Can pubertal boys with Klinefelter syndrome benefit from spermatogonial stem cell banking?

D. Van Saen1,*, I. Gies2, J. De Schepper1,2, H. Tournaye1,3, and E. Goossens1

1Research Group Biology of the Testis, Department of Embryology and Genetics, Vrije Universiteit Brussel, Laarbeeklaan 103, 1090 Brussels, Belgium 2Department of Pediatrics, UZ Brussel, Laarbeeklaan 101, 1090 Brussels, Belgium 3Centre for Reproductive Medicine, UZ Brussel, Laarbeeklaan 101, 1090 Brussels, Belgium

*Correspondence address. Tel: +32-2-477-46-44; Fax: +32-2-477-44-32; E-mail: dorien.van.saen@vub.ac.be

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BACKGROUND: Although early development of testes appears normal in boys with Klinefelter syndrome (KS), spermatogonial stem cell (SSC) depletion occurs in mid puberty, leading to infertility. Cryopreservation of SSCs prior to stem cell loss is an option that is currently offered to boys who have to undergo gonadotoxic treatments. This study aimed to explore the possibility of preserving SSCs in pubertal KS adolescents by testicular tissue banking.

METHODS: A retrospective study was conducted in seven non-mosaic 47,XXY adolescents, aged 13–16 years, who were invited for an experimental testicular tissue banking programme during their follow-up at the Paediatric Endocrinology Department of the UZ Brussel between 2009 and 2011. Paraffin-embedded testicular tissue was sectioned and stained with haematoxylin-eosin, and immunostainings were performed for Mage-A4, anti-Mullerian hormone, Inhibin α and steroidogenic acute regulatory protein. The presence of spermatogenesis and/or spermatogonia was evaluated.

RESULTS: Massive fibrosis and hyalinization was observed in all but one KS patients. Although spermatogonia were seen in five patients, spermatogonia were only present in tubules showing normal architecture in the youngest patient who also had normal follicle-stimulating hormone and inhibin B concentrations.

CONCLUSIONS: Testicular tissue cryopreservation in KS adolescents should be recommended as soon as possible, probably before hormonal changes of failing Sertoli cell function are detected.

Key words: fertility preservation / immunohistochemistry / Klinefelter syndrome / spermatogonial stem cells

Introduction

Klinefelter syndrome (KS), or the XXY condition, is the most common sex chromosome abnormality in humans (1/600 live births). KS is characterized by gynaecomastia, tall stature, small testes, low testosterone levels, learning disabilities and behavioural problems (Klinefelter et al., 1942). It is amongst the most frequent genetic causes of human infertility: 11% of azoospermic men have a 47,XXY karyotype (Van Assche et al., 1996). Since germ cell depletion starts with the onset of puberty, testicular tissue banking at early puberty may be a strategy to preserve the fertility of these patients.

Cryopreservation of spermatogonial stem cells (SSCs) prior to stem cell loss is currently offered to boys undergoing gonadotoxic treatments, which may render them sterile (Tournaye and Goossens, 2011). After chemo- or radiotherapy, the frozen–thawed SSCs can be reintroduced in the patient’s own testis by SSC transplantation. However, since KS testes are characterized by extensive fibrosis and hyalinization of the seminiferous tubules, the ultimate use of the frozen tissue will be different. For KS boys, in vitro maturation of SSCs might be considered. So far, in vitro spermatogenesis of human SSCs has not been possible, but this technique might become an option in the near future since the in vitro differentiation of mouse SSCs up to mature sperm cells has recently been reported (Sato et al., 2001; Stukenborg et al., 2008).

Unfortunately, <10% of KS patients are diagnosed before puberty, explaining the limited experience on testicular tissue banking in KS adolescents (Bojesen et al., 2003).

To explore whether SSCs can be recovered in pubertal KS adolescents, a detailed histological study of a fragment of testicular tissue obtained at the time of banking was performed. We hypothesized...
that the youngest KS adolescents without biological signs of testicular failure, i.e. declining serum inhibin B and/or rising serum follicle-stimulating hormone (FSH) concentrations, would present with the highest number of SSCs.

Materials and Methods

Patients and samples
From 2009, we initiated an experimental study protocol to offer testicular cryopreservation in KS adolescents with clinical (arrest or regression of testicular volume) or biological (serum FSH >10 IU/l or declining serum inhibin B) signs of testicular failure. All KS boys, who ever had been counselled at our hospital and were in the peripubertal age, were informed about the study’s experimental protocol by the paediatric endocrinologist at their annual follow-up or contacted directly, if older than 13 years. Up to now, seven pubertal non-mosaic 47,XXY boys, showing azoosperma either after masturbation or after penile vibrostimulation or electroejaculation, have had testicular tissue banking performed under general anaesthesia. In two out of the seven boys, diagnosis was made prenatally (through karyotyping for maternal age). In four boys, chromosomal analysis was performed during childhood because of minor neurological and/or cognitive perturbations (mostly in association with behavioural changes). In the remaining patient, karyotyping was performed because of gynaecomastia and small testes.

Patients were followed up every 4 months by assessing the testicular volume, the FSH and inhibin B concentrations and spermaturia with the aim of detecting early spermatogenesis in these adolescent patients. When no further testicular growth or a decreasing inhibin B or increasing FSH concentration was observed or when azoosperma was observed in a semen sample after masturbation or after penile vibrostimulation or electroejaculation, testicular tissue banking was proposed. Written informed consent was obtained from both the parents and the teenager. Exclusion criteria were previous testosterone therapy and present or previously informed about the study’s experimental protocol by the paediatric endocrinologist at their annual follow-up or contacted directly, if older than 13 years. From 2009, we initiated an experimental study protocol to offer testicular cryopreservation in KS adolescents with clinical (arrest or regression of testicular volume) or biological (serum FSH >10 IU/l or declining serum inhibin B) signs of testicular failure. All KS boys, who ever had been counselled at our hospital and were in the peripubertal age, were informed about the study’s experimental protocol by the paediatric endocrinologist at their annual follow-up or contacted directly, if older than 13 years. Up to now, seven pubertal non-mosaic 47,XXY boys, showing azoosperma either after masturbation or after penile vibrostimulation or electroejaculation, have had testicular tissue banking performed under general anaesthesia. In two out of the seven boys, diagnosis was made prenatally (through karyotyping for maternal age). In four boys, chromosomal analysis was performed during childhood because of minor neurological and/or cognitive perturbations (mostly in association with behavioural changes). In the remaining patient, karyotyping was performed because of gynaecomastia and small tests.

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A testicular biopsy was taken from the lower pole of the largest testis under general anaesthesia. The technique of a single large volume biopsy instead of a multiple biopsy sampling method was chosen to reduce the risk of post-operative fibrosis. The majority of the testicular tissue was cut in 6 mm³ fragments and frozen, for potential later use in fertility treatments, according to the non-controlled freezing method, as already described by Van Saen et al., 2011. A randomly taken single small biopsy was frozen for histological purposes of this study.

The control tissues were testicular biopsies, which are routinely performed at the time of banking, from three adolescent patients (aged 10.2, 12.3 and 12.4 years) who banked their testicular tissue prior to gonadotoxic treatments. These patients were diagnosed with idiopathic bone marrow aplasia, sickle-cell anaemia and thalassaemia major, respectively.

Histological examination
Testicular tissue was fixed in hydrosafe fixative (R10 S7-16-60, Labonord, Rekkem, Belgium) for at least 1 h. After embedding in paraffin, 5 μm-thick sections were made at three different depths. If no SSCs were observed in these three sections, an additional fragment was thawed, fixed and examined. All histological examinations were performed on an inverted light and fluorescence microscope (Olympus IX81). Digital images were made using a digital camera (CC12 Soft Imaging System).

Structural integrity and presence of SSCs
Structural integrity was evaluated by haematoxylin-eosin staining. The amount of fibrosis was semi-quantified by giving them a score on a scale from 0 to 4 with 0 being no fibrosis and 4 being complete fibrosis. If fibrosis was observed in one quarter of the section surface, the Score 1 was given, while Scores 2 and 3 were given if fibrosis was observed in, respectively, half of the section or three quarters of the section. The definitive score for each patient was the median score from the different cross sections. The percentages of normal, degenerated and hyalinized tubules were calculated. When the tubular morphology was well-preserved (intercellular adhesion, attachment of cells to the basal lamina, intact basement membrane), the tubules were scored as normal. Tubules were considered degenerated when the following changes were observed: (i) degenerative Sertoli cells with pyknotic nuclei; (ii) detachment of cells from the basement membrane; (iii) loss of intercellular contacts and/or (iv) moderate thickened basal lamina. Hyalinized tubules showed massive thickening of the basement membrane and complete loss of seminiferous epithelial cells.

To assess the number of spermatogonia, slides were stained for melanoma-associated antigen 4 (MAGE-A4; provided by Dr Giulio Spagnoli, University of Basel, Switzerland), as described before (Van Saen et al., 2011). Histomorphometric analysis was performed by light microscopy at a total magnification of × 400. Per patient, three cross sections (six image fields per cross section) were evaluated. For each cross section, the percentages of MAGE-A4-positive and -negative tubules were determined in normal, degenerated and hyalinized seminiferous tubules, as well as the total number of MAGE-A4-positive cells, i.e. spermatogonia. Descriptive data are reported as mean ± SD.

Sertoli cell maturation and function
Sertoli cells were stained by the maturation marker anti-Mullerian hormone (AMH; MCA2246, AbD Serotec, Düsseldorf, Germany) and by the secretion marker inhibin α (M3609, Dako, Heverlee, Belgium). Immunohistochemical staining was performed as described above with few changes. Antigen retrieval was performed using unmasking solution (H-3301, Vector laboratories, Brussels, Belgium) and non-specific antibody binding was blocked using Cas Block (No.00-8120, Invitrogen, Merelbeke, Belgium). Wash steps were performed with phosphate-buffered saline containing 0.1% Tween (P1379, Sigma-Aldrich, Bornem, Belgium).

Leydig cell hyperplasia
Leydig cells were stained using the marker steroidogenic acute regulatory protein (STAR) (sc-25806, Santa Cruz Biotechnology, Boechout, Belgium). Immunohistochemical staining was performed as described above with few changes. Antigen retrieval was performed using unmasking solution (H-3301, Vector laboratories, Brussels, Belgium) and non-specific antibody binding was blocked using Cas Block (No.00-8120, Invitrogen, Merelbeke, Belgium). Wash steps were performed with phosphate-buffered saline containing 0.1% Tween (P1379, Sigma-Aldrich, Bornem, Belgium).

Results
Up to now, seven KS teenagers had testicular tissue banking. At the moment of testicular tissue sampling, they were aged between 13 and 16 years (Table 1). Their testes were in the scrotal position and their testis volume ranged from 4 to 12 ml. Serum FSH ranged from 1.1 to 33.7 IU/l, serum inhibin B ranged from undetectable to 146.5 ng/l and testosterone concentrations ranged from 0.17 to 6.63 μg/l.
Structural integrity and presence of SSCs

The overall structural integrity was evaluated by haematoxylin-eosin staining and compared with normal adolescent control tissue (aged 10.2, 12.3 and 12.4 years) (Fig. 1A). In all KS biopsies, interstitial fibrosis was present. Histological quantification showed that the degree of fibrosis of the seminiferous tubules ranged between 1 and 4 in the KS subjects, compared with no fibrosis (0) in normal adolescents. In five KS patients, fibrosis was abnormally high (Table I). The tissue from the youngest patient showed the least disturbed morphology with 71% of the tubules showing a normal architecture (Fig. 1B). Nevertheless, degeneration had already started as seen in areas with degenerated (18%) and hyalinized tubules (11%) (Table II). All other KS patients had very few normal tubules (ranging 0–18%) and mainly degenerated and hyalinized tubules (Fig. 1C). In Patients 2 and 4–6, areas with extensive hylainization were observed (Fig. 1D), making it impossible to distinguish remnants of tubules (marked as not definable in Table II). Consequently, percentages of MAGE-A4-positive and -negative tubules could not be calculated (marked with an asterisk in Table II).

In a first evaluation (three depths per patient), MAGE-A4+ cells could be detected in four out of seven KS (Patients 1, 3, 4 and 7). For the KS in which no spermatogonia were observed in the first biopsy, a second biopsy was evaluated. After performing this additional analysis, the presence of spermatogonia could be shown in one more KS patient (Patient 5). However, the spermatogonia were found in tubules with a normal architecture only in Patient 3 (Fig. 1F). In the others, the spermatogonia were within degenerated (Fig. 1G) or hyalinized (Fig. 1H) tubules or in fibrotic tissue. In none of the biopsies, spermatids or spermatooza were observed (Table II). The number of spermatogonia was severely reduced compared with those of normal adolescent boys (ranging from 1 to 438 in the Klinefelter boys, compared with 312–610 in normal young adolescents in all evaluated tubules). Only in Patient 3, who had normal serum FSH and inhibin B levels, a significant number of spermatogonia was observed.

Sertoli cell maturation and function

AMH is a marker for immature Sertoli cells and thus loss of AMH expression is an indication of Sertoli cell maturation (Wikström et al., 2007). In the youngest control (Figs 1C and 2A), almost 90% of the tubules showed strong AMH expression, while in the older controls (Figs 2B, C and 3C) most of the tubules showed intermediate or no AMH expression, indicating that Sertoli cells in the older controls had already started maturing. Down-regulation of AMH expression was observed in all KS patients (Fig. 2C–E), suggesting Sertoli cell maturation. However, a great inter- and intra-variability was seen in Sertoli cell maturation. Different staining intensities within tubules from the same patient could be observed. Surprisingly, no correlation was found with the age of the patients. For example, Patient 6 (almost 16 years old) had more immature tubules compared with younger patients.

To evaluate Sertoli cell function, testis tissue from all KS was stained for inhibin α. In all patients Sertoli cells stained positive for this marker (Fig. 2E and F) and no obvious differences in staining intensity were observed.

Leydig cell hyperplasia

Leydig cells were stained for STAR to detect Leydig cell hyperplasia. In Patients 3 and 6, no Leydig cell hyperplasia was observed, while in Patients 2, 5 and 7 numerous Leydig cells were observed between tubules. These Leydig cells were arranged in patches between the seminiferous tubules. Numerous Leydig cells also stained in Patient 1, but less cells were stained compared with the patches observed in Patients 2, 5 and 7. No staining was performed in Patient 4 because there was no paraffin-embedded tissue left (Table I; Fig. 3).

Discussion

In a population of seven pubertal KS adolescents, undergoing testicular banking in a research setting, extensive tubular fibrosis was observed in six patients. Only in the youngest boy (aged 13), spermatogonia were found in non-degenerating seminiferous tubules.

Experience with testicular tissue sampling in KS teenagers is very limited (for review; Damani et al., 2001; Wikström and Dunkel, 2008). In 14 KS adolescents who had a testicular biopsy between the age of 10 and 14 years, Wikström et al. (2004) found spermatogonia in the testicular biopsies of seven boys, who were all younger than 12 years of age, and still had prepubertal-sized testes and normal serum inhibin B and FSH concentrations at testicular sampling. In our study, none of the KS patients was prepubertal. Our results are

Table I Characteristics of Klinefelter patients at the time of testicular sampling.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (year)</th>
<th>TV (ml)</th>
<th>Serum FSH (IU/l)</th>
<th>Serum inhibin B (ng/l)</th>
<th>Serum testosterone (µg/l)</th>
<th>Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hormonal parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Spzoa</td>
</tr>
<tr>
<td>1</td>
<td>15.3</td>
<td>8/10</td>
<td>12.6</td>
<td>21.7</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>14.2</td>
<td>12/12</td>
<td>30</td>
<td>&lt;15</td>
<td>4.95</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>13.3</td>
<td>4/4</td>
<td>1.1</td>
<td>146.5</td>
<td>0.17</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>15.3</td>
<td>8/8</td>
<td>11.9</td>
<td>&lt;15</td>
<td>0.30</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>4/4</td>
<td>33.7</td>
<td>&lt;10</td>
<td>6.63</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>15.9</td>
<td>6/6</td>
<td>16.4</td>
<td>&lt;15</td>
<td>3.28</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>15.9</td>
<td>6/6</td>
<td>20.7</td>
<td>28.3</td>
<td>2.23</td>
<td>–</td>
</tr>
</tbody>
</table>

LCHP, Leydig cell hyperplasia; ND, not determined; TV, testicular volume.
in accordance with the Finnish study, where none of the adolescents with very low serum inhibin B concentrations had spermatogonia at biopsy. Damani et al. (2001) found spermatogonial cells in the testicular tissue of a 15-year-old KS boy, presenting with an elevated FSH concentration (39 IU/l).

Our data suggest that, for an optimal preservation of SSCs, testicular tissue preservation should preferentially be proposed before hyalinization occurs. To offer a maximum preservation of SSCs, an early detection of the syndrome, i.e. before adolescence, is thus necessary. On the other hand, it is not known whether in KS adolescents, in
Spermatogonial stem cells in pubertal KS boys

Histological evaluation of testicular tissue integrity and presence of germ cells.

Table II

<table>
<thead>
<tr>
<th>Klinefelter patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evaluated tubule sections</td>
<td>274</td>
</tr>
<tr>
<td>Normal tubules</td>
<td>18%</td>
</tr>
<tr>
<td># MAGE-A4 cells</td>
<td>126</td>
</tr>
<tr>
<td>Degenerated tubules</td>
<td>51%</td>
</tr>
<tr>
<td># MAGE-A4 cells</td>
<td>26</td>
</tr>
<tr>
<td>Hyalinized tubules</td>
<td>31%</td>
</tr>
<tr>
<td># MAGE-A4 cells</td>
<td>13</td>
</tr>
</tbody>
</table>

Nd, not definable.

aNo calculation possible.

Within the normal range until the onset of puberty (Aksglaede et al., 1996). Unfortunately, there are no serum parameters to screen for KS at birth. Klinefelter patients would require a neonatal screening programme. Blood lymphocytes remains the gold standard for diagnosing KS. Banking sperm. Karyotyping of metaphase spreads from cultured peripheral blood lymphocytes or testicular tissue in KS adolescents outside study purposes, since future fertility may be guaranteed by this strategy at this moment. There is a need for, besides increasing awareness in medical officers, a national neonatal screening programme. Therefore, new less expensive genetic screening methods like fluorescence in situ hybridization and real-time PCR are suggested (Tu¨ttelmann et al., 2006). Early detection of the syndrome would allow proper follow-up and treatment at all stages and would also be favourable for maximum preservation of the spermatogonial population. At present, we cannot recommend cryopreservation of testicular tissue in KS adolescents outside study purposes, since future fertility cannot be guaranteed by this strategy at this moment. Therefore, new less expensive genetic screening methods like fluorescence in situ hybridization and real-time PCR are suggested (Tu¨ttelmann et al., 2006). The best practice would be part of a national screening programme. The recent discovery that these spermatogenic foci originated from spermatogonia that were of normal diploid status, that is, of normal genetic material and could thus form normal haploid gametes, is of great importance. It was suggested that, in non-mosaic adult KS, most of the tubules in foci with sperm- atogenesis contained spermatozoa displaying a normal karyotype, whereas focal spermatogenesis are detected, where focal spermatogenetical

progresses, according to spermatogonial depleting a normal karyotype.

In a recent report, the possibility of obtaining spermatozoa from 47,XXY KS patients showed no abnormality of the X or Y chromosomes (Staessen et al., 2009). The outcome of these findings may be regarded as an absolute rule to all those that these spermatogenic foci originated from spermatogonia that were of normal diploid status, that is, of normal genetic material and could thus form normal haploid gametes, is of great importance. It was suggested that, in non-mosaic adult KS, most of the tubules in foci with sperm- atogenesis contained spermatozoa displaying a normal karyotype, whereas focal spermatogenesis are detected, where focal spermatogenetical

progresses, according to spermatogonial depleting a normal karyotype.

In a recent report, the possibility of obtaining spermatozoa from 47,XXY KS patients showed no abnormality of the X or Y chromosomes (Staessen et al., 2009).
although 47,XXY spermatogonia were also able to undergo and complete the spermatogenic process leading to mature spermatozoa (Foresta et al., 1999). These observations may suggest that, although diagnosed as non-mosaic on a lymphocyte level, a mosaic pattern in the testis might exist. One might speculate that the surviving spermatogonia in the adolescent patients in our study have a normal karyotype because it is reasonable that euploid spermatogonia have a higher chance of surviving compared with aneuploid cells.

AMH is produced by immature Sertoli cells and expression is decreased around the time when the first spermatocytes appear. Figure 2 Sertoli cell maturation in non-mosaic Klinefelter boys assessed by AMH staining. (A) Tests from a 10.6-year-old adolescent control; in most tubules strong AMH expression is observed. The inserted picture is from an adult control testis in which no AMH staining is detected. (B) Tests from a 12.3-year-old adolescent control; tubules show intermediate or no AMH staining. (C) Tests from Patient 3; most of the tubules show intermediate AMH staining. (D) Tests from Patient 4; most of the tubules show no AMH staining. (E and F) Inhibin-α staining in, respectively, Patients 3 and 7. All tubules show positive staining. (G) Graph showing the distribution of staining intensity for AMH in seven KS and three adolescent controls. Pictures were taken at magnification ×200. Scale bars represent 100 μm.
However, it was observed that AMH expression is generally decreased in normal testes from boys older than 14 years, even if tubules are without germ cells (Raipert-De Meyts et al., 1999). In our study, the youngest boy was 13.3 years old while the other patients were older than 14. AMH staining was still detected in all KS patients, although mostly intermediate staining was observed. These results are in agreement with a study by Wikström et al. (2007) in which loss of AMH expression was also observed to occur at a later age in 14 KS patients.

Although inhibin B levels were low or undetectable in all but one patient, all patients showed staining for inhibin α in the Sertoli cells. Inhibin B is a heterodimer and consists of an α subunit and the βB subunit. Sertoli cells produce both the α subunit and the βB subunit before puberty. However, after puberty, only the α subunit continues to be expressed by Sertoli cells, whereas the maturing germ cells are responsible for the production of the βB subunit (Luisi et al., 2005). The absence of maturing germ cells can thus explain the low levels of inhibin B, while the expression of inhibin α suggests that the Sertoli cells remain functional.

Leydig cells were stained for STAR. Patches of numerous Leydig cells were observed in four KS patients, indicating that Leydig cell hyperplasia has already started at puberty. Our results should be interpreted with caution; first, because only a small number of KS adolescents were studied. Furthermore, the KS adolescents studied may not be representative for the general KS population since none of our patients were referred for cryptorchidism or small genitalia, although these abnormalities may be a regular finding in young KS patients. Since no longitudinal data exist, it is currently far from clear whether this population of KS boys are comparable to the population of KS patients diagnosed at adult age and undergoing TESE for ICSI. Finally, the age of the controls differs from the age of the KS boys. Unfortunately, testicular tissue from age-matched controls was not available. Control tissue was used from routinely stored samples from boys who were assigned for testicular tissue cryopreservation. In boys who can provide a semen sample for cryopreservation, no testicular biopsy is taken.

In conclusion, SSCs within architecturally normal tubules were only observed in the youngest KS adolescent (13 years). In this patient, serum inhibin B levels were normal. In four other KS adolescents SSCs were found in degenerated tubules or fibrotic tissue. Yet more data are certainly needed to make recommendations on early fertility preservation in KS boys by testicular tissue sampling. This, however, will be difficult, because apart from practical problems in conducting larger studies, most KS boys are not physically different from 46,XY boys in the prepubertal and the early pubertal years.

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Authors’ roles

D.V.S. contributed to the conception and design of experiments, acquisition of data, analysis and interpretation of data, drafting of the

Figure 3 Detection of Leydig cell hyperplasia in non-mosaic KS boys by STAR staining. (A) Testis from a 12.3-year-old control boy; few (functional) Leydig cells were detected. (B) Testis from an adult control; Leydig cells are arranged in clusters between the seminiferous tubules. (C) Testis from Patient 3; few Leydig cells are detected, comparable with the situation in the adolescent control. (D) Testis from Patient 5; Leydig cell hyperplasia is observed between the seminiferous tubules. Pictures were taken at magnification ×200. Scale bars represent 100 μm.
manuscript and final approval of the version to be published. I.G. participated in the conception and design of experiments, revision of the manuscript and final approval of the version to be published. J.D.S. participated in the conception and design of experiments, revision of the manuscript and final approval of the version to be published. H.T. participated conception and design of experiments, revision of the manuscript and final approval of the version to be published. E.G. contributed to the conception and design of experiments, analysis and interpretation of data, revision of the manuscript and final approval of the version to be published.

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Conflict of interest

None declared.

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