant values.
[§]c-TESE versus m-TESE: $P = 0.505$.

We cannot be sure that our results would have been similar with microsurgical m-TESE. However, testicular biopsy for TESE was performed by the same experienced surgeon (B.C.) throughout the study. Since the amount of tissue removed for TESE was similar in all patients, we can reasonably assume that the comparison between the groups was valid.

Previous testosterone treatment, withdrawn at least 9 months before TESE to avoid a possible decrease in gonadotropin secretion, did not decrease SRR in our study. This lack of a deleterious effect of a previous testosterone treatment was also found in five out of six other studies (Mehta *et al.*, 2013; Plotton *et al.*, 2015; Rohayem *et al.*, 2015; Garolla *et al.*, 2018; Boeri *et al.*, 2020). Schiff *et al.*, (2005) reported a deleterious effect, but they included only five patients with previous testosterone therapy. Since testosterone treatment could have decreased AMH secretion, we checked that AMH plasma levels did not differ according to prior testosterone therapy. Treatments designed to increase testosterone secretion were reported in six out of 28 studies (Schiff *et al.*, 2005; Ramasamy *et al.*, 2009; Rohayem *et al.*, 2015; Majzoub *et al.*, 2016; Ozer *et al.*, 2018; Guo *et al.*, 2020), without consistent results for SRR. Only patients with low testosterone levels were treated, which introduces a selection bias that prevents a comparison of SRR between treated and non-treated patients. No treatment was administered before TESE in our study.

Among hormonal parameters, the FSH plasma level was significantly lower in TESE+ KS patients in only two out of 28 studies (Vernaeve *et al.*, 2004; Ozer *et al.*, 2018), LH was significantly lower in only one out of 23 (Rohayem *et al.*, 2015), testosterone was higher in seven out of 26 (Ramasamy *et al.*, 2009; Sabbaghian *et al.*, 2014; Majzoub *et al.*, 2016; Chehrazi *et al.*, 2017; Ozer *et al.*, 2018; Vloeberghs *et al.*, 2018; Guo *et al.*, 2020) and testicular volume was higher in two out of 19 (Madgar, 2002; Özkan *et al.*, 2022). None of these factors affected TESE outcome in the present study.

AMH and inhibin B plasma levels were the only factors correlating with SRR in our study. They are both secreted by Sertoli cells. Plasma levels are normal in infants with non-mosaic KS and decline after puberty (Bastida *et al.*, 2007; Aksglaede *et al.*, 2010). The inhibin B plasma level is lower than the limit of quantification in the majority of adult KS patients. This is also the case for AMH plasma levels, in a smaller proportion of cases. This induced difficulties in comparing AMH, and especially inhibin B, in KS patients according to TESE outcome and according to age group. We compared the percentage of patients with AMH and inhibin B plasma levels below the limit of quantification and also compared plasma levels by non-parametric methods. As expected, AMH and inhibin B plasma levels correlated with age and were significantly higher in our Young group. AMH plasma levels were clearly higher in TESE+ patients in the whole population ($P = 0.001$). The differences were also significant in the Young group, and even in the Adult group. A weaker difference was found for inhibin B, with slightly higher plasma levels in TESE+ than in TESE− patients in the overall population; the difference did not remain significant taking each age group separately. The large proportion of patients with inhibin B plasma levels below the limit of quantification, especially in Adults, could prevent a significant difference emerging. AMH and inhibin B plasma levels correlated with each other; this probably reflects their common origin in Sertoli cells.

Rohayem *et al.* measured AMH and inhibin B plasma levels only in their teenage group. As in the present study, the majority of patients had an undetectable inhibin B plasma level. The authors found that AMH plasma levels did not differ according to TESE outcome, but their series included patients younger than 15 years, who had high AMH plasma levels and a low SRR, as already mentioned. This could explain the lack of difference in AMH plasma levels in their study.

ROC curves showed that AMH had a better predictive value than inhibin B, although the shape of the ROC curves did not reveal a clear cutoff value for AMH plasma level that could rule out TESE. Nevertheless, SRRs were clearly lower when AMH plasma levels were below the limit of quantification or in the lower quartile (SRR about 30%) than in the upper quartile (SRR about 70%). The difference was less clear for inhibin B. Nevertheless, even if the AMH or inhibin B plasma level is below the limit of quantification, TESE may be positive. Thus, we did not confirm the results of Benderradji *et al.* (2021), for whom a serum concentration of AMH under 2.5 pmol/l predicted negative TESE in a cohort of 19 non-mosaic KS patients.

Aboukhshaba *et al.* (2021) showed that the AMH plasma level was moderately predictive of m-TESE success in a cohort of 46 patients with NOA, including three with KS.

Higher AMH and inhibin B plasma levels in TESE+ cases support the hypothesis that, in seminiferous tubule foci with conserved spermatogenesis, Sertoli cells remain more functional due to improved Sertoli-germ cell paracrine cross-talk. However, since AMH and inhibin B plasma levels decrease with age (Aksglaede *et al.*, 2006), age must be taken into account for interpretation. In addition, AMH and inhibin B plasma levels were found to be lower in case of history of cryptorchidism (Hamdi *et al.*, 2017) but, given the low proportion of history of cryptorchidism in our cohort because of the selection criteria, this would probably not impact our result. Recently, a genome-wide association study by Greiber *et al.* (2018) found that an *AMH* variant (minor allele frequency 16%) was associated with higher AMH levels in prepubertal and adolescent boys. It could be interesting to know whether this variant is associated with a better SRR in our population. A further study would be required.

Discrepancies between TESE results and maximal Johnsen's score, as seen in Fig. 6, could be explained by the mosaicism of seminiferous tubules. Testicular samples containing seminiferous tubules with focal spermatogenesis may be present or not in the testicular samples destined for TESE or for histopathological study. In addition, there seems to be a correlation between AMH and inhibin B plasma levels with Johnsen's score. This suggests a possible link between the level of gonadal dysgenesis and the level of hormone production.

The strength of the present study lies in its prospective design and the relatively large number of patients. As this was a single-center study and patient recruitment was made in parallel, management was homogeneous across age groups, enabling good comparison.

The weakness of the study was that the hormonal markers, inhibin B and AMH, were at low levels in the KS population and below the limit of quantification in several patients. Development of assays with improved sensitivity would increase the prognostic value of these markers in the future.

Conclusion

The present cohort of 119 prospectively included patients showed higher AMH and inhibin B plasma levels in KS patients with successful

sperm retrieval after c-TESE. The predictive value for TESE outcome was far from absolute and no threshold value could be defined to rule out TESE. Nevertheless, higher AMH and inhibin B plasma levels seemed to be related to the presence of foci of spermatogenesis, in which Sertoli cell functions are improved, in contact with germ cells with a 46, XY chromosomal complement, which are able to enter into meiosis and form spermatozoa.

In addition, we confirmed the results of our previous study of the impact of age. The SRR did not differ between young (15–22 years) and adult (23–43 years) KS patients. We also confirmed that testosterone treatment did not reduce the chances of sperm retrieval when discontinued at least 9 months before surgery.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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Authors' roles

H.L. and I.P. designed the study. I.P. was responsible for the logistical aspects of the study. L.R. extracted data and wrote the manuscript. B.C. performed all the testicular biopsies for TESE. E.L., S.G.d.E., M.B., J.L. and G.S. performed sperm analysis, sperm extraction and cryopreservation. M.L., E.F., L.P.-C., A.B. and B.S. referred patients from infertility consultations in our department. A.d.S. did the psychological evaluation of all the patients of the Young group and some of the Adult group. F.D. did the pathological analysis of the biopsies. D.S. and C.S.-B. performed the genetic analysis to confirm non-mosaic KS. R.E. and H.L. did the statistical analysis. All named authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship for this article, take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.

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Conflict of interest

The authors have no conflicts of interest to disclose.

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