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Determining the expression levels of *CSF-1* and *OCT4*, *CREM-1*, and protamine in testicular biopsies of adult Klinefelter patients: Their possible correlation with spermatogenesis

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Abstract

Klinefelter syndrome (KS) is the most prevalent genetic disorder of infertile males. This study aimed to determine in Klinefelter patients (KS) the expression levels of spermatogenic markers and testicular growth factors that might predict spermatogenesis based on conventional testicular sperm extraction (TESE). The expression levels of the pre-meiotic (*OCT4*, *CD9*, *GFR-α1*, *α-6-INTEGRIN*, *SALL4*, *C-KIT*), meiotic (*CREM-1*), and post-meiotic (protamine) markers, as well as the colony stimulating factor-1 (*CSF-1*) were examined in testicular biopsies with and without mature sperm of KS and normal karyotype of azoospermic patients (AZO) with complete spermatogenesis. In the biopsies of AZO, the expression levels (fold of expression compared to the PPI of the same sample) of *OCT4* were 9.68± 7.93, *CREM* 42.78± 28.22, *CSF-1* 3.07 ± 3.19, and protamine 78498.12 ± 73214.40. Biopsies from KS included 7 with sperm and 17 without sperm. Among the biopsies with sperm, the expression levels of *OCT4* were 7.27± 9.29, *CREM* 3.13± 7.89, *CSF-1* 35.5 ± 48.01, and protamine 902.97 ± 2365.92. In 14 biopsies without sperm, we found low expression levels of *OCT4*, *CREM* and *CSF-1*, and no expression of protamine. However, in three of the biopsies without sperm that highly expressed *OCT4* and *CSF-1*, the expression levels of *CREM*-1 and protamine were high. These results may be used for further consulting with patients considering repeating conventional TESE or micro TESE and cryopreservation for possible future in-vitro spermatogenesis.

KEYWORDS
Klinefelter patients, male infertility, NOA patients, *OCT4*, spermatogenesis

1 | INTRODUCTION

Klinefelter syndrome (KS) is the most prevalent genetic disorder, occurring in about 0.2% of males, 3%–4% of those who are barren (infertile), and consists of 11% of azoospermic (AZO) cases (Forti et al., 2010; Jacobs & Strong, 1959; Klinefelter et al., 1942; Morris et al., 2008; Nielsen & Wohlert, 1991). It is caused by non-disjunction at the meiotic or mitotic stage. The karyotype of KS patients is non-

mosaic (47, XXY) in about 90% of the cases. More critical aneuploidies (48, XXYY; 48, XXXY; 49, XXXXY), in addition to the mosaicism (46, XY/47, XXY), comprise approximately 10% of cases (Kim et al., 2017; Lanfranco et al., 2004; Rajender & Oates, 2018).

The phenotypic characterizations of KS patients vary, but usually include small testes, hypogonadism, high gonadotrophins levels, relatively low testosterone (T) and AZO (Bonomi et al., 2017; Groth et al., 2013; Kamischke et al., 2003; Kim et al., 2017; Lanfranco et al., 2004). The levels of testosterone reflect the number and function of Leydig cells. Aksglaede et al., 2007 reported that around 66% of KS patients between 20 and 40 years old had normal testosterone levels.

Throughout puberty, the testes of KS patients start to deteriorate, changing to abnormal seminiferous tubules that show hyalinization and fibrosis. This process leads to Leydig cell hyperplasia and severe germ cell diminution (or depletion) in 95% of KS patients (Aksglaede et al., 2007; Gordon et al., 1972; Groth et al., 2013; Klinefelter et al., 1942; Rohayem et al., 2015; Wikström et al., 2004; Wikström & Dunkel, 2011). Nevertheless, spermatogenesis spots can be found in the seminiferous tubules of KS patients, enabling the presence of sperm in the ejaculate in around 8.4% of patients and the presence of sperm in testicular sperm extraction (TESE) in around 40% of KS patients (Corona et al., 2017; Lanfranco et al., 2004; Van Saen et al., 2018; Wikström & Dunkel, 2008). However, factors predicting successful TESE are still controversial (Corona et al., 2017). Also, arguments still exist concerning the aetiology and mechanism of impaired spermatogenesis, which includes genetic, epigenetic, and environmental testicular factors (Franik et al., 2016; Gies et al., 2016).

Spermatogenesis is a complicated process involving spermatogonial stem cells (SSCs) that proliferate and differentiate to generate mature sperm (De Rooij & Grootegoed, 1998; Fayomi & Orwig, 2018; Phillips et al., 2010; Tagelenbosch & de Rooij, 1993). The number of SSCs is very low (Phillips et al., 2010; Tagelenbosch & de Rooij, 1993). Under normal conditions, SSCs proliferate, and their progeny are highly proliferative cells that differentiate to generate meiotic and post-meiotic cells, and thereafter by spermiogenesis, develop millions of mature sperm daily (De Rooij & Grootegoed, 1998; Fayomi & Orwig, 2018; La & Hobbs, 2019; Phillips et al., 2010; Tagelenbosch & de Rooij, 1993). This process is under the regulation of endocrine/autocrine/paracrine factors produced mainly by the Sertoli cells (SCs), other somatic cells in the testes, and other endocrine organs. Sertoli cells are considered to be the major cellular component in the seminiferous tubules that provides a suitable niche for spermatogonial stem cell (SSC) development (Meng et al., 2000). They produce crucial growth factors sustaining SSCs division and maturation. One of these factors is glial cell line-derived neurotropic factor (GDNF), which regulates SSCs proliferation. In addition, SCs and Leydig cells produce colony-stimulating factor-1 (*CSF-1*). SCs also produce fibroblast growth factor 2 (FGF2) and other factors that are suggested to be essential for SSCs self-renewal. In addition, SCs secrete factors important for SSCs differentiation, such as stem cell factor (SCF) and others (De Rooij, 2009; Meng et al., 2000; Oatley et al., 2009; Oliver & Stukenborg, 2020; Takashima et al., 2015).

Spermatogonial cells express specific molecular markers such as glial cell line-derived nerve factor family receptor alpha 1 (GFR-α1), promyelocytic leukaemia zinc finger protein (PLZF), octamer-binding transcription factor 4 (*OCT4*), cluster of differentiation 9 (CD9), *α6*-integrin, b1-integrin, G protein-coupled receptor 125 (GPR125), c-KIT, CD133, SSEA4, VASA, DAZL, and MAGEA4. Meiotic and post-meiotic cells express different markers including BOULE, CREM, acrosin, protamine, and others (Conrad et al., 2008; Dym et al., 2009; Fayomi & Orwig, 2018; Hermann et al., 2010; Huleihel et al., 2015; Zohni et al., 2012).

However, knowledge about the presence of different spermatogonial cells in the testes of KS patients is still limited (Braye et al., 2019; Deebel et al., 2020; Gies et al., 2012; Goossens et al., 2013; Van Saen et al., 2012; Wikström et al., 2004). Van Saen et al., 2018 reported the detection of spermatogonia in testicular tissues of some KS patients. The estimation of their presence was based on the expression of *OCT4* and MAGE-A4 markers. Moreover, it was suggested that the spermatogenic arrest that occurs at the spermatogonium or primary spermatocyte level in KS boys, undergoes an apoptotic process at puberty instead of development (Gies et al., 2012; Wikström & Dunkel, 2008). In a study by Kleiman et al., 2016 of KS patients in which some had positive sperm retrieval and some did not, they showed that the expression levels of spermatogenic markers *DAZ*, *RBM*, *BOLL*, were identified in 45% of the KS patients, while *CDYI*, which is a post-meiotic spermatogenic marker, was identified in only 19%. In a systematic review and meta-analysis, Deebel et al., 2020 reported around 49% of spermatogonia were positive among adult KS patients in whom sperm was not retrieved. It is expected that in those KS patients in whom sperm was retrieved using TESE, premeiotic/miotic and post-miotic markers will be present.

Difficulty in having biological offspring is a very important issue among KS patients and their partners, as shown by Maiburg et al., 2011. Thus, among those in whom no sperm was found in testicular sperm extraction, some will consider re-TESE or micro TESE even without having good prognostic markers. Moreover, cryopreservation of these tissues for future in-vitro maturation is also a dilemma in these cases. In the present study, we examined the expression levels of different markers of premeiotic spermatogonial cells and cells of meiotic and post-meiotic stages, and the growth factor *CSF-1* in testicular biopsies with and without the sperm of KS patients, as well as several cases with normal karyotype and complete spermatogenesis, in order to detect markers that may help to predict hidden spermatogenesis.

2 | MATERIALS AND METHODS

2.1 | Testicular biopsies of patients

The present study was approved by the local institutional ethical committees [Tel-Aviv Medical Center (TLV-01-262)]. All patients signed informed consent documentation to use their testicular biopsy for research (normal AZO were from 2006 to 2008. XXY patients from

2002–2012). Testicular biopsies were obtained from 5 AZO with normal karyotype (two patients agreed to perform analysis for AZF, and no microdeletions were found; while the others did not agree to this analysis) who had undergone testicular sperm extraction, and histology revealed complete spermatogenesis (average of ≥15 sperm cells per tubule) and from 24 non-mosaic KS (47, XXY) men who were referred for conventional TESE due to absence of sperm in their ejaculate after centrifugation and a meticulous search of sperm cells. In seven of them, sperm cells were retrieved from testicular tissue to be used in an IVF- intracytoplasmic sperm injection (ICSI) program. One to three samples, depending on testicular size, were taken from each testis under general anaesthesia (Hauser et al., 1998). The age of the patients ranged from 28 to 40 years. The TESE specimens were histologically characterized and identified as described in Tables 1–3. Testicular biopsies were divided for histological evaluation and for RNA extraction (Kleiman et al., 2011; Kleiman et al., 2016). None of the patients underwent surgery or chemotherapy/radiotherapy prior to the retrieval procedure. All patients underwent karyotype analysis.

2.2 | RNA evaluation

Evaluation of RNA for spermatogenic markers and growth factors was performed. The expression of the premeiotic markers *OCT4*, *CD9*, *GFR-α*, *α-6-INTEGRIN*, *SALL4*, *C-KIT*, the meiotic marker *CREM*, and the post-meiotic marker protamine were examined. The expression levels of the growth factor *CSF-1* were also examined. The examination was performed using reverse transcriptase with poly-dT oligonucleotides (Kleiman et al., 2016). 1.5 μl of the cDNA product was assessed by a quantitative real-time polymerase chain reaction (qPCR), as described by Kleiman et al., 2011. Briefly, total RNA was extracted from testicular tissue samples (8–15 mg) and stored at −80°C until used. RNA was extracted from tissue using TRI Reagent (Sigma, MO, USA) except for five tissue samples that were extracted with a GeneEluteTM Mammalian Total RNA Kit (Sigma, MO). Reverse transcription (RT) was performed in 30 μl aliquots containing 0.7 μg RNA and oligo(dT)15 using a RevertAidTM First strand cDNA Synthesis Kit (Fermentas, Burlington, CA). The designed primers were tested by RT-PCR, gel polymerase, and Real Time PCR. They were checked for genomic DNA, and the RT negative control (DDW was used) was always negative in all of the steps above. Gene expression was normalized with PPI (as a reference gene) of the same sample.

Gene amplification of the markers and growth factors was handled using the specific oligonucleotide sequences of the primers, as presented in Table S1.

3 | RESULTS

3.1 | Testicular findings of patient biopsies

The histology of five AZO with normal karyotypes who had undergone testicular sperm revealed complete spermatogenesis (Table 1). In seven testicular biopsies of KS men, sperm cells were detected in the cytological search, but the histological findings were similar to those without sperm cells. With few tubules with SCs and many obliterated ones, as well as hyperplasia and hyalinization of the

TABLE 1 Hormone levels from AZO with complete spermatogenesis (CS)

Pt#	Age (Years)	FSH (IU/L)	LH (IU/L)	Total T (nmol/L)	Histology
1	33	NA	NA	NA	CS
2	24	11.3	3.56	14.1	CS
3	35	18.5	6.7	NA	CS
4	30	4	3	17.6	CS
5	30	3.3	3	22	CS
Mean ± SD	30.4 ± _4.16	9.27 ± 7.13	4.06 ± 1.77	17.9 ± 3.96	

TABLE 2 Hormone levels from KS patients with mature sperm on TESE

Pt#	Age (years)	FSH (IU/L)	LH (IU/L)	Total, *T* (nmol/L)	Histology
1	32	31.0	14.0	NA	Mixed of focal spermatogenesis and hyalinized tubules (MFSHT)
2	33	30.6	10.5	7.8	MFSHT
3	31	24.4	17.0	15.4	MFSHT
4	31	27.3	17.3	NA	MFSHT
5	37	36.0	13.5	17.5	MFSHT
6	36	15.9	8.9	9.0	MFSHT
7	31	33.0	27.8	14.2	MFSHT
Mean ± SD	33.0± 2.5	28.3 ± 6.6	15.6 ± 6.2	12.8 ± 4.2	

TABLE 3 Hormone levels from KS patients without mature sperm on TESE

Pt#	Age	FSH (mIU/ml)	LH mIU/ml)	Total testosterone (nmol/L)	Histology
1	28	15.9	12.5	8.5	SCO
2	35	26.1	17.8	13.58	SCO
3	35	26.2	20.1	NA	SCO
4	40	33.7	16.4	NA	SCO
5	30	43.2	32	16.30	SCO
6	39	36	23	15	SCO
7	33	60	33.8	9.7	SCO
8	28	49.6	18.6	9.7	SCO
9	28	24	NA	13.0	SCO
10	37	14.3	14.9	18.35	SCO
11	31	40.9	26	NA	SCO
12[a]	34	15.9	NA	NA	SCO
13	35	19.3	18.2	8.3	SCO
14	31	27.4	29.9	10.82	SCO
15	31	41.5	23.7	12.9	SCO
16	28	25.6	16.50	11	SCO
17	35	48.52	27.7	13.4	SCO
Mean ± SD	32.8 ± 3.9	32.2 ± 12.6	22.8 ± 5.8	11.4± 4.1	

Note: The levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone (T) were examined in the blood of KS patients (without sperm in their testicular biopsies) by radioimmunoassay.
[a]Under hCG treatment; NA, not available; SCO, Sertoli cell only.

interstitial tissue. Those with mature sperm cells were classified as mixed atrophy (Table 2). Testicular biopsies of the KS men ($n = 17$), who were without sperm following meticulous cytological search by biologist technicians in the andrology and IVF lab, presented concordant histological findings of Sertoli Cell Only (SCO) with severe atrophy of the tubules, extensive hyalinization, and hyperplasia in eosin (H&E) staining of testicular sections, and were classified as SCs only (Table 3).

3.2 | Hormone levels of patients

The hormone levels of FSH, LH and testosterone (T) were examined in the blood of KS patients, as well as among the AZO in whom histology revealed complete spermatogenesis (Tables 1–3). The FSH and LH levels of all KS men with sperm (Table 2) and without sperm (Table 3) were highly abnormal with mean and standard deviation levels 28.2 ± 6.4, 32.2 ± 12.6, and 16.0± 6.0, 22.8 ± 5.8 mIU/ml respectively. Mean T levels were12.8± 4.2 and 11.4 ± 4.1 nmol/ml respectively. Only one KS patient without sperm was under hCG treatment (Table 3; #12). Among the AZO in whom histology revealed complete spermatogenesis, two had normal LH and FSH levels, and two had elevated FSH levels and normal LH levels with mean and standard deviation levels of FSH 9.3 ± 7.1, and LH 4.1 ± 1.8 mIU/ml. All four had normal testosterone levels with mean levels of 17.9± 4.0.

3.3 | Expression of spermatogenic markers and growth factors in testicular biopsies

3.3.1 | Spermatogenic markers

Testicular biopsies from AZO with normal karyotypes, in which histology revealed complete spermatogenesis, at different levels, expressed the examined premeiotic cells markers *OCT4* (5/5), *CD9* (5/5), *GFR*-α (5/5), α-*6-INTEGRIN* (5/5), *SALL4* (5/5), and *C-KIT* (5/5) (Table 4). The meiotic marker *CREM*-1 was expressed in all 5/5 and the post-meiotic marker protamine was expressed only in 4/5 (Table 4).

Most of the testicular biopsies from KS patients with sperm expressed the examined pre-meiotic cells markers [*OCT4* (6/7), *CD9* (7/7), *GFR*-α (7/7), α-*6-INTEGRIN* (7/7), *SALL4* (7/7), and *C-KIT* (7/7)] at different levels (Table 5). Only part of these biopsies expressed the meiotic marker *CREM* (2/7 biopsies) and the post-meiotic marker protamine (3/7) (Table 5).

Variable number of testicular biopsies from KS patients without sperm expressed the examined pre-meiotic cells markers [*OCT4* (10/17), *CD9* (17/17), *GFR*-α (16/17), α-*6-INTEGRIN* (17/17), *SALL4* (17/17) and *C-KIT* (14/17)], and at remarkably different levels (Table 6). The meiotic marker *CREM*-1 was expressed only in 6/17 testicular biopsies of KS patients, and the post-meiotic marker protamine was expressed only in 3/17 (Table 6). It should be noted that all testicular biopsies that did not express *OCT4* (patients 1–7; Table 6), also did not express the meiotic and post-meiotic markers (*CREM* and

TABLE 4 Expression levels of specific pre-meiotic markers is correlated with the expression levels of meiotic and post-meiotic markers in biopsies with complete spermatogenesis.

	Markers of spermatogenic stages								
	Pre-meiotic						Meiotic	Post-meiotic	Growth factor
Pt. #	*OCT4*	*CD9*	*GFR-α*	*α-6-INTEGRIN*	*SALL4*	*C-KIT*	*CREM*	*PROTAMINE*	*CSF-1*
1	3.627	17.862	2.343	0.363	22.144	0.317	36.856	59428.040	3.928
2	5.116	161.888	19.012	0.286	41.179	3.326	48.632	22597.196	ND
3	5.183	16.840	4.450	0.489	36.729	2.343	0.550	0	7.694
4	11.784	25	2.156	0.935	40.895	1.584	78.730	152542.972	3.614
5	22.687	16.898	2.987	2.956	69.737	0.010	49.141	157922.388	0.095
Mean	9.679	47.697	6.189	1.005	42.136	1.516	42.781	78498.12	3.832
STD	7.926	63.924	7.224	1.118	17.271	1.384	28.220	73214.40	3.105

TABLE 5 Expression levels of specific pre-meiotic markers is correlated with the expression levels of meiotic and post-meiotic markers in biopsies with sperm from KS patients.

	Markers of spermatogenic stages								
	Pre-meiotic						Meiotic	Post-meiotic	Growth factor
Pt. #	*OCT4*	*CD9*	*GFR-α1*	*α-6-INTEGRIN*	*SALL4*	*C-KIT*	*CREM*	*PROTAMINE*	*CSF-1*
1	0.0	0.2152	0.173	0.278	27.547	0.000	0	19.75	0.71393082
2	1.04	212.137	3.781	1.651	2.618	0.142	0	32.75	4.70389609
3	2.52	440.762	10.438	4.239	16.666	0.701	0	0	19.4791145
4	3.0	232.946	4.404	0.083	2.393	0.099	0.92	0	0.59207678
5	4.18	103.526	2.127	6.163	8.391	0.254	0	0	13.3971683
6	15.17	177.768	6.121	19.888	19.479	2.171	0	6268.28	97.9420298
7	25	247.941	15.712	3.280	1.369	0.670	21	0	111.728714
Mean	7.272	202.185	6.108	5.083	11.209	0.576	3.131	902.968	35.508
STD	9.298	136.124	5.328	6.882	10.176	0.754	7.886	2365.915	48.013

protamine, respectively). The highest expression levels of *OCT4* were associated with high expression levels of *CREM* and protamine (Table 6). The highest levels of *GFR-α* and *α-6-INTEGRIN* in all examined biopsies (1–17) were positively associated with the highest *OCT4* levels and the meiotic and post-meiotic markers (*CREM* and protamine, respectively) (Table 6).

3.3.2 | Growth factors

CSF-1 showed low levels of expression in parallel to undetectable or low levels of *OCT4*, *CREM*, and protamine (patients 1–14; Table 6), and high expression levels in parallel to high expression levels of *CREM* and protamine (patients 15–17; Table 6).

Thus, our results show a positive correlation between the expression levels of the pre-meiotic marker *OCT4*, *CD9*, *GFR-α*, the growth factor *CSF-1*, and the meiotic and post-meiotic markers *CREM* and protamine respectively.

In all five patients with complete spermatogenesis (Table 7, Group A) *OCT4* was detected and in 4/5, *CSF-1* was also detected. In 4/5 of patients, protamine was detected at high levels (>22,000), and in only in one patient, protamine was undetectable (Table 7, Group A).

In 6/7 of the KS patients in whom mature sperm cells were retrieved (Table 7, Group B1, 2, 3), the expression of *OCT4* was detected, and *CSF-1* was observed in 7 out of 7 patients (Table 7, Group B1, 2, 3). The expression of protamine (a post-meiotic marker) was observed at variable extent, 3 with expression levels and 4 with undetectable levels (Table 7, Group B1, 2, 3).

In only 3 of the 17 KS patients in whom no sperm was found (Table 7, Group C2), the expression levels of *OCT4* detected were relatively high (42.9 ± 13.5), and in 14, the expression was rather low (0.968 ± 1.49) (Table 7, Group C1). The expression levels of *CSF-1* were variably detected in this group of patients (Table 7, Group C1, 2). Among the 17 KS patients in whom no mature sperm was retrieved, but in whom OCT 4 was relatively high, 3 patients (Table 7, Group C2) exhibited distinct expression of protamine (616.6 ± 559.2).

TABLE 6 Expression levels of specific pre-meiotic markers is correlated with the expression levels of meiotic and post-meiotic markers in biopsies without sperm from KS patients.

	Markers of spermatogenic stages								
	Pre-meiotic						Meiotic	Post-meiotic	Growth factor
Pt. #	*OCT4*	*CD9*	*GFR-α*	*α-6-INTEGRIN*	*SALL4*	*C-KIT*	*CREM*	*PROTAMINE*	*CSF-1*
1	0	24.741	0.492	2.119	0.533	0.001	0	0	0
2	0	0.386	0.117	1.782	25.436	0.004	0	0	1.530
3	0	1.801	0.590	11.033	12.543	0.00	0	0	2.700
4	0	0.229	0.088	0.404	21.991	0.003	0	0	0.200
5	0	0.127	0	0.137	4.343	0	0	0	0.296
6	0	9.773	0.330	1.02E-10	0.421	0	0	0	0.680
7	0	53.403	0.286	0.179	0.296	0	0	0	0.099
8	0.33	100.695	1.964	0.199	0.270	0.019	0	0	0.689
9	0.63	46.009	5.831	1.897	5.111	0.123	0	0	9.340
10	0.861	63.287	3.491	0.955	6.983	0.126	0.600	0	17.920
11	1.728	137.078	7.748	0.961	1.692	0.045	0	0	8.400
12	2.393	56.448	6.886	1.651	17.253	0.033	0	0	1.277
13	2.538	252.275	2.555	0.091	1.364	2.401	2.451	0	0.830
14	5.076	155.832	5.771	0.243	17.862	1.417	5.953	0	9.940
15	28.817	718.505	11.149	4.736	10.584	2.040	50	1364.216	162.45
16	38.689	581.589	8.391	3.467	3.201	0.668	21.315	19.614	86.450
17	61.132	783.536	20.518	6.886	20.096	0.475	28.126	465.893	88.270
Mean	8.364	175.630	4.482	2.161	8.822	0.432	6.379	108.807	23.004
STD	17.530	259.057	5.432	2.961	8.684	0.768	13.909	342.531	45.586

Note: The RNA expression levels of the spermatogenic cell markers and the growth factor *CSF-1* were examined by qPCR analysis as described in material and methods section. The levels are expressed as fold of increase compare to the PPI of the same sample.

TABLE 7 Mean age, endocrine and paracrine/autocrine factors expression levels are in correlation with the mean expression levels of premeiotic and postmeiotic markers in biopsies with and without sperm from KS patients and from AZO with complete spermatogenesis.

N	Subgroup	Karyotype	Sperm search	Protamine (fold)	*OCT4* (fold)	*CSF-1* (fold)	Age (years)	FSH (mIU/ml)	LH (mIU/ml)	Testosterone (nmol/ml)
5	A	46XY	+	78498.12 ± 732	9.679 ± 7.92	3.070 ± 3.19	30.4 ± 4.2	9.3 ± 7.1	4.1 ± 1.8	17.9 ± 4.0
4	B1	47XXY	+	Undetectable	10.6 ± 12.5	48.2 ± 55.1	33.0 ± 3.5	30.8 ± 5.6	19.4 ± 7.4	15.7 ± 1/7
2	B2	47XXY	+	19.7	Undetectable	0.7	32	31.0	14.0	NA
1	B3	47XXY	+	3150.5 ± 4409.2	8.1 ± 10.0	4.7 ± 97.9	34.5 ± 2.1	23.3 ± 10.4	11.4 ± 4.6	8.4 ± 0.8
14	C1	47XXY	−	Undetectable	0.968 ± 1.49	3.85 ± 5.42	33.1 ± 3.97	30.8 ± 13.7	21.9 ± 6.99	12.3 ± 3.46
3	C2	47XXY	−	616.6 ± 559.2	42.9 ± 13.5	112.4 ± 35.4	31.3 ± 3.5	38.5 ± 9.6	22.0 ± 4.6	12.4 ± 4.5

Note: The levels of hormones and the RNA expression levels of *OCT4* and *CSF-1* were examined as described in Section 2.

In the other 14 with relatively low *OCT4*, protamine expression was undetectable (Table 7, Group C1).

4 | DISCUSSION

In the present study, we showed that some testicular biopsies without sperm from KS patients expressed markers of the premeiotic stage such as *OCT4*, *CD9*, *GFR-α*, *α-6-INTEGRIN*, and the differentiating marker *C-KIT* even though their pathological findings were of SCO. In some testicular biopsies without sperm, we also found the expression of the meiotic marker *CREM* and the post-meiotic marker protamine. A very interesting observation was the positive association between high expression levels of *OCT4*, and the high expression levels of *CREM* and protamine, as well as with *CSF-1* in patients without sperm in their testicular tissue. Previous studies showed that the presence of *OCT4* and *MAGE-4* correlates with positive germ cells in testicular biopsies of KS patients, as well as *DAZ*, *RBM*, *BOLL* and *CDY1*

(Kleiman et al., 2016). It is suggested that, at birth, KS patients have a normal number of germ cells in their testes, but the period in which germ cells diminution occurs is still under discussion. Recent studies suggest germ cells loss occurs before the age of 4, and even prenatally (Braye et al., 2019; Deebel, et al., 2020; Gies et al., 2012; Goossens et al., 2013; Van Saen et al., 2012, 2018; Wikström et al., 2004). In a study from D'Aurora et al. (2015), a KS testis transcriptome analysis showed overexpression of SOCS3, suggesting that the development of protective mechanisms against inflammatory response is likely taking place in the testis of KS patients.

According to different authors, the testicular biopsies in around 50%–60% of KS patients who undergo TESE or micro TESE will have no mature sperm that could be used in an IVF-ICSI program (Aksglaede & Juul, 2013; Corona et al., 2017). Due to the fact that the seminiferous tubules in KS patients are not homogenous and the desire of couples to have their own biological child, many of these couples will consider re-TESE or micro TESE rather than sperm donation. Consultation with these cases is rather difficult since there are no good predictors of mature sperm cells presence yet established.

Controversy still exists whether young age could influence sperm retrieval rates in KS patients (Ferhi et al., 2009; Plotton et al., 2015; Ramasamy et al., 2009). Deebel et al., 2020 in their meta-analysis could not determine a correlation between age and the presence of spermatogonia. However, they could demonstrate that about 49% of postpubertal adults without mature testicular sperm had spermatogonial cells, while prepubertal boys had 83% spermatogonia cells present in their testicular tissue. Our group of patients included only adult KS patients, with no age difference between sub-groups.

Hormone patterns were also suggested as prognostic factors for sperm retrieval in KS patients (Cissen et al., 2016). In a study by Rohayem et al., 2015, it was suggested that KS patients with LH levels below 17.5 and testosterone levels above 7.5 nmol/L have higher sperm retrieval rates. Deebel et al., 2020 in their systematic review on testicular tissue from KS, showed that those without spermatogonia had higher FSH, LH and testosterone levels than those who did have spermatogonia in their testicular tissue; however, there were no significant differences in Inhibin B levels found between biopsies with and without spermatogonia (Deebel et al., 2020). The authors themselves and others (Aksglæde et al., 2011) suggest that none of the hormone levels are a good predictor for mature sperm retrieval or for the presence of spermatogonia in the unsuccessful TESE group. Our study also showed no correlation between FSH, LH or testosterone levels to the expression of premeiotic and post-meiotic markers.

In the present study, we looked at different premeiotic, meiotic, and post-meiotic markers and found *OCT4* and the testicular growth factor *CSF-1* as possible predictors of the presence of meiotic/post-meiotic cells in biopsies in which mature sperm cells were not retrieved. Assessment of premeiotic markers *OCT4* and *CSF-1*, in conjunction with post-meiotic protamine, might further improve the prediction of mature sperm cells beyond conventional histological analysis.

In the present study, we used only conventional TESE with multiple biopsies. It is possible to suggest a repeated sperm retrieval with the micro TESE technique, but it is still controversial whether this is advantageous (Corona et al., 2017). In a recent study by Corona et al., 2017, they were unable to demonstrate any advantage of micro TESE compared to conventional TESE. This might be due to the low testicular volume among KS patients (Corona et al., 2017). In the study by Haliloglu et al., 2014, in only 3 out of 18 KS patients (16.7%) who failed sperm retrieval using micro TESE was repeated micro TESE successful.

Since no predictors exist concerning re-TESE or microTESE, and medical treatment has not been proven effective among KS patients, we suggest evaluating RNA expression using RT-multiplex PCR analysis in order to better classify those KS patients in whom no sperm cells were retrieved. According to our study, if *OCT4* is not expressed or showed very low expression, the chance of finding post-meiotic germ cells is negligible; therefore, the chance of retrieving sperm in re-TESE or micro TESE is also probably low. On the other hand, if protamine, the post-meiotic marker or *CSF-1* growth factor is expressed at high levels, we would assume that there would be a good chance of successful sperm retrieval in a second TESE or micro TESE. This assumption, of course, must be tested in future studies.

The differentiation of spermatogonial cells in a 3D-culture system has demonstrated by us and others (Abu Elhija et al., 2012; Huleihel et al., 2015; Khajavi et al., 2014) in murine and monkey models, as well as recently by us (Abofoul-Azab et al., 2018) in prepubertal cancer patients. However, due to the fact that only very low numbers of spermatogonia exist in KS patients, we will probably have to proliferate them in vitro before differentiation to post-meiotic cells. Sadri-Ardekani et al. (2009) demonstrated their ability to increase the number of human spermatogonia in culture up to 18,450-fold (Sadri-Ardekani et al., 2009). Development of expanding the number of spermatogonia and further differentiating them to post-meiotic cells might be used by ICSI to produce healthy babies from KS patients with negative mature sperm retrieval. This future option should be kept in mind and might also be discussed with KS patients with negative sperm retrieval and the presence of *OCT4*, a premeiotic marker.

In conclusion, we suggest that larger scale studies are needed to verify pathological results using *OCT4* and *CSF-1* markers to try to increase the precision diagnosis among NOA patients in whom sperm was not retrieved. These results may be used for further consulting patients considering re-TESE or re-micro TESE and cryopreservation of testicular tissue for possible future use.

AUTHOR CONTRIBUTIONS

Mahmoud Huleihel and Eitan Lunenfeld made substantial contributions to the conception and design of the study. Mahmoud Huleihel, Maram Abofoul-Azab, Sandra Kleiman and Shimi Barda designed the experiments. Maram Abofoul-Azab, Sandra Kleiman and Shimi Barda performed the experiments and analyses. Mahmoud Huleihel, Eitan Lunenfeld and Maram Abofoul-Azab wrote the manuscript. Mahmoud Huleihel, Eitan Lunenfeld, Maram Abofoul-Azab, Sandra Kleiman, Shimi Barda and Ron Hauser participated in interpreting the data and their presentation, and critically revising the paper for key intellectual content. All of the authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Abofoul-Azab, M., Abumadighem, A., Lunenfeld, E., Kapelushnik, J., Shi, Q., Pinkas, H., & Huleihel, M. (2018). Development of postmeiotic cells in vitro from spermatogonial cells of prepubertal cancer patients. *Stem Cells and Development*, *27*(15), 1007–1020. https://doi.org/10.1089/SCD.2017.0301

Abu Elhija, M., Lunenfeld, E., Schlatt, S., & Huleihel, M. (2012). Differentiation of murine male germ cells to spermatozoa in a soft agar culture system. *Asian Journal of Andrology*, *14*(2), 285–293. https://doi.org/10.1038/AJA.2011.112

Aksglaede, L., & Juul, A. (2013). Testicular function and fertility in men with Klinefelter syndrome: A review. *European Journal of Endocrinology*, *168*(4), R67–R76. https://doi.org/10.1530/EJE-12-0934

Aksglæde, L., Skakkebæk, N. E., Almstrup, K., & Juul, A. (2011). Clinical and biological parameters in 166 boys, adolescents and adults with non-mosaic Klinefelter syndrome: A Copenhagen experience. *Acta Paediatrica (Oslo, Norway: 1992)*, *100*(6), 793–806. doi:10.1111/J.1651-2227.2011.02246.X

Aksglaede, L., Andersson, A. M., Jørgensen, N., Jensen, T. K., Carlsen, E., McLachlan, R. I., Skakkebaek, N. E., Peterson, J. H., & Juul, A. (2007). Primary testicular failure in Klinefelter's syndrome: The use of bivariate luteinizing hormone-testosterone reference charts. *Clinical Endocrinology*, *66*(2), 276–281. https://doi.org/10.1111/J.1365-2265.2006.02722.X

Bonomi, M., Rochira, V., Pasquali, D., Balercia, G., Jannini, E. A., & Ferlin, A. (2017). Klinefelter syndrome (KS): Genetics, clinical phenotype and hypogonadism. *Journal of Endocrinological Investigation*, *40*(2), 123–134. https://doi.org/10.1007/S40618-016-0541-6

Braye, A., Tournaye, H., & Goossens, E. (2019). Setting up a cryopreservation programme for immature testicular tissue: Lessons learned after more than 15 years of experience. *Clinical Medicine Insights. Reproductive Health*, *13*, 117955811988634. https://doi.org/10.1177/1179558119886342

Cissen, M., Meijerink, A. M., D'Hauwers, K. W., Meissner, A., Van Der Weide, N., Mochtar, M. H., … VanWely, M. (2016). Prediction model for obtaining spermatozoa with testicular sperm extraction in men with non-obstructive azoospermia. *Human Reproduction (Oxford, England)*, *31*(9), 1934–1941. https://doi.org/10.1093/HUMREP/DEW147

Conrad, S., Renninger, M., Hennenlotter, J., Wiesner, T., Just, L., Bonin, M., Aicher, W., Buhring, H-J., Mattheus, U., Mack, A., Wanger, H-J., Minger, S., Matzkies, M., Reppel, M., Hescheler, J., Sievert, K-D., Stenzl, A., & Skutella, T. (2008). Generation of pluripotent stem cells from adult human testis. *Nature*, *456*(7220), 344–349. https://doi.org/10.1038/NATURE07404

Corona, G., Pizzocaro, A., Lanfranco, F., Garolla, A., Pelliccione, F., Vignozzi, L., Ferlin, A., Foresta, C., Jannini, E. A., Maggi, M., Lenzi, A., Pasquali, D., & Francavilla, S. (2017). Sperm recovery and ICSI outcomes in Klinefelter syndrome: A systematic review and meta-analysis. *Human Reproduction Update*, *23*(3), 265–275. https://doi.org/10.1093/HUMUPD/DMX008

D'Aurora, M., Ferlin, A., Di Nicola, M., Garolla, A., De Toni, L., Franchi, S., Palka, G., Foresta, C., Stuppia, L., & Gatta, V. (2015). Deregulation of sertoli and leydig cells function in patients with Klinefelter syndrome as evidenced by testis transcriptome analysis. *BMC Genomics*, *16*(1), 156. https://doi.org/10.1186/S12864-015-1356-0

De Rooij, D. G. (2009). The spermatogonial stem cell niche. *Microscopy Research and Technique*, *72*(8), 580–585. https://doi.org/10.1002/JEMT.20699

De Rooij, D. G., & Grootegoed, J. A. (1998). Spermatogonial stem cells. *Current Opinion in Cell Biology*, *10*(6), 694–701. https://doi.org/10.1016/S0955-0674(98)80109-9

Deebel, N. A., Galdon, G., Zarandi, N. P., Stogner-Underwood, K., Howards, S., Lovato, J., Kogan, S., Atala, A., Lue, Y., & Sadri-Ardekani, H. (2020). Age-related presence of spermatogonia in patients with Klinefelter syndrome: A systematic review and meta-analysis. *Human Reproduction Update*, *26*(1), 58–72. https://doi.org/10.1093/HUMUPD/DMZ038

Dym, M., Kokkinaki, M., & He, Z. (2009). Spermatogonial stem cells: Mouse and human comparisons. *Birth Defects Research. Part C, Embryo Today: Reviews*, *87*(1), 27–34. https://doi.org/10.1002/BDRC.20141

Fayomi, A. P., & Orwig, K. E. (2018). Spermatogonial stem cells and spermatogenesis in mice, monkeys and men. *Stem Cell Research*, *29*, 207–214. https://doi.org/10.1016/J.SCR.2018.04.009

Ferhi, K., Avakian, R., Griveau, J. F., & Guille, F. (2009). Age as only predictive factor for successful sperm recovery in patients with Klinefelter's syndrome. *Andrologia*, *41*(2), 84–87. https://doi.org/10.1111/J.1439-0272.2008.00875.X

Forti, G., Corona, G., Vignozzi, L., Krausz, C., & Maggi, M. (2010). Klinefelter's syndrome: A clinical and therapeutical update. *Sexual Development: Genetics, Molecular Biology, Evolution, Endocrinology, Embryology, and Pathology of Sex Determination and Differentiation*, *4*(4–5), 249–258. https://doi.org/10.1159/000316604

Franik, S., Hoeijmakers, Y., D'Hauwers, K., Braat, D. D. M., Nelen, W. L. M., Smeets, D., Claahsen-van der Grinten, H. L., Ramos, L., & Fleischer, K. (2016). Klinefelter syndrome and fertility: Sperm preservation should not be offered to children with Klinefelter syndrome. *Human Reproduction (Oxford, England)*, *31*(9), 1952–1959. https://doi.org/10.1093/HUMREP/DEW179

Gies, I., De Schepper, J., Goossens, E., Van Saen, D., Pennings, G., & Tournaye, H. (2012). Spermatogonial stem cell preservation in boys with Klinefelter syndrome: To bank or not to bank, that's the question. *Fertility and Sterility*, *98*(2), 284–289. https://doi.org/10.1016/J.FERTNSTERT.2012.04.023

Gies, I., Oates, R., De Schepper, J., & Tournaye, H. (2016). Testicular biopsy and cryopreservation for fertility preservation of prepubertal boys with Klinefelter syndrome: A pro/con debate. *Fertility and Sterility*, *105*(2), 249–255. https://doi.org/10.1016/J.FERTNSTERT.2015.12.011

Goossens, E., Van Saen, D., & Tournaye, H. (2013). Spermatogonial stem cell preservation and transplantation: From research to clinic. *Human Reproduction (Oxford, England)*, *28*(4), 897–907. https://doi.org/10.1093/HUMREP/DET039

Gordon, D. L., Krmpotic, E., Thomas, W., Gandy, H. M., & Paulsen, C. A. (1972). Pathologic testicular findings in Klinefelter's syndrome: 47,XXY vs 46,XY/47,XXY. *Archives of Internal Medicine*, *130*(5), 726–729. https://doi.org/10.1001/ARCHINTE.1972.03650050054009

Groth, K. A., Skakkebæk, A., Høst, C., Gravholt, C. H., & Bojesen, A. (2013). Clinical review: Klinefelter syndrome—a clinical update. *The Journal of Clinical Endocrinology and Metabolism*, *98*(1), 20–30. https://doi.org/10.1210/JC.2012-2382

Haliloglu, A. H., Tangal, S., Gulpinar, O., Onal, K., & Pabuccu, R. (2014). Should repeated TESE be performed following a failed TESE in men with Klinefelter syndrome? *Andrology*, *2*(1), 42–44. https://doi.org/10.1111/J.2047-2927.2013.00157.X

Hauser, R., Botchan, A., Amit, A., Ben Yosef, D., Gamzu, R., Paz, G., Lessing, J. B., Yogev, L., & Yavetz, H. (1998). Multiple testicular sampling in non-obstructive azoospermia—Is it necessary? *Human Reproduction (Oxford, England)*, *13*(11), 3081–3085. https://doi.org/10.1093/HUMREP/13.11.3081

Hermann, B. P., Sukhwani, M., Hansel, M. C., & Orwig, K. E. (2010). Spermatogonial stem cells in higher primates: Are there differences from those in rodents? *Reproduction (Cambridge, England)*, *139*(3), 479–493. https://doi.org/10.1530/REP-09-0255

Huleihel, M., Nourashrafeddin, S., & Plant, T. M. (2015). Application of three-dimensional culture systems to study mammalian spermatogenesis, with an emphasis on the rhesus monkey (Macaca mulatta). *Asian Journal of Andrology*, *17*(6), 972–980. https://doi.org/10.4103/1008-682X.154994

Jacobs, P. A., & Strong, J. A. (1959). A case of human intersexuality having a possible XXY sex-determining mechanism. *Nature*, *183*(4657), 302–303. https://doi.org/10.1038/183302A0

Kamischke, A., Baumgardt, A., Ju, J., Horst, J., & Nieschlag, E. (2003). Clinical and diagnostic features of patients with suspected Klinefelter syndrome. *Journal of Andrology*, *24*(1), 41–48. https://doi.org/10.1002/J.1939-4640.2003.TB02638.X

Khajavi, N., Akbari, M., Abolhassani, F., Dehpour, A. R., Koruji, M., & Habibi Roudkenar, M. (2014). Role of somatic testicular cells during mouse spermatogenesis in three-dimensional collagen gel culture system. *Cell Journal (Yakhteh)*, *16*(1), 90.

Kim, S. Y., Lee, B. Y., Oh, A. R., Park, S. Y., Lee, H. S., & Seo, J. T. (2017). Clinical, hormonal, and genetic evaluation of idiopathic nonobstructive azoospermia and Klinefelter syndrome patients. *Cytogenetic and Genome Research*, *153*(4), 190–197. https://doi.org/10.1159/000487039

Kleiman, S. E., Lehavi, O., Hauser, R., Botchan, A., Paz, G., Yavetz, H., & Yogev, L. (2011). CDY1 and BOULE transcripts assessed in the same biopsy as predictive markers for successful testicular sperm retrieval. *Fertility and Sterility*, *95*(7), 2297–2302. https://doi.org/10.1016/J.FERTNSTERT.2011.03.020

Kleiman, S. E., Yogev, L., Lehavi, O., Yavetz, H., & Hauser, R. (2016). Distinctive pattern of expression of spermatogenic molecular markers in testes of azoospermic men with non-mosaic Klinefelter syndrome. *Journal of Assisted Reproduction and Genetics*, *33*(6), 807–814. https://doi.org/10.1007/S10815-016-0698-0

Klinefelter, H. F., Reifenstein, E. C., & Albright, F. (1942). Syndrome characterized by gynecomastia, aspermatogenesis without A-Leydigism, and increased excretion of follicle-stimulating hormone. *The Journal of Clinical Endocrinology & Metabolism*, *2*(11), 615–627. https://doi.org/10.1210/JCEM-2-11-615

La, H. M., & Hobbs, R. M. (2019). Mechanisms regulating mammalian spermatogenesis and fertility recovery following germ cell depletion. *Cellular and Molecular Life Sciences: CMLS*, *76*(20), 4071–4102. https://doi.org/10.1007/S00018-019-03201-6

Lanfranco, F., Kamischke, A., Zitzmann, M., & Nieschlag, P. E. (2004). Klinefelter's syndrome. *Lancet (London, England)*, *364*(9430), 273–283. https://doi.org/10.1016/S0140-6736(04)16678-6

Maiburg, M. C., Hoppenbrouwers, A. C., van Stel, H. F., & Giltay, J. C. (2011). Attitudes of Klinefelter men and their relatives towards TESE-ICSI. *Journal of Assisted Reproduction and Genetics*, *28*(9), 809–814. https://doi.org/10.1007/S10815-011-9603-Z

Meng, X., Lindahl, M., Hyvönen, M. E., Parvinen, M., De Rooij, D. G., Hess, M. W., Raatikainen-hokas, A., Sainio, K., Rauvala, H., Lakso, M., Pichel, J. G., Westphal, H., Saarma, M., & Sariola, H. (2000). Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science*, *287*(5457), 1489–1493. https://doi.org/10.1126/SCIENCE.287.5457.1489

Morris, J. K., Alberman, E., Scott, C., & Jacobs, P. (2008). Is the prevalence of Klinefelter syndrome increasing? *European Journal of Human Genetics: EJHG*, *16*(2), 163–170. https://doi.org/10.1038/SJ.EJHG.5201956

Nielsen, J., & Wohlert, M. (1991). Chromosome abnormalities found among 34,910 newborn children: Results from a 13-year incidence study in Arhus, Denmark. *Human Genetics*, *87*(1), 81–83. https://doi.org/10.1007/BF01213097

Oatley, J. M., Oatley, M. J., Avarbock, M. R., Tobias, J. W., & Brinster, R. L. (2009). Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. *Development (Cambridge, England)*, *136*(7), 1191–1199. https://doi.org/10.1242/DEV.032243

Oliver, E., & Stukenborg, J. B. (2020). Rebuilding the human testis in vitro. *Andrology*, *8*(4), 825–834. https://doi.org/10.1111/ANDR.12710

Phillips, B. T., Gassei, K., & Orwig, K. E. (2010). Spermatogonial stem cell regulation and spermatogenesis. *Philosophical Transactions of the Royal Society of London Series B, Biological Sciences*, *365*(1546), 1663–1678. https://doi.org/10.1098/RSTB.2010.0026

Plotton, I., Giscard D'Estaing, S., Cuzin, B., Brosse, A., Benchaib, M., Lornage, J., Ecochard, R., Dijoud, F., & Lejeune, H. (2015). Preliminary results of a prospective study of testicular sperm extraction in young versus adult patients with nonmosaic 47,XXY Klinefelter syndrome. *The Journal of Clinical Endocrinology and Metabolism*, *100*(3), 961–967. https://doi.org/10.1210/JC.2014-3083

Rajender, A., & Oates, R. (2018). AUA Update Series 37: Evaluation and management of Klinefelter syndrome, AUA University. Retrieved December 26, 2021, from https://auau.auanet.org/content/update-series-2018-lesson-24-evaluation-and-management-klinefelter-syndrome.

Ramasamy, R., Ricci, J. A., Palermo, G. D., Gosden, L. V., Rosenwaks, Z., & Schlegel, P. N. (2009). Successful fertility treatment for Klinefelter's syndrome. *The Journal of Urology*, *182*(3), 1108–1113. https://doi.org/10.1016/J.JURO.2009.05.019

Rohayem, J., Fricke, R., Czeloth, K., Mallidis, C., Wistuba, J., Krallmann, C., Zitzmann, M., & Kliesch, S. (2015). Age and markers of Leydig cell function, but not of Sertoli cell function predict the success of sperm retrieval in adolescents and adults with Klinefelter's syndrome. *Andrology*, *3*(5), 868–875. https://doi.org/10.1111/ANDR.12067

Sadri-Ardekani, H., Mizrak, S. C., van Daalen, S. K., Korver, C. M., Roepers-Gajadien, H. L., Koruji, M., Hovingh, S., de Reijke, T. M., de la Rosette, J. J., van der Veen, F., de Rooij, D. G., Repping, S., & van Pelt, A. M. (2009). Propagation of human spermatogonial stem cells in vitro. *JAMA*, *302*, 2127–2134. https://doi.org/10.1001/jama.2009.1689

Tagelenbosch, R. A. J., & de Rooij, D. G. (1993). A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. *Mutation Research*, *290*(2), 193–200. https://doi.org/10.1016/0027-5107(93)90159-D

Takashima, S., Kanatsu-Shinohara, M., Tanaka, T., Morimoto, H., Inoue, K., Ogonuki, N., … Shinohara, T. (2015). Functional differences between GDNF-dependent and FGF2-dependent mouse spermatogonial stem cell self-renewal. *Stem Cell Reports*, *4*(3), 489–502. https://doi.org/10.1016/J.STEMCR.2015.01.010

Van Saen, D., Tournaye, H., & Goossens, E. (2012). Presence of spermatogonia in 47,XXY men with no spermatozoa recovered after testicular sperm extraction. *Fertility and Sterility*, *97*(2), 319–323. https://doi.org/10.1016/J.FERTNSTERT.2011.11.009

Van Saen, D., Vloeberghs, V., Gies, I., Mateizel, I., Sermon, K., De Schepper, J., Tournaye, H., & Goossens, E. (2018). When does germ cell loss and fibrosis occur in patients with Klinefelter syndrome? *Human Reproduction*, *33*(6), 1009–1022. https://doi.org/10.1093/HUMREP/DEY094

Wikström, A. M., & Dunkel, L. (2008). Testicular function in Klinefelter syndrome. *Hormone Research*, *69*(6), 317–326. https://doi.org/10.1159/000117387

Wikström, A. M., & Dunkel, L. (2011). Klinefelter syndrome. *Best Practice & Research. Clinical Endocrinology & Metabolism*, *25*(2), 239–250. https://doi.org/10.1016/J.BEEM.2010.09.006

Wikström, A. M., Raivio, T., Hadziselimovic, F., Wikström, S., Tuuri, T., & Dunkel, L. (2004). Klinefelter syndrome in adolescence: Onset of puberty is associated with accelerated germ cell depletion. *The Journal of Clinical Endocrinology and Metabolism*, *89*(5), 2263–2270. https://doi.org/10.1210/JC.2003-031725

Zohni, K., Zhang, X., Tan, S. L., Chan, P., & Nagano, M. (2012). CD9 is expressed on human male germ cells that have a long-term repopulation potential after transplantation into mouse testes. *Biology of Reproduction*, *87*(2). 27, 1–8 https://doi.org/10.1095/BIOLREPROD.112.098913

SUPPORTING INFORMATION

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