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

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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 azoospermia 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, karyoptyping 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 azoospermia 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 operated cryptorchidism. Only non-mosaic 47,XXY KS patients were recruited since spermatogenesis in mosaic cases may be less affected (Kaplan *et al.*, 1963; Cozzi *et al.*, 1994).

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 $mm^3$ 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 fixed for histological purposes of this study.

The control tissues were testicular biopsies, which are routinely preserved for histology 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) as previously described (Van Saen *et al.*, 2011). Per cross section the tubules were evaluated based on staining intensity and subsequently scored as strong (+), intermediate (±) or no staining (−) for AMH. For inhibin α, the presence of staining was evaluated.

## 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 I). 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.

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

| Patient | Hormonal parameters | | | | | Histology | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Age (year) | TV (ml) | Serum FSH (IU/l) | Serum inhibin B (ng/l) | Serum testosterone (μg/l) | Spzoa | Spgia | Fibrosis | LCHP |
| 1 | 15.3 | 8/10 | 12.6 | 21.7 | ND | − | + | 3 | + |
| 2 | 14.2 | 12/12 | 30 | <15 | 4.95 | − | − | 3 | ++ |
| 3 | 13.3 | 4/4 | 1.1 | 146.5 | 0.17 | − | + | 1 | − |
| 4 | 15.3 | 8/8 | 11.9 | <15 | 0.30 | − | + | 4 | ND |
| 5 | 16 | 4/4 | 33.7 | <10 | 6.63 | − | + | 3 | ++ |
| 6 | 15.9 | 6/6 | 16.4 | <15 | 3.28 | − | − | 4 | − |
| 7 | 15.9 | 6/6 | 20.7 | 28.3 | 2.23 | − | + | 2 | ++ |

LCHP, Leydig cell hyperplasia; ND, not determined; TV, testicular volume.

## 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 hyalinization 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 spermatozoa 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 maturity. 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

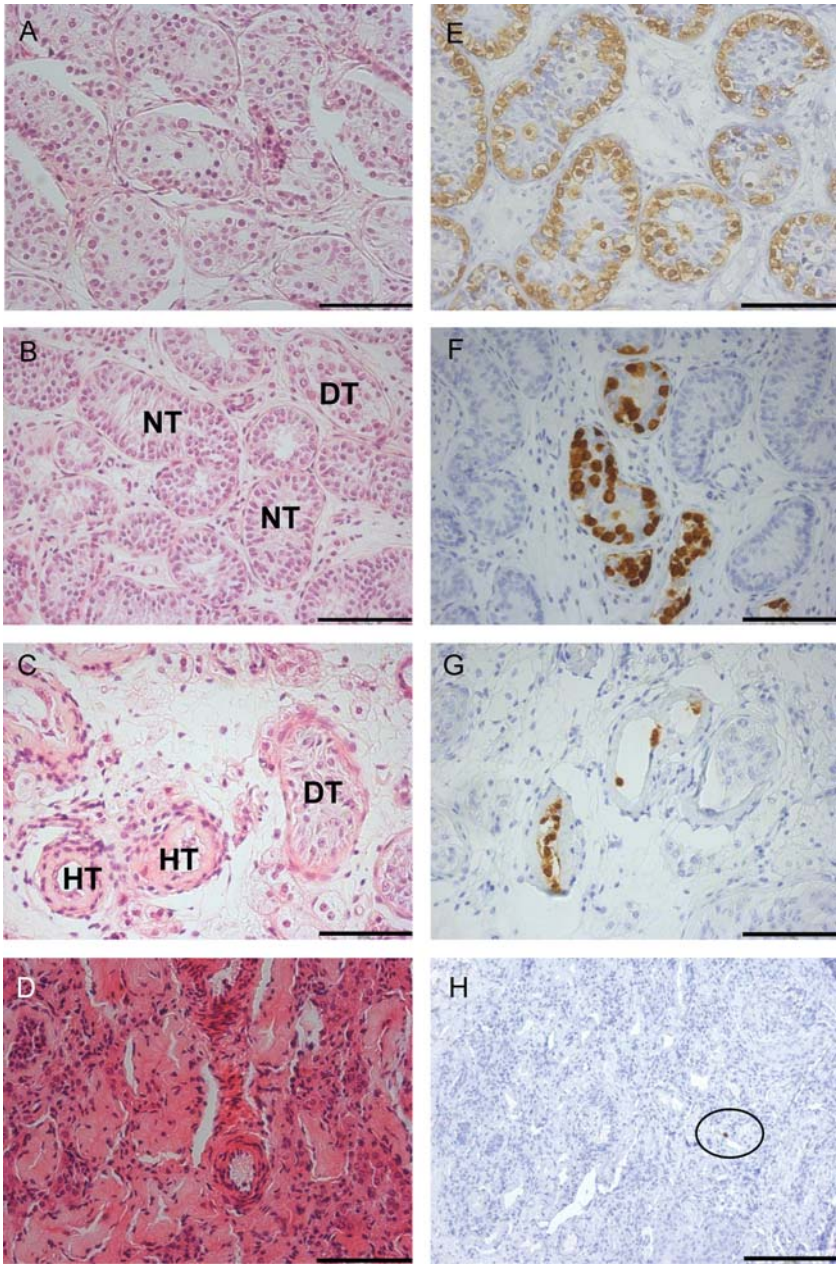


**Figure 1** Overall integrity of testicular tissue in control and non-mosaic Klinefelter boys (haematoxylin-eosin staining) (**A**–**D**). (A) Testis from a 12.3 years old adolescent boy; spermatogenesis has already started in some tubules up to the spermatocyte stage. (B) Testis from Patient 3 (13.3 years old) showing mostly tubules with normal architecture. (C) Testis from Patient 1 (15.3 years old) showing degenerated and hyalinized tubules. (D) Testis from Patient 4 (15.3 years old) showing massive fibrosis and hyalinization; normal or degenerated tubules were not detected. Detection of spermatogonia by MAGE-A4 staining in control and non-mosaic Klinefelter boys (**E**–**H**). (E) Testis from a 12.3 years old adolescent boy; spermatogonia are observed in most of the tubules. (F) testis from Patient 3 (13.3 years old) showing spermatogonia in some normal tubules. (G) Testis from Patient 1 (15.3 years old) showing some spermatogonia in degenerated tubules. (H) Testis from Patient 4 (15.3 years old) showing one spermatogonium in hyalinized tissue. Pictures were taken at magnification ×200. Scale bars represent 100 μm. NT, normal tubules; DT, degenerated tubules; HT, hyalinized tubules.

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

**Table II Histological evaluation of testicular tissue integrity and presence of germ cells.**

| | Klinefelter patients | | | | | | | | Controls | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 1C | 2C | 3C | |
| Evaluated tubule sections | 274 | 430 | 366 | nd | 55 + nd | 86 + nd | 279 | 193 | 123 | 125 | |
| Normal tubules | 18% | 0% | 71% | 0% | nd | nd | 17% | 93% | 84% | 93% | |
| # MAGE-A4$^-$ tubules | 50 | 100% | 247 | 95% | 2 | 8 | 100% | 48 | 100% | 25 | 14% | 9 | 9% | 1 | 1% |
| # MAGE-A4$^+$ tubules | | | 14 | 5% | | | | | | 155 | 86% | 94 | 91% | 115 | 99% |
| # MAGE-A4$^+$ cells | | | 126 | | | | | | | 572 | | 272 | | 410 | |
| Degenerated tubules % | 51% | nd | 18% | 0% | nd | nd | 30% | | | 5% | | 12% | | 5% | |
| # MAGE-A4$^-$ tubules | 130 | 94% | 136 | 100% | 44 | 69% | 53 | 100% | 78 | 100% | 81 | 98% | 2 | 22% | 7 | 47% | 2 | 33% |
| # MAGE-A4$^+$ tubules | 9 | 6% | | | 20 | 31% | | | | | 2 | 2% | 7 | 78% | 8 | 53% | 4 | 67% |
| # MAGE-A4$^+$ cells | 26 | | | | 195 | | | | | | 12 | | 38 | | 30 | | 29 | |
| Hyalinized tubules % | 31% | nd | | | 11% | | 100% | nd | | | 53% | | 2% | | 4% | | 2% | |
| # MAGE-A4$^-$ tubules | 83 | 98% | nd | 100% | 7 | 17% | nd | [a] | nd | [a] | nd | 100% | 148 | 100% | 4 | 100% | 2 | 40% | 1 | 33% |
| # MAGE-A4$^+$ tubules | 2 | 2% | | | 34 | 83% | 1 | [a] | 3 | [a] | | | | | 3 | 60% | 2 | 67% |
| # MAGE-A4$^+$ cells | 13 | | | | 117 | | 1 | | 3 | | | | | | 10 | | 7 | |

Nd, not definable.
[a]No calculation possible.

whom spermatogonia are detected, whether focal spermatogenesis may persist until young adulthood, providing the possibility of banking sperm. The overall recovery of spermatozoa by testicular sperm extraction (TESE) in adult KS patients is reported to be 44%, but an even higher success rate of 55% was achieved when micro-TESE was applied (for review, see Fullerton *et al.*, 2010). However, given the limited number of KS adolescents investigated up to now and the lack of longitudinal histological data, we cannot exclude that in some KS adolescents the hyalinization process might progress very rapidly, making it unlikely to find residual spermatozoa when sampled at an adult age. Klinefelter patients can present a wide spectrum of the classical symptoms. It is possible that the adolescent patients in this study belong to a different spectrum compared with the adult KS patients who are diagnosed as part of an infertility work-up. KS patients in our study were mainly diagnosed because of learning or behaviour problems. It is not known if KS boys with neurological signs have a more severe form of testicular failure.

Gathering the ideal cohort to study the whole clinical picture within Klinefelter patients would require a neonatal screening programme. Unfortunately, there are no serum parameters to screen for KS at early infancy, since levels of testosterone, FSH, LH and inhibin B are within the normal range until the onset of puberty (Aksglaede *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 by testicular tissue banking. Karyotyping of metaphase spreads from cultured peripheral blood lymphocytes remains the gold standard for diagnosing KS. However, new less expensive genetic screening methods like fluorescence *in situ* hybridization and real-time PCR are suggested (Tüttelmann and Gromoll, 2010). However, this molecular approach is probably too expensive and thus does not fulfil all requirements to be part of a national neonatal screening programme. Therefore, the most productive strategy would be to increase awareness, not only in general practitioners and paediatricians, but also in school medical officers. KS should be suspected if clinical signs such as smaller testicular and penile size, taller stature, learning disabilities and verbal cognitive deficits are observed during infancy and childhood (Radicioni *et al.*, 2010).

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. Germ cell transplantation or tissue grafting may not be feasible at an adult age due to the ongoing destruction of the tubular and interstitial architecture that occurs from adolescence onwards. Hunt *et al.* (1998) studied the proliferative potential of XXY-mouse germ cells *in vitro* and found no significant changes. Thus, in the human too, *in vitro* maturation techniques may evolve and become feasible in the future.

In a recent report, it was observed that all spermatocytes analysed from foci of spermatogenesis in non-mosaic patients were euploid 46,XY and can thus form normal haploid gametes. It was suggested that these spermatogenic foci originated from spermatogonia that have randomly lost one of the X chromosomes (Sciurano *et al.*, 2009). Analysis of embryos obtained after fertilizing oocytes with sperm cells from 47,XXY KS patients showed no abnormality of the X or Y chromosomes (Staessen *et al.*, 1996). Another study reported that, in non-mosaic adult KS, most of the tubules in foci with spermatogenesis contained spermatozoa displaying a normal karyotype,

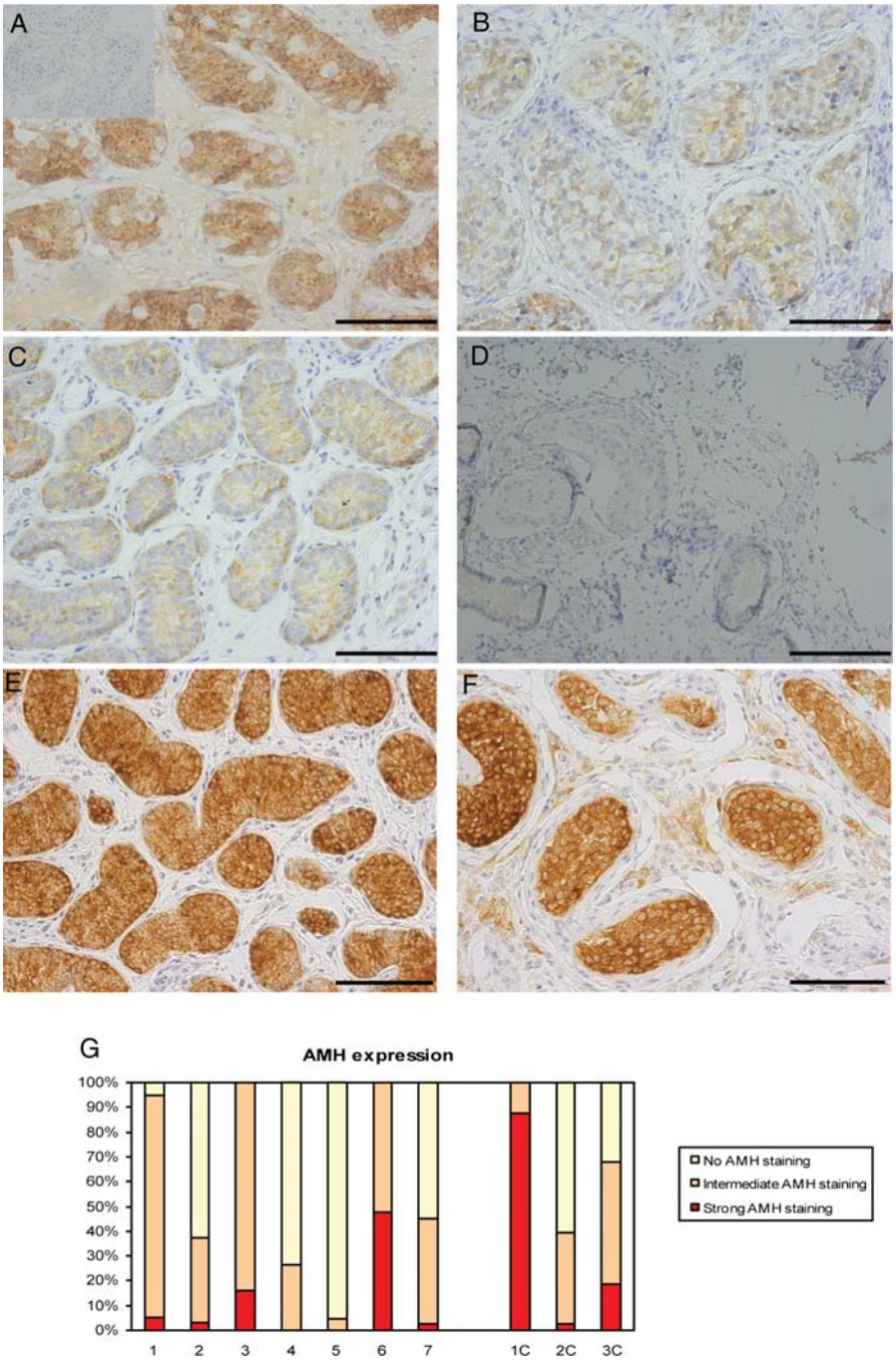


**Figure 2** Sertoli cell maturation in non-mosaic Klinefelter boys assessed by AMH staining. (**A**) Testis from a 10.6-year-old adolescent control; in most tubules strong AMH expression is observed. The inserted picture is from an adult control testis in which no AMH staining is detected. (**B**) Testis from a 12.3-year-old adolescent control; tubules show intermediate or no AMH staining. (**C**) Testis from Patient 3; most of the tubules show intermediate AMH staining. (**D**) Testis from Patient 4; most of the tubules show no AMH staining. (**E** and **F**) Inhibin $\alpha$ 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 $\times 200$. Scale bars represent 100 μm.

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.

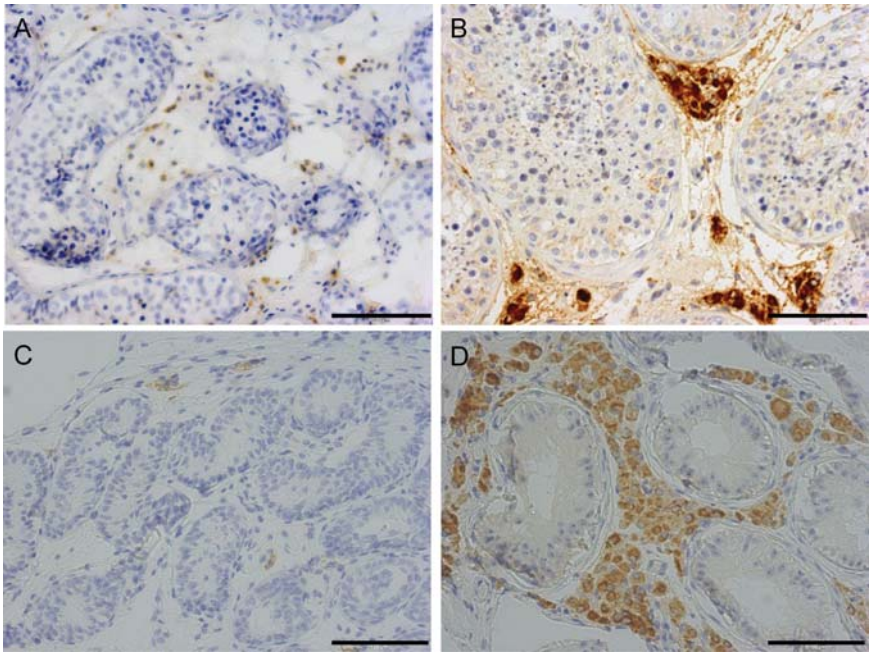


**Figure 3** Detection of Leydig cell hyperplasia in non-mosaic KS boys by STAR staining. (**A**) Testis from a 12.3-year-old control boy; few (functional) Leydig cells were detected. (**B**) Testis from an adult control; Leydig cells are arranged in clusters between the seminiferous tubules. (**C**) 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.

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 $\beta_B$ subunit. Sertoli cells produce both the α subunit and the $\beta_B$ 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 $\beta_B$ 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

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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