The feasibility of fertility preservation in adolescents with Klinefelter syndrome

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There is little information on the feasibility of fertility preservation in adolescent Klinefelter syndrome (KS) patients. We conducted an observational study to evaluate the feasibility of fertility preservation in this population. The study was conducted in eight KS adolescents, aged between 15 and 17 years, who were referred for counseling about their future fertility to the center CECOS (Centre d’Etude et de Conservation des Oeufs et du Sperme humain) at Rouen University Hospital between October 2008 and December 2011. The patients were first seen with their parents and then separately. It was proposed to them that they should provide a semen sample, if this was azoospermic, two other semen samples spaced by 3 months were collected. If azoospermia was confirmed, a bilateral testicular biopsy was proposed for sperm retrieval and testicular tissue preservation. Each adolescent met the psychologist before undergoing testicular biopsy. Paraffin-embedded testicular tissue was evaluated after staining with hematoxylin–eosin and saffron and immunostaining using vimentin, anti-Müllerian hormone, androgen receptor and MAGE-A4 antibodies. Sertoli cell maturity, germ cell identification and lamina propria alteration were assessed on seminiferous tubules. KS adolescents were not deeply concerned about their future fertility and only became involved in the process of fertility preservation after at least three medical consultations. The parents agreed immediately that fertility preservation should be attempted. Seven non-mosaic XXY adolescents presented with azoospermia and one XXY/XY adolescent had oligozoospermia. Increased plasma levels of FSH and LH as well as bilateral testicular hypotrophy were observed in all patients. The XXY/XY adolescent banked four semen samples before testosterone replacement therapy. Two patients refused testicular biopsy. Five patients accepted a bilateral testicular biopsy. Spermatozoa were retrieved in one patient, elongated spermatids and spermatocytes I in a second patient. The number of patients enrolled in our study was low because the diagnosis of KS is only rarely made before or at the onset of puberty. Most XXY males are diagnosed in adulthood within the context of male infertility.

Wider Implications of the Findings: Spermatozoa can be retrieved in semen sample and in testicular tissue of adolescent Klinefelter patients. Furthermore, the testis may also harbor spermatogonia and incompletely differentiated germ cells. However, the physician should discuss with the patient and his parents over a period of several months before collecting a semen sample and performing bilateral testicular biopsy. Fertility preservation might best be proposed to adolescent Klinefelter patients just after the onset of puberty when it is possible to collect a semen sample and when the patient is able to consider alternative options to achieve fatherhood and also to accept the failure of spermatozoa or immature germ cell retrieval.

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Fertility preservation in XXY adolescents

Introduction

Klinefelter syndrome (KS) is the commonest sex chromosome disorder observed at birth in the general male population, with an incidence varying from 1/500 to 1/1000 in newborns (Bojesen et al., 2003; Morris et al., 2008; Tüttelmann et al., 2010). Diagnosis is mostly performed in adulthood within the context of male infertility and only 10% of XXY males are diagnosed before 14 years (Bojesen et al., 2003; Tüttelmann et al., 2010). In all, ~3% of infertile males and 10% of patients with non-obstructive azoospermia exhibit KS (Mau-Holzmann, 2005).

The extra X chromosome in non-mosaic XXY males appears as a result of non-disjunctions occurring equally during the first paternal meiotic division and the first or second maternal meiotic divisions (Hassold and Hunt, 2001; Thomas and Hassold, 2003; Tüttelmann et al., 2010). An increased paternal or maternal age has been observed in parents of XXY males at the time of conception (Bojesen et al., 2003; Lanfranco et al., 2004). Non-mosaic XXY individuals form 90% of the population of XXY males (Garcia-Quevedo et al., 2011). The mosaic XY/XXY form results from mitotic instability occurring during the first divisions of human embryo (Tüttelman et al., 2011).

Males with KS are infertile due to severe spermatogenesis impairment responsible for azoospermia in ~90% of homogeneous XXY males and 74.7% of mosaic form (Mau-Holzmann, 2005). Nevertheless, testicular sperm extraction (TESE) combined with ICSI has completely modified the management of infertility in KS and offers the opportunity to these patients to father children with their own spermatozoa. TESE can detect residual foci of active spermatogenesis in 27–72% of azoospermic adult XXY males (Fullerton et al., 2010). Normal fertilization and embryo development, pregnancies and births have been achieved in XXY males after ICSI using testicular spermatozoa (Fullerton et al., 2010).

As in males with non-obstructive azoospermia and normal karyotype, conventional non-invasive parameters such as testicular volume, FSH, LH and inhibin B plasmatic levels are very poor predictive factors for TESE outcome in KS patients. However, the potential for successful sperm retrieval decreases with age in XXY patients (range 30.5–35 years) (Okada et al., 2005; Emre Bakircioglu et al., 2006; Ferhi et al., 2009; Bakircioglu et al., 2011; Wikström and Dunkel, 2011). This observation may be related to data obtained after histological evaluation of testicular tissue in XXY males revealing that degeneration of seminiferous epithelium (germ cell depletion, Sertoli and Leydig cell dysfunction), fibrosis and hyalinization of seminiferous tubules progress with age after puberty in KS patients (Aks-glaede et al., 2006). Considering that germ cell depletion occurs throughout life in KS males (Lin et al., 2004; Aks-glaede et al., 2006; Emre Bakircioglu et al., 2006; Ichioka et al., 2006), one could argue that these patients should benefit from fertility preservation at the onset or just after the onset of puberty and before testosterone replacement therapy to increase the chance of retrieving spermatozoa in their ejaculate or in case of azoosperma after TESE.

Here we report on a small group of adolescent KS patients to demonstrate the acceptability of fertility preservation in these patients and the possibility either to retrieve spermatozoa in their ejaculate or after TESE or to identify the presence of immature germ cells in testicular tissue in case of TESE failure.

Materials and Methods

Subjects

The study included Klinefelter adolescents, aged between 15 and 17 years, 7 subjects with a 47.XXY karyotype and 1 subject with a mosaic form 47.XXY(12)/46,XY[6] who attended the unit between October 2008 and December 2011. Karyotypes were performed on metaphase spreads from cultured peripheral blood lymphocytes for each patient. If the diagnosis was performed during prenatal diagnosis, the numerical chromosome abnormality was confirmed after birth. These patients were referred by the pediatrician’s endocrinologist to our Reproductive Biology Laboratory specifically for counseling about their future fertility and to evaluate the possibility of collecting a semen sample. For six of them, the endocrinologist discussed hormone replacement therapy. Therefore, the patients were seen before introducing the treatment. Our center belongs to the national French network CECOS (Centre d’Etude et de Conservation des Oeufs et du Sperme humain) in charge, of fertility preservation in males in France, since 1983 and more recently in females. Considering that age may reduce the chance of spermatozoa retrieval in XXY males (Lin et al., 2004; Emre Bakircioglu et al., 2006; Ichioka et al., 2006), XXY adolescents may benefit from fertility preservation. This proposition is in accordance with the current French bioethics law that allows fertility preservation (gametes or germinal tissue) in males or females considered at ‘risk of premature failure of gametogenesis process’.

The young adolescents were seen first with their parents, then alone. We began by asking about their knowledge of KS and its impact on male fertility. Then, we provided specific and complementary information about the effect of an extra X chromosome on spermatogenesis, leading progressively to the conclusion that it may have a negative impact on spermatogenesis and that XXY males are at high risk of infertility due to azoosperma. Then, we questioned them about the different ways it is possible to build a family in case of male infertility. Taking into account their response, different options for family building were suggested: (i) to use their own spermatozoa that might be retrieved in a semen sample or after TESE in an assisted reproductive procedure; bearing in mind that if spermatozoa were retrieved they could be banked for the future; (ii) sperm donation or adoption (iii) spermatogenesis could be re-evaluated in adulthood and further options for family building might be possible in the future. Next, we questioned the mother, the father and the adolescent separately about their own opinion on the fertility preservation techniques on offer (collecting semen samples or performing testicular biopsy for TESE and testicular tissue freezing). After these explanations,
we questioned the young adolescents privately, without the presence of his parents, about his point of view on his future fertility and the feasibility to produce a semen sample. Young adolescents generally experience some difficulties in talking about erection, masturbation and ejaculation.

After the first consultation, we proposed to the young adolescents that they produce a semen sample. The young adolescents decided themselves when they would prefer to produce their first semen sample. Some of them came back several weeks later to do so. When azoospermia was detected in the first semen analysis, one or two further semen samples at least 3 months after were collected. The result of each semen sample was revealed to the young adolescent and his parents during a consultation conducted as described above. If azoospermia was confirmed, a bilateral testicular biopsy was proposed: a part of which would be used for TESE and another for testicular tissue preservation in case of TESE failure. The parents and the adolescents were informed that no biological or clinical parameters could predict if mature or immature germ cells would be retrieved and that the use of frozen–thawed testicular tissue containing immature germ cells was at an experimental stage in animals and not yet available for human use. Then, the adolescents met the psychologist to confirm the acceptability of fertility preservation procedure. Before undergoing a bilateral testicular biopsy for sperm retrieval and testicular tissue preservation, an informed consent was signed by both the patient and his parents for spermatozoa or testicular tissue banking and also for analysis and publication of the collected data. After testicular biopsy, the adolescent was seen once again with his parents to address the result of the biopsy.

The patient’s medical history, body mass index (BMI), clinical examination and testicular volume assessed clinically and by ultrasound examination, were recorded in combination with semen analysis. Furthermore, plasma LH and FSH were measured by chemiluminescent immunoassay (Immune 2500, Semes Healthcare Diagnostics), testosterone by radioimmunoassay (in duplicate) (Immunotech Beckman Coulter, Marseille, France), inhibin B by ELISA (Ge II ELISA Beckman Coulter) as well as anti-Mullerian hormone (AMH) by immunoassay (EIA Immunotech Beckman Coulter). The lower limit of sensitivity for LH and FSH was 0.1 IU/l. The detection limit was 0.1 ng/ml for testosterone, 2.6 pg/ml for inhibin B and 1 ng/ml for AMH. All blood samples were collected within 2 months before the testicular biopsy and analyzed in the same Biochemistry Laboratory of Rouen University Hospital.

Four other boys, aged between 1 and 17 years, who cryobanked testicular tissue within the context of non-malignant disease, were considered as controls (C) for testicular tissue histological evaluation. Controls (C1, C2, C3 and C4) presented thrombopenia (C1), drepanocytosis (C2), idio-pathic medular aplasia (C3) and suspicion of testis tumor on the left testis (C4). Testicular tissue preservation was performed before gonadotoxic treatment (C1, C2, C3) or after ejaculated spermatozoa preservation failure (C4). C1 and C2 were Tanner stage 1, C3 was Tanner stage 2 and C4 was Tanner stage 5. Control C4 underwent simultaneously left tests orchietomy and testicular biopsy on the right tests for fertility preservation. Histological analysis revealed complete necrosis of his left tests without any abnormal cells. These controls are critical because their age differs from the Klinefelter patient group but these controls were the only patients who banked testicular tissue in our laboratory within the context of non-malignant disease and before any treatment potentially toxic to the male gonad. An informed consent was both signed by the patients and their parents for testicular tissue banking and for analysis and publication of the collected data.

**Semen samples**

Semen samples were produced in the laboratory by masturbation. In young adolescents, it is difficult to obtain precise information concerning the duration of sexual abstinence indeed, some of them may have their first experience of ejaculation in our laboratory.

Semen samples were analyzed after liquefaction for 20 min at 37°C according to World Health Organization guidelines (WHO, 1999). However, semen parameters (volume, seminal pH, sperm concentration (10⁹/ml), progressive motility (WHO grades a + b combined; %), vitality (%)) were assessed based on values quoted in the next edition of the guidelines (WHO, 2010). Furthermore, sperm morphology (% normal forms) was also assessed according to David’s modified classification (Auger et al., 2001). The diagnosis of azoospermia or cryptozoospermia was confirmed after centrifugation of the remaining ejaculate at (300g, 15 min).

**Testicular biopsy**

For adolescent Klinefelter patients, a bilateral testicular biopsy representing approximately a quarter of the global tests volume was performed on each testis convexity under general anesthesia. The same urologist surgeon performed all the testicular biopsies. After incision of the tunica albuginea, seminiferous tubules were excised and transported to the laboratory at 37°C in culture medium (IVF medium™, Orglio, Lyon, France). Each testicular fragment was weighed. For testicular spermatozoa extraction, two-thirds of the testicular biopsy was shredded with insulin liberated, at 4°C, 10 min. A third observation was performed on the pellet after migration.

Spermatozoa identified in the supernatant before migration or in the pellet after migration were diluted (v/v) with a sperm cryoprotectant medium (Spermfreeze™, JCD, Lyon, France) and were automatically frozen in straws (Cryobiosystem, L’Aigle, France) with an automatic apparatus (Freezall™, Air Liquide Santé, France) before being plunged into liquid nitrogen.

**Testicular tissue freezing**

For Klinefelter adolescents, one-third of the testicular biopsy of each testis was cut in 10 mg fragments. Each fragment was individually placed into cryovials (Nunc®. Roskilde, Denmark) containing 1.3 ml of cryoprotective medium. The freezing medium consisted of Leibovitz™ L-15 medium (Eurobio, Courtaboeuf, France) supplemented with 1.5 M dimethyl-sulphoxide (Sigma-Aldrich, Ville, France), plus 0.05 mol/l sucrose (Sigma-Aldrich) and 10% (v/v) patient serum. Testis fragments were equilibrated, at 4°C for 30 min in freezing solution and then were frozen using a programmable freezer (Freezall™, Air Liquide, Paris, France) and a controlled slow freezing protocol without seeding (start at 5°C, 2°C/min to −9°C, 7 min of soaking, 0.3°C/min to −40°C, 10°C/min to −140°C). Cryovials were transferred into liquid nitrogen and stored until use as proposed by Milazzo et al. (2008, 2010).

For controls (C1, C2 and C3), testicular biopsy was only used for testicular tissue freezing according to the procedure mentioned above. For control C4, testicular biopsy on the right tests was used for TESE and testicular tissue freezing as performed for Klinefelter adolescents.

**Histological analysis of testicular tissue**

One small fragment of each testis was sent by the urologist directly to the pathology department of Rouen University Hospital. Another small fragment (close to 3 mg) of each testis randomly selected among testicular
Seminiferous tubule assessment

The mean diameter and area of 30 cross-sectioned tubules were assessed using a digital imaging analysis system (LAS 2.8.1, Leica, Germany). A cross-sectioned seminiferous tubule was considered dilated if its diameter was ≥400 μm (Volkmann et al., 2011). A tubule was defined as cross-sectioned when the ratio between the longest diameter and the diameter perpendicular to the longest one was evaluated between 1 and 1.5. Four different measures of the lamina propria of each cross-sectioned seminiferous tubule were performed and the mean value was used to define the thickness. The lamina propria was considered as thickened if >10 μm (Volkmann et al., 2011). The morphology of the lamina propria was also assessed according to its regularity. A qualitative assessment of the lamina propria was performed as follows: (i) score 1 for thin and regular lamina propria, (ii) score 2 for thickened and regular lamina propria, (iii) score 3 for thickened and irregular lamina propria (degenerative seminiferous tubule). We also explored the relationship between the morphology scoring of lamina propria and seminiferous tubule epithelium.

For each seminiferous tubule, intratubular cells were identified (Sertoli and germ cells). The percentage of empty seminiferous tubules, tubules containing only Sertoli cells or tubules containing germ cells whatever the differentiation stage was determined. When germ cells were observed, the stage of differentiation observed in each tubule—spermatogonia only, spermatocytes I, round or elongated spermatids as well as spermatozoa—was assessed. For each section, the total area of the tissue (At), area with tubules (A+) and area without tubules representing interstitial tissue (A−) were measured in order to evaluate Leydig cell hyperplasia.

In complement, the presence of spermatogonia was assessed using immunostaining with melanoma-associated antigen 4 (MAGE-A4 generously provided by Dr Giulio Spagnoli, University of Basel, Switzerland). Three-micrometer sections of paraffin-embedded tissue were deparaffinized in xylene and rehydrated in an ethanol bath series. Slides were boiled for 40 min in 0.01 M citrate buffer, pH 6 (S2031, Dako, Trappes, France). Background staining was blocked by incubation 5 min in HP boiling for 40 min in 0.01 M citrate buffer, pH 6 (S2031, Dako, France). Slides were dehydrated in ascending ethanol series and mounted in Eukitt medium (Eukitt C, EUK 100, CML 717, France). Negative controls were obtained by omitting primary antibody.

For AMH, labelling was scored according to the staining intensity and the proportion of intratubular stained area. For AR, labelling was scored for Sertoli cells according to the intensity and the proportion of stained cells. Sertoli cell maturation assessment after anti-AMH and AR immunostaining was performed as follow: (i) after AMH labelling, slides were scored as 0 for complete, as 1 for partial and as 2 for negative intratubular area staining; (ii) after AR labelling as 0 for negative staining, as 1 for cytoplasmic staining and as 2 for intense Sertoli cell nuclei staining; (iii) after Vimentin labelling, Sertoli cell morphology was considered as ‘infantile’ and scored as 0 if nuclei were round to ovoid with one or two small nucleoli and as ‘adult’ and scored as 1 if nuclei were many often triangle shaped, with a large, centrally located nucleolus (Nistal et al., 1998).

The global score for Sertoli cell maturity was the sum of AMH, AR and vimentin morphological scores and was consequently comprised between 0 (infantile), 1–4 (intermediate) and 5 (adult). Sertoli cell maturity score was established for each seminiferous tubule according to germ cell epithelium.

Descriptive data are reported as: (i) percentage of germ cells at different stages of maturity for seminiferous tubule content, (ii) mean and standard deviation for seminiferous diameter and (iii) mean and range for Sertoli cell maturity scoring and lamina propria scoring.

Results

Patient clinical characteristics

Patient biological and clinical characteristics are shown in Table 1. Most of the patients (five out of eight) were diagnosed within the context of painless gynecomastia that occurred at mid-puberty. All the patients had entered puberty spontaneously with normal progression and had developed satisfactory secondary sexual characteristics. Bilateral descended and small firm testes were observed in all patients, with a testicular volume varying from 1.3 to 3.5 ml. One patient (P6) had a history of bilateral maldescended testes with a spontaneous descent that occurred at 6 years. One patient (P8) had an incomplete foreskin without hypospadias diagnosed and surgically cured at the age of 10 years. Six patients (P1, P2, P3, P5, P6 and P8) would require
<table>
<thead>
<tr>
<th>Probands</th>
<th>Karyotype</th>
<th>Age at diagnosis (years)</th>
<th>Context of diagnosis</th>
<th>Age at fertility preservation (years)</th>
<th>Tanner stage</th>
<th>BMI</th>
<th>Right testis volume (ml)a</th>
<th>Left testis volume (ml)a</th>
<th>FSH (IU/l)</th>
<th>LH (IU/l)</th>
<th>Testosterone (µg/l)</th>
<th>SHBG (nmol/l)</th>
<th>AMH (µg/l)</th>
<th>Inhibin B (pg/ml)</th>
<th>Semen volume (ml)</th>
<th>Spermogram</th>
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</thead>
<tbody>
<tr>
<td>P1</td>
<td>47,XXY[12]/46,XY[6]</td>
<td>In utero</td>
<td>Prenatal diagnosis (maternal age)</td>
<td>15.5</td>
<td>A3P3G3</td>
<td>21.4</td>
<td>2.3</td>
<td>3.4</td>
<td>18.3</td>
<td>4.7</td>
<td>2.35</td>
<td>24</td>
<td>7.0</td>
<td>44</td>
<td>0.5</td>
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<td>P2</td>
<td>47,XXY</td>
<td>15</td>
<td>Gynecomastia</td>
<td>16.5</td>
<td>A3P3G2</td>
<td>18.9</td>
<td>3.5</td>
<td>2.3</td>
<td>42.6</td>
<td>9.5</td>
<td>2.44</td>
<td>28</td>
<td>6.2</td>
<td>&lt;15</td>
<td>0.5</td>
<td>Azoospermia</td>
</tr>
<tr>
<td>P3</td>
<td>47,XXY</td>
<td>At birth</td>
<td>Micropenis</td>
<td>16</td>
<td>A3P5G2</td>
<td>24.3</td>
<td>1.9</td>
<td>2.1</td>
<td>43.6</td>
<td>10.3</td>
<td>2.44</td>
<td>36</td>
<td>2.0</td>
<td>&lt;15</td>
<td>0.4</td>
<td>Azoospermia</td>
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<td>47,XXY</td>
<td>17</td>
<td>Gynecomastia</td>
<td>17</td>
<td>A2P4G2</td>
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<td>1.3</td>
<td>1.3</td>
<td>33.9</td>
<td>18.6</td>
<td>4.78</td>
<td>43</td>
<td>1.3</td>
<td>&lt;15</td>
<td>0.9</td>
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<td>47,XXY</td>
<td>17</td>
<td>Gynecomastia</td>
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<td>A3P5G2</td>
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<td>1.5</td>
<td>77.3</td>
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<td>A2P4G3</td>
<td>18.8</td>
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<td>Gynecomastia</td>
<td>15.5</td>
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<td>18.5</td>
<td>2.4</td>
<td>2.2</td>
<td>44.9</td>
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<td>4.92</td>
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<td>47,XXY</td>
<td>In utero</td>
<td>Prenatal diagnosis (previous trisomy 21)</td>
<td>15</td>
<td>A2P4G3</td>
<td>19.4</td>
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<td>45.8</td>
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<td>1.74</td>
<td>25</td>
<td>1.0</td>
<td>&lt;15</td>
<td>0.6</td>
<td>Azoospermia</td>
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</table>

AMH, anti-Müllerian hormone; FSH, normal values between 1 and 10.5 IU/l; LH, normal values between 1 and 8 IU/l; OAT, oligoasthenoteratozoospermia; SHBG, sex hormone binding globulin; yr, years.
aTesticular volume measured by ultrasound examination.
testosterone replacement therapy, as proposed by the pediatric endocrinologist. The basal serum concentrations of FSH (range: 18.3–77.3 UI/l) (normal values between 1 and 10.5 UI/l) and LH (range: 4.7–47.5 UI/l) (normal values between 1 and 8 UI/l) were significantly higher than normal values for age. Serum inhibin B was undetectable in five patients (P2, P3, P4, P5 and P8) and detectable but decreased in the others. Testosterone concentrations reached values observed in adult in only three patients (P4, P5 and P7) (>3 ng/ml).

Seven patients were accompanied by both parents and one by just his mother. All the KS adolescents presented since infancy, communication limitation with difficulties concerning social adaptation, clearly recognized by their parents and the patients themselves. Seven patients had difficulties in learning language and reduced scholar performance.

Concerning fertility preservation, seven patients had not really thought about their future fertility and were only involved in the procedure proposed, after at least three medical consultations. The parents immediately welcomed the procedure and considered fertility preservation as a potential chance for the future fertility of their sons. One patient (P5) stressed the point immediately on performing all the fertility preservation procedure—semen sample and testicular biopsy if necessary—and talked also about sperm donation in case of germ cell retrieval failure. Three patients (P2, P4 and P8) raised the question of adoption.

**Semen parameters and testicular biopsy**

All the patients agreed to produce a semen sample and collected from two to five samples spaced at least by 3 months. P2 had his first experience of masturbation in our laboratory and failed twice to produce a semen sample. Hyposperma was detected in all patients. Non-mosaic XXY patients presented azoosperma and P1 with a XXY/XY constitution presented oligoasthenoteratozoospermia (Table I). P1 banked four semen samples before hormone replacement therapy. The sperm concentration varied from 0.1 to 1.0 × 10⁶/ml, prefreeze motility (WHO a+b combined) varied from 15 to 40% and the percentage of spermatozoa normal forms was 32%. A total of 22 straws were banked, with 5% of motile and progressive spermatozoa after thawing.

Two patients (P4 and P5) did not perform testicular biopsy and expressed their refusal during the consultation with the psychologist, although P4 initially agreed with the possibility to undergo testicular biopsy. P5 refused testicular biopsy because he was afraid of the general anesthesia necessary for surgical testicular biopsy. Five patients accepted a bilateral testicular biopsy (Table II). The mean delay between the first consultation and testicular biopsy was 11 months (range: 6–13 months). The fragment weight varied between 100 and 382 mg. One patient (P3) had successful spermatozoa recovery on his right testis. Another patient (P7) achieved collection of spermatoocytes I and elongated spermatids on the right testis.

Overall, two out of the six adolescent KS patients who attempted the procedure of fertility preservation banked spermatozoa and a third banked immature germ cells (spermatocytes I and elongated spermatids).

One month after the testicular biopsy and after a complete historical evaluation of testis biopsy, the TESE result was discussed with the young adolescents during another consultation. In cases of TESE failure and when immature germ cells were not retrieved, each patient was met separately to obtain his own opinion about the question of his future parenthood. The several consultations organized before testicular biopsy helped the patient to express a proposition. Two patients talked about sperm donation and one patient thought of adoption. No surgical, medical or psychological complications occurred for the different patients involved in the procedure.

**Testicular tissue analysis**

Histological evaluation of testicular tissue confirmed the presence of spermatozoa on the right testis of P3 (Table II). Immature germ cells were also observed on the right testis of P7 (spermatocytes I or elongated spermatids). However, the percentage of tubules with germ cells remained very low. The concentration of spermatogonia was reduced in KS patients compared with controls whatever the age of the controls.

A large number of seminiferous tubules were empty and degenerative even in patients with mature or immature germ cells (Fig. 1). None of the patients had dilated seminiferous tubules. However, the mean diameter was heterogeneous between the different patients and for each patient between the two testicles. Furthermore, the seminiferous tubule diameter did not vary between seminiferous tubules with or without germ cells. The mean diameter was lower in KS patients with germ cells compared with pubertal normal boy (C4), more specifically for P3 with mature spermatozoa after TESE.

Lamina propria alterations were more severe in adolescent Klinefelter patients compared with controls and increased when germ cells and Sertoli cells disappeared from seminiferous tubules even in the youngest patient (P8). Regular and thin lamina propria was observed in controls (score 1) and >50% of KS seminiferous tubules had thickened or irregular lamina propria (score 2 or 3). Sertoli cell maturity scoring revealed an intermediate maturity profile, between infantile and adult profiles, in most KS patients. However, Sertoli cell maturity was close to adult profile in the two patients with mature or immature germ cells in their seminiferous tubules (P3 and P7) and preferentially in tubules containing germ cells. Sertoli cell maturity was not in agreement with the clinical phenotype and the physiological age of the KS patients compared with controls.

**Discussion**

Our data show the feasibility of obtaining semen samples from adolescent KS patients after the onset of puberty in order to retrieve spermatozoa for fertility preservation. However, testicular biopsy for TESE or testicular tissue freezing is not acceptable for all adolescents. In our population, 1 out of 8 patients (12%) had a KS with a mosaic form 47,XXY/46,XY confirming that KS is generally associated with a homogeneous karyotype 47,XXY (Tüttelmann et al., 2010). Most of our XXY adolescents were diagnosed after the onset of puberty (5 out of 8, Table I) because of gynecomastia (3 out of 8), puberty retardation (1 out of 8) or short stature (1 out of 8). Our population was consistent with the observation that XXY subjects are seldom diagnosed before the onset of puberty (Radicioni et al., 2010; Lahloul et al., 2011). Two subjects were diagnosed prenatally because the pregnancy was at risk of Down syndrome (maternal...
Table II Histological evaluation of seminiferous tubule and characterization of lamina propria alterations, in testicular tissue of five XXY adolescents.

<table>
<thead>
<tr>
<th>Probands</th>
<th>Age at testis biopsy (year)</th>
<th>Right testis</th>
<th>Left testis</th>
<th>ST with GC</th>
<th>SCO</th>
<th>ST with GC</th>
<th>SCO</th>
<th>Empty ST</th>
<th>ST with GC</th>
<th>SCO</th>
<th>ST with GC</th>
<th>SCO</th>
<th>Empty ST</th>
<th>ST with GC</th>
<th>SCO</th>
<th>ST with GC</th>
<th>SCO</th>
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<th>ST with GC</th>
<th>SCO</th>
<th>ST with GC</th>
<th>SCO</th>
<th>Empty ST</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2</td>
<td>16.5</td>
<td>300</td>
<td>SGO: 33%</td>
<td>69.8 ± 26.9</td>
<td>Inf/Int/A</td>
<td>1.75 (1–3)</td>
<td>3</td>
<td>0</td>
<td>241</td>
<td>SGO: 77%</td>
<td>Empty ST:</td>
<td>23%</td>
<td>Inf/Int/A</td>
<td>1.83 (1–3)</td>
<td>3</td>
<td>0</td>
<td>147.4 ± 37.4</td>
<td>A</td>
<td>31 569</td>
<td>17</td>
<td>101</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td>16</td>
<td>172</td>
<td>SGO: 3%</td>
<td>44.2 ± 55.2</td>
<td>A</td>
<td>Int</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>8789</td>
<td>193</td>
<td>SGO: 50%</td>
<td>Empty ST:</td>
<td>50%</td>
<td>Int/A</td>
<td>1.8 (1–2)</td>
<td>3</td>
<td>0</td>
<td>149.2 ± 46.1</td>
<td>Inf/Int</td>
<td>1.43 (1–2)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>P6</td>
<td>16</td>
<td>240</td>
<td>SGO: 100%</td>
<td>97.3 ± 34.7</td>
<td>Inf/Int</td>
<td>1.2 (1–2)</td>
<td>—</td>
<td>0</td>
<td>210</td>
<td>SGO: 100%</td>
<td>Empty ST:</td>
<td>50%</td>
<td>Inf/Int</td>
<td>1.43 (1–2)</td>
<td>0</td>
<td>0</td>
<td>93 ± 27.7</td>
<td>Inf/Int</td>
<td>2</td>
<td>3</td>
<td></td>
<td></td>
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<tr>
<td>P7</td>
<td>15.5</td>
<td>101</td>
<td>SGO: 14%</td>
<td>98 ± 25.3</td>
<td>Inf/Int</td>
<td>1.8 (1–2)</td>
<td>1.89 (1–3)</td>
<td>3</td>
<td>1504</td>
<td>382</td>
<td>SGO: 47%</td>
<td>Empty ST:</td>
<td>53%</td>
<td>Inf/Int</td>
<td>1.8 (1–2)</td>
<td>3</td>
<td>0</td>
<td>136.6 ± 5.6</td>
<td>2.7</td>
<td>193</td>
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<td></td>
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<tr>
<td>P8</td>
<td>15</td>
<td>110</td>
<td>SGO: 100%</td>
<td>98.7 ± 11.8</td>
<td>Inf</td>
<td>—</td>
<td>2</td>
<td>—</td>
<td>100</td>
<td>SGO: 100%</td>
<td>Empty ST:</td>
<td>50%</td>
<td>Int</td>
<td>1.64 (1–2)</td>
<td>0</td>
<td>0</td>
<td>136.6 ± 18.3</td>
<td>Inf</td>
<td>1.64 (1–2)</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>1</td>
<td>SGO: 13%</td>
<td>63 ± 8.8</td>
<td>Inf/Inf</td>
<td>1</td>
<td>1</td>
<td>12 494</td>
<td>1</td>
<td></td>
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<tr>
<td>C2</td>
<td>9</td>
<td>SGO: 100%</td>
<td>49.4 ± 6.3</td>
<td>Inf</td>
<td>—</td>
<td>1</td>
<td>19 820</td>
<td>1</td>
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<tr>
<td>C3</td>
<td>12</td>
<td>SGO: 100%</td>
<td>80.6 ± 10.3</td>
<td>Inf</td>
<td>—</td>
<td>1</td>
<td>36 866</td>
<td>1</td>
<td></td>
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<tr>
<td>C4</td>
<td>17</td>
<td>SGO: 7%</td>
<td>147.4 ± 37.4</td>
<td>Inf</td>
<td>—</td>
<td>1</td>
<td>31 569</td>
<td>1</td>
<td></td>
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Data for seminiferous diameter are reported as mean (± sd) and data for lamina propria scoring are presented as mean and range values. Sertoli cell maturity was qualitatively assessed and characterized as infantile, intermediate or adult stage. A, Sertoli cell adult stage; GC, germ cells; Inf, Sertoli cell infantile stage; Int, Sertoli cell intermediate stage; SC, Sertoli cells; Sc I, Spermatocytes I at leptotene or pachytene stage; SCO, Sertoli cell only; Sg, spermatogonia; Sd, elongated spermatid; ST, seminiferous tubule; Sz, spermatozoa; yr, years.
age, previous pregnancy with 21 trisomy) also in agreement with reports that <10% of XXY subjects are diagnosed during prenatal cytogenetic examination (Bojesen et al., 2003; Radicioni et al., 2010). Furthermore, XXY subjects are mostly clinically normal at birth (Lahlou et al., 2011). However, genital anomalies may be a reason for referral: conditions including micropenis (Zinn et al., 2005; Lee et al., 2007), as seen in our patient (P3, Table 1) and maldescended testis (Lee et al., 2007; Ferlin et al., 2008; Cabrol et al., 2011). One of our adolescents (P6, Table 1) had a history of maldescended testes but the karyotype was only performed because of painless gynecomastia occurring after the onset of puberty. A very small number of XXY subjects are diagnosed during infancy for cognitive and language deficit (Verri et al., 2010). In our population, five XXY adolescents who were diagnosed at the onset of puberty exhibited developmental delays with learning difficulties but these did not indicate cytogenetic analysis. Furthermore, our patients entered puberty

**Figure 1** Sertoli cell maturity scoring assessed after immunostaining of vimentin (VIM), anti-Müllerian hormone (AMH) and AR in Klinefelter patients (P3, P7 and P8) and controls (C1 and C4) and counterstaining with HES. Photomicrographs were captured at ×400 magnification and scale bars represent 30 μm. Brown staining indicates Sertoli cells with a positive cytoplasmic expression of VIM, AMH or AR. (A) Prepubertal testis from controls C1. Sertoli cells presented an infantile morphology, a strong expression of AMH and an undetectable expression of AR. (B) Testis from P8 showing seminiferous tubule with Sertoli cells presenting an intermediate phenotype, with a weak expression of AMH and AR. (C) Testis from P7 with elongated spermatids and Sertoli cells presenting an adult phenotype with an undetectable expression of AMH and a strong nuclear expression of AR. (D) Testis from P3 with seminiferous tubule presented elongated spermatids and Sertoli cells with an adult phenotype. (E) Post-pubertal testis from control C4 with adult and mature Sertoli cells: Sertoli cells presented an adult morphology, an undetectable expression of AMH and a strong expression of AR. AMH, anti-Müllerian hormone; AR, androgen receptor; C, control; Es, elongated spermatid; P, patient; Sg, spermatogonia; Sp I, spermatocyte I; St, Sertoli cell; Spz, spermatozoa; Vim, vimentin; y, years.
spontaneously and at the expected time as it has been generally reported in XXY males (Lahlou et al., 2011). Our KS patients presented the typical clinical findings associated with KS in that their testes had a firm consistency but a low volume (less than 2 ml for most of patients) (Lanfranco et al., 2004). Furthermore, elevated levels of gonadotrophins were also observed in all of them. All the patients that accompanied the adolescents during the procedure accepted fertility preservation. The opinion of the mother and father was taken separately but both completely agreed to perform a testicular biopsy for sperm retrieval and testicular tissue freezing in case of azoospermia. The parents were fully convinced of the possibility of fertility preservation even if no biological or clinical parameters might predict objectively the chance of mature or immature germ cell retrieval. The fathers also wanted information about the ability of their son to have a normal sexual life in the future. Our young adolescents considered the question of their future fertility as a low priority for their age except for one patient (P8). However, they all agreed to produce a semen sample, considered as a non-invasive procedure and for five of them accepted testicular biopsy. Our KS adolescents first priority was for their physical appearance to be as virilized as other adolescents. The consultation with the psychologist was fundamental to enable these patients to express their refusal to accept treatment and to obtain information about their future status of infertile man. There is only one report in the literature concerning parents’ and KS patient’s objective opinion on fertility preservation: the family of a 15-year-old adolescent undergoing TESE considered that the procedure might secure the possibility for him to become a father in later life (Damani et al., 2001). We believe it to be an absolute necessity to discuss with the adolescent, over a several-month period, the issues of sperm production and also the question of unsuccessful spermatozoa recovery. The adolescent should be involved in the fertility preservation process only if he is completely aware that a negative result is possible and if he has considered alternative options to become a father. Such counselling is vital to evaluate the acceptability of the concept of fertility preservation before its technical feasibility is considered and may influence the chance for the adolescent to succeed in producing a semen sample. In our study, spontaneous semen collection was possible in all the adolescents as also reported in a population of 13 adolescents aged between 15 and 19.5 years (Aksglaede et al., 2008). We did not use any penile vibrostimulation to provoke ejaculation, as suggested by Van Saen et al. (2012). Furthermore, in the series of Gies et al. (2012), only 1 out of 7 patients agreed to attempt semen sampling. The discrepancy between the different studies may be related to the fact that the population of Gies et al. (2012) was younger and that pubertal development might not be sufficient to allow erection and ejaculation. Spermaturia was not evaluated in our study because all the patients succeeded in performing semen sampling. One of our patients failed to collect twice, but succeeded thereafter. This patient did not have any experience of masturbation before his first appointment in our laboratory. Spermaturia might be present in younger KS adolescents because spermarche appears early during pubertal development but this hypothesis was not verified either in a population of 7 KS boys aged between 12 and 15.6 years (Gies et al., 2012) nor in a population of 12 pubertal boys with KS aged >16 years (Ratcliffe, 1982). A low semen volume was observed in all the patients included in our study, even in patient with normal testosterone plasmatic level (P4 and P5; Table 1), also observed in a population of 13 KS adolescents aged <20 years (Aksglaede et al., 2008). These data suggested a probable androgen insufficient despite testosterone plasmatic level within the normal range (Aksglaede et al., 2008). Spermatozoa have occasionally been identified in the ejaculate of KS men (Futterweit, 1967; Foss et al., 1971; Kamischke et al., 2003; Lin et al., 2004; Lanfranco et al., 2004; Aksglaede et al., 2008). The prevalence reported varies from, from 4.3% (Aksglaede et al., 2008) to 8.4% (Kamischke et al., 2003). Furthermore, the population studied was generally a population of adult males aged more than (i) 20 years (Kamischke et al., 2003), (ii) 22 years (Lanfranco et al., 2004) or (iii) 29 years (Aksglaede et al., 2008). Furthermore, in the report of Kamischke et al. (2003), 2 patients out of 4 who presented spermatozoa in the ejaculate were 18.6 and 19.7 years old, respectively. None of the 13 adolescents with KS who were >20 years old (Aksglaede et al., 2008) or of 7 patients <16 years old (Gies et al., 2012), had spermatozoa in their ejaculate. Our patient with spermatozoa in his ejaculate had a mosaic form of KS that might alter the spermatogenesis process less severely than non-mosaic KS (Gies et al., 2012). However, the sperm concentration was very low and spermatozoa banking was proposed in view of the progressive decline in spermatogenesis with age in XXY males. Furthermore, the patient required hormone replacement therapy that is known to affect spermatogenesis and spermatozoa production (McLachlan et al., 2002; Schiff et al., 2005).

Two positions may be adopted when considering the possibility of spermatozoa recovery after TESE in Klinefelter adolescents: the positive one supported by our study and the case reported by Damani et al. (2001) and the negative one following from two recent reports of Klinefelter adolescents in whom TESE did not retrieve any spermatozoa (Van Saen et al., 2012; Gies et al., 2012). In our study, a bilateral testicular biopsy was performed while only one testis was explored in the two above-mentioned studies. This parameter is well known to influence the success of TESE, also confirmed by the fact that mature spermatozoa were only detected in one testis in our patient (P3). Furthermore, in a population of 112 adult azoospermic Klinefelter males who underwent bilateral TESE and from whom spermatozoa were recovered in our laboratory spermatozoa were isolated from one testis only in ~40%, (unpublished data). In the situation of an unsuccessful unilateral TESE in non-obstructive azoospermia, it remains possible that spermatozoa could be retrieved from the other testis. The same conclusion applies to the possibility of immature germ cell recovery in adolescent Klinefelter patients. An alternative proposal to obtain the best chance of sperm retrieval is first to remove a small amount of tissue from one testis and only if sperm recovery is unsuccessful, to perform a bilateral testicular biopsy at a later stage (Damani et al., 2001). However, this procedure is not adapted for the identification of immature germ cells. Taking these conclusions into account, we felt it is best to propose a bilateral testicular biopsy to our adolescent Klinefelter patients to optimize the chance of mature or immature germ cell recovery. No complication occurred in our population after the bilateral testicular biopsy.

Another aspect of fertility preservation in Klinefelter patients is the question of clinical or biological predictive factors of successful recovery of spermatozoa. Gonad failure in KS is established early during testis development and leads after the puberty to an intensive hyalinization of seminiferous tubules. Germ cell degeneration starts during
mid-term fetal life (Akseslaede et al., 2006), progresses during early infancy (Mikamo et al., 1968) and childhood (Müller et al., 1995) and is accelerated at the onset of puberty (Akseslaede et al., 2006). Therefore, one could argue that fertility preservation should be proposed as soon as the diagnosis of KS is known even during the infantile period when the relative number of spermatogonia seems to be higher and the seminiferous tubule degeneration process is not complete (Mikamo et al., 1968; Wikström et al., 2004). However, it has been suggested that focal active spermatogenesis in XXY males is only possible when spermatogonial stem cell lineage has a 46,XY constitution and that all spermatocytes I also have a 46,XY constitution (n = 11, Scirano et al., 2009; n = 10, Vialard et al., 2012) while on the other hand, Foresta et al. (1999) observed only 47,XXY spermatogonia and spermatocytes I in two 47,XXY males. The first hypothesis supposed that XXY spermatogonia have difficulty entering meiosis (or are unable to) and that XXY spermatogonia degenerate via apoptosis during mitotic divisions at the onset of puberty rather than during the meiotic process (Scirano et al., 2009; Vialard et al., 2012). The presence of spermatogonia in prepubertal testicular tissue that might be only XXY cannot predict the ability of these cells to go through meiosis and to achieve spermatogenesis. Even if the preservation of prepubertal testicular tissue from Klinefelter patients allowed the preservation of seminiferous tubules with normal architecture and higher number of spermatogonia, we are not sure to be able to prevent the normal degenerative process of germ cells and seminiferous tubules even in the conditions of in vitro maturation, germ cell transplantation or tissue grafting. Therefore, fertility preservation in Klinefelter boys might be preferably proposed just after the onset of puberty to possibly retrieve more numerous mature gametes than in adult Klinefelter males or survivor 46,XY spermatogonia.

However, after the onset of puberty, extensive tubular degeneration occurs. In our study, thickening and regularity of lamina propria were normal in controls and severely impaired in adolescent Klinefelter patients whatever the age of the patient and whatever the testis contained germ cells or not (Table II). Fibrosis of the lamina propria of seminiferous tubules is associated with accumulation of collagen fibers and extracellular components as well as an increase in the thickness of myofibroblast layers (Völkmann et al., 2011). Morphological alterations of the lamina propria of seminiferous tubules in KS patients were associated with spermatogenesis defects. Lamina propria was less impaired in seminiferous tubules containing germ cells or Sertoli cells compared with empty seminiferous tubules. Initial germ cell defects could represent the first step in lamina propria degeneration and thickening (Völkmann et al., 2011). However, Sertoli cell dysfunction may also participate to lamina propria degeneration. At the onset of puberty, Sertoli cells normally switch from an immature, proliferative state to a mature, non-proliferative state. In Klinefelter patients, Sertoli cells might fail to switch to a functionally mature status. The patchy nature of the testicular histology, with more or less affected areas (Akseslaede et al., 2006), appears to be also associated with a patchy maturation of Sertoli cells. During the infantile period, Sertoli cells show typically immature features with oval and elongated nuclei associated with a regular shape (Nistal et al., 1982), a high level of AMH expression (Rajpert-de Meyts et al., 1999) and no or slight cytoplasmic expression of AR (Rey et al., 2009). At the onset of puberty, Sertoli cells undergo physiologically morphological and functional maturation: the nucleus enlarges and becomes tripartite with a more prominent nucleolus (Chemes et al., 1979), AMH expression is severely down-regulated at around 14 years (Rey et al., 1993; Rajpert-de Meyts et al., 1999), and AR is highly expressed in Sertoli cell nuclei (Rey et al., 2009). We used a combined score taking into account these different parameters in order to obtain a global evaluation of Sertoli cell maturation for each patient and for the different seminiferous tubules with or without germ cells and to be able to compare the score between our patients. In our adolescent Klinefelter patients, a heterogeneous phenotype of Sertoli cells was observed between adolescent Klinefelter patients and in a same patient, between seminiferous tubules. However, Sertoli cell maturation was close to the adult phenotype in patients with persistent differentiating germ cells with a higher degree of maturity in seminiferous tubules containing germ cells. In seminiferous tubules with Sertoli cell only, mixed patterns of Sertoli cell maturation but preferentially immature phenotype were detected, as described previously (Wikström et al., 2007; Van Saen et al., 2012). The occurrence of immature Sertoli cells might be the consequence of a maturation defect or due to the re-emergence of an immature phenotype due to de-differentiation of previously mature Sertoli cells. However, the preferential occurrence of Sertoli cell mature phenotype in seminiferous tubules containing differentiating germ cell is more in agreement with a probable failure of maturation. One of the fundamental questions in Klinefelter patients is whether the abnormal karyotype affects originally germ cells or somatic cells, not only Sertoli cells but also Leydig cells. We also observed a high degree of interstitial tissue hyperplasia in adolescent Klinefelter patients compared with controls, as observed in adult Klinefelter patients confirming that the testicular phenotype in Klinefelter patient is a result of impaired function and interaction of several cell types that has begun before the onset of puberty (Akseslaede et al., 2006; Wikström et al., 2007; Van Saen et al., 2012).

Neither clinical features nor hormonal parameters could predict germ cell recovery in our adolescent Klinefelter patients. No difference was observed between patients presenting spermatозoa in their ejaculate or spermatозoa recovered by TESE, the patient with immature germ cells in testicular biopsy and those patients who did not have any germ cells. Furthermore, most of them (five out of eight) had undetectable serum inhibin B concentrations, even in one (P3) in whom spermatозoa retrieval after TESE was possible. Among the three patients (P1, P6 and P7) who had detectable serum inhibin B: ejaculated spermatозoa were obtained from one (P1) and immature germ cells (spermatocytes I, round and elongated spermatids) were recovered after testicular biopsy in the second (P7), whereas Sertoli cell only was observed for the third patient (P6). From mid- to late puberty, Sertoli cell dysfunction is reflected by decreased AMH and inhibin B and increased FSH (Lah lou et al., 2011). It has also been suggested that the decline in inhibin B after puberty may reflect germ cell dysfunction or depletion (Gies et al., 2012). Therefore, early diagnosis of KS would increase the chance of spermatозoa retrieval since the semen quality of KS patients may decline with age (Lin et al., 2004; Emre Bakircioglu et al., 2006; Ichikawa et al., 2006; Gies et al., 2012; Van Saen et al., 2012). Furthermore, Leydig cell dysfunction may be another factor that affects spermatogenesis process in XXY adolescents (Lah lou et al., 2011). However, the plasma testosterone level was low in the two patients with mature spermatозoa, normal for the patient with immature germ cells and varied from normal to decreased in patients
without any germ cells. In conclusion, even if the number of patients included in our study was small, it appears that no clinical or hormonal factors are predictable of germ cell retrieval in young KS adolescents, as also reported in other published data (Aksгляede et al., 2008; Gies et al., 2012; Van Saen et al., 2012).

In conclusion, although our study was small and more data are required to confirm the results, we have shown that spermatozoa can be retrieved in semen samples or from testicular tissue of a significant minority of adolescent Klinefelter patients. Furthermore, the testis may also contain spermatogonia and non-completely differentiated germ cells—spermatocytes and elongated spermatids. However, before collecting a semen sample or performing bilateral testicular biopsy, it is vital that the physician should discuss fertility preservation with the patient and his parents over a period of several months. Given such counselling, the patient will decide himself whether or not to accept the whole procedure (semen sample and testicular biopsy). We believe that the optimum time to propose fertility preservation in adolescent Klinefelter patients might be just after the onset of puberty when it is possible to collect a semen sample and when the patient is mature enough to be able to consider alternative options to become a father and to accept the failure of germ cell retrieval. Early diagnosis of KS has also to be considered because that may not only improve the possibility of fertility preservation after the onset of puberty but also the medical care and the quality of life of these patients (Radiocini et al., 2010).

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Authors’ roles

N.R. managed fertility preservation in XXY adolescents, contributed to the design of the study, the choice of experiments, data collection and analysis, drafting of the manuscript. J.-P.M., A.B. and A.W. performed testicular tissue cryopreservation, histological evaluation and revised the manuscript. L.S., urologist, carried out testicular tissue biopsy and revised the manuscript. A.P. also managed fertility preservation in adolescents and critically revised the manuscript. M.C., pediatrician endocrinologist, recruited the patients and critically revised the manuscript. All authors have read and approved the final manuscript.

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Conflict of interest

None declared.

References


