Failure of a combined clinical- and hormonal-based strategy to detect early spermatogenesis and retrieve spermatogonial stem cells in 47,XXY boys by single testicular biopsy

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BACKGROUND: Although germ cells in boys with Klinefelter syndrome (KS) are reduced in number as early as infancy, a severe germ cell loss occurs during mid-puberty. Therefore, we wanted to detect spermatogenesis at an early stage and investigate the strategy of preserving spermatozoa and/or testicular spermatogonial stem cells in adolescents with KS when signs of deteriorating spermatogenesis are observed.

METHODS: Tanner staging, testicular size, serum inhibin B and spermaturia were assessed every 4 months before the attempt to procure gametogenic cells in seven non-mosaic 47,XXY adolescents, aged between 10 and 16 years.

RESULTS: Despite an increasing testis volume in the youngest and a Tanner staging of more than three in the oldest patients, no spermaturia was observed. In two patients serum inhibin B increased gradually, while in all others a rather rapid but variable decline was observed at different ages. No spermatozoa were observed after electroejaculation. No spermatocytes or spermatids were found at microscopic examination of single biopsies, while spermatogonia were identified in four subjects, three of whom had measurable serum inhibin B. Massive fibrosis and hyalinization were observed in all biopsies.

CONCLUSION: No spermatogenesis was documented in non-mosaic 47,XXY adolescents either by spermaturia, electroejaculation or testicular biopsy. Neither clinical nor hormonal parameters were of value in determining the timing for optimal spermatogonial stem cell retrieval. More data are needed to elucidate the potential role of testicular tissue cryopreservation in adolescents with KS. Therefore, at present, the cryopreservation of testes tissue for clinical reasons should not be recommended.

Key words: Klinefelter syndrome / fertility / testicular tissue sampling / cryopreservation

Introduction

While previously Klinefelter syndrome (KS) patients with non-mosaic 47,XXY karyotype were invariably considered sterile, the finding of focal spermatogenesis and the recovery of spermatozoa in young adulthood used for ICSI have changed their fertility outcome dramatically (Tournaye et al., 1996). In couples with men with KS undergoing testicular sperm extraction (TESE) and ICSI, pregnancies resulting in live births were reported (Staessen et al., 1996; Vernaeve et al., 2004; Schiff et al., 2006). However, in our experience and in that of most other andrology/fertility centres, sperm for ICSI can only be recovered in about half of the TESE procedures in patients with KS (Friedler et al., 2001; Vernaeve et al., 2004). Unfortunately, no clinical parameters are available to predict successful TESE in order to select young adults with KS who are eligible for this procedure (Vernaeve et al., 2004). Since germ cell depletion starts with the onset of puberty, it has been proposed to offer freezing of semen samples containing low numbers of spermatozoa from boys with KS in early puberty, before initiating testosterone substitution. Early testicular tissue sampling, before starting testosterone supplementation, might also offer a greater chance of retrieving gametogenic cells than in adulthood (Wikström et al., 2004).
Therefore, the present study aimed at exploring the possibility of detecting spermatogenesis in early adolescence by repetitive assessments for spermaturia and investigating whether semen collection by masturbation, vibro- or electrostimulation and/or testicular tissue cryopreservation might be a possible strategy to preserve future biological fertility in most adolescents with KS. In adolescents with KS from the age of 10 years on, a 4 monthly follow-up of gonadal function by measurement of testicular size, serum inhibin B and FSH, and of spermaturia at a testis volume of 6 ml or more was proposed. We hypothesized that spermaturia might be present in the early stages of puberty in adolescents with KS and that whenever spermaturia was absent and ejaculation did not provide spermatozoa, spermatogonial stem cells (Ap and Ad cells) could be found at biopsy and cryopreserved.

Materials and Methods

Inclusion criteria were non-mosaic 47,XXX karyotype as assessed from peripheral lymphocytes, age older than 10 years and written informed consent of the parents and assent of the teenager. Exclusion criteria were previous testosterone therapy and present or previously corrected cryptorchidism. Only non-mosaic 47,XXX patients were recruited since spermatogenesis in mosaic cases is reported to be less affected in some of these individuals, with some even having a complete normal spermatogenesis (Kaplan et al., 1969; Cozzi et al., 1994). Pubertal development was assessed according to Marshall and Tanner (1969) staging and testicular volume was assessed with the Prader orchidometer. Serum inhibin B was measured using a commercial enzyme-linked immunosorbent assay as previously described (Belva et al., 2010). FSH was measured with the Elecsys electrochemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany) with a sensitivity of <0.1 IU/l and a total imprecision (% coefficient of variation) of <6%. Testosterone was measured using the Spectria Testosterone Coated Tube Radioimmunoassay (Orion Diagnostica, Finland).

When testicular volume was 6 ml or more, spermaturia in five consecutive first-voided morning urine samples was investigated. Spermaturia was assessed by microscopic examination of the sediment after 10 min of centrifugation at 3000g. The urine samples were mixed well and 50 ml was placed in a conical-shaped tube. After centrifugation and decantation of the supernatant, two 10 µl aliquots of the resuspended pellet were placed on a slide under 22 × 22 mm coverslips. The preparations were then examined using phase-contrast optics at ×200 magnification. Both coverslips were examined field-by-field to make a complete scan of the entire aliquot and the presence of spermatozoa was recorded.

Vibro-or electrostimulation, followed by testicular biopsy, was only performed in case no further testicular growth was observed during follow-up, when a decreasing serum inhibin B concentration and/or an increasing FSH concentration (at least above 10 mU/l) was documented; and/or when initial serum inhibin B concentration was below the lower reference value or when azoospermia was observed in a semen sample obtained after masturbation.

Penile vibrostimulation was performed using two penile vibrators (Ferticare Personal, Multicept, Albertslund, Denmark) applying a ‘sandwich-technique’ (Brackett, 1999). Electroejaculation was performed using the Seager electroejaculator equipped with a 1-inch rectal probe (Dalzell USA Medical Systems, Marshall, VA, USA) according to standard protocols (Seager Halstead, 1993). All these interventions were performed under general anaesthesia and were followed by a testicular tissue sampling.

Testicular tissue recovery was performed from the lower pole of the largest testis. The technique of a single large volume biopsy instead of the multiple biopsy sampling method was chosen to avoid the risk of post-operative fibrosis and to preserve maximal endocrine testicular function. A small testis fragment (6 mm³) was fixed in hydroxylapatite for at least 1 h. After embedding in paraffin, 5 µm-thick sections were cut. To assess the number of spermatogonia, slides were stained for melanoma-associated antigen 4 (MAGE-A4; provided by Giulio Spagnoli, University of Basel, Switzerland), as described previously (Van Saen et al., 2011). Histomorphometric analysis was performed by light microscopy at a magnification of ×400. The amount of sclerosis was semi-quantified (one ‘+’ per fibrotic section quadrant) and the tubular diameter was determined. Descriptive data are reported as mean ± SD. All historical examinations were performed by a single experienced researcher (E.G.).

Even if no spermatozoa were observed in the biopsy, the testicular tissue was frozen as there may well be germ cells elsewhere in the tissue sample that could be cultured/manipulated in the future (at a time appropriate for conception), since diagnostic biopsies in adult men with KS were found not to predict accurately the results of TESE (Schiess et al., 2006). Tissue freezing was performed according to standard spermatogonial stem cell freezing protocols (Izadiyar et al., 2002; Van Saen et al., 2011). The study was approved by the ethical review board of the University Hospital of Brussels and written consent was obtained in all patients and their parents.

Results

Seven non-mosaic 47,XXX teenagers with KS were included in the study protocol and eventually all seven had testicular tissue recovery. At inclusion, they were aged between 10 and 16 years (Table I). In two of the patients, KS diagnosis was made prenatally (karyotyping for maternal age) and in four chromosome analysis was performed during childhood because of minor neurological and/or cognitive perturbations (mostly in association with behavioural changes). In the oldest boy, karyotyping had been carried out because of gynecomastia and small testes. None of the patients was diagnosed with or treated for cryptorchidism or microepispadias. At inclusion, all testes were in the scrotal position and their single volume ranged between 2 and 12 ml. Penis length was within normal limits for age in all subjects. Clinical and hormonal follow up ranged between 36 months for the youngest patient and 4 months for the oldest patient.

As shown in Fig. 1, a gradual increase in testicular volume was observed in those boys younger than 13 years, whereas no further increase was noted after the age of 14.5 years. In only two patients serum inhibin B increased gradually, while in all others a rather rapid decline, occurring at different ages (between 12 and 15 years), was observed (Fig. 2).

Only the oldest adolescent consented for delivery of a semen sample by masturbation, which showed a low volume (1 cc) and no spermatozoa at examination. In all other patients spermatozoa were not observed, either in the collected urine samples or in the ejaculates obtained by vibrostimulation and electroejaculation. Thus eventually, all boys underwent testicular tissue recovery.

As shown in Table II, at the moment of testicular tissue sampling, testicular volume was between 4 and 12 ml; serum FSH was elevated (> 10 IU/l) in all but one boy and inhibin B concentration was below the lower reference or detection limit (<10 µg/l) in four out of seven boys, of whom only one also had a normal FSH value. There appeared
to be no relationship between serum testosterone concentration and inhibin B or FSH levels.

At follow-up 4 weeks later, all patients had healed well and had no residual testicular swelling or pain.

In all biopsies, degeneration of seminiferous tubules in addition to interstitial fibrosis was present. Histological quantification (Table II) showed that the degree of fibrosis of the seminiferous tubules ranged between (+) and ++++, compared with no sclerosis in normal adolescents (Figs. 3 and 4). In five subjects with KS, fibrosis was abnormally high (Fig. 4). Mean tubule diameter was in the normal range in boys with KS, except in one patient, where no tubules could be detected at all. In only four patients with KS, the presence of spermatogonia could be shown in some of the tubules (Fig. 3). However, the spermatogonia were found in tubules with normal architecture in only one patient. In the other patients, the spermatogonia were seen in hyalinized tubules or in fibrotic tissue.

### Table I Clinical and biological characteristics of patients with KS at inclusion.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (year)</th>
<th>Tanner stage</th>
<th>Testes volume (ml)</th>
<th>Serum FSH (IU/l)</th>
<th>Serum inhibin B (ng/l)</th>
<th>Serum testosterone (µg/l)</th>
</tr>
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<tr>
<td>1</td>
<td>13.6</td>
<td>A1P1G2</td>
<td>4/4</td>
<td>1.5</td>
<td>118.4</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>2</td>
<td>13.6</td>
<td>A2P5G5</td>
<td>10/12</td>
<td>29</td>
<td>15</td>
<td>4.28</td>
</tr>
<tr>
<td>3</td>
<td>10.2</td>
<td>A1P2G1</td>
<td>2/2</td>
<td>0.4</td>
<td>23.4</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>A1P2G2</td>
<td>5/5</td>
<td>0.5</td>
<td>150.6</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>A1P2G2</td>
<td>6/6</td>
<td>1.2</td>
<td>181</td>
<td>0.31</td>
</tr>
<tr>
<td>6</td>
<td>15.6</td>
<td>A1P4G4</td>
<td>6/6</td>
<td>13.6</td>
<td>20.1</td>
<td>3.5</td>
</tr>
<tr>
<td>7</td>
<td>15.6</td>
<td>A2P3G5</td>
<td>8/8</td>
<td>19</td>
<td>27.7</td>
<td>1.58</td>
</tr>
</tbody>
</table>

Figure 1 Testes volume in adolescents with Klinefelter syndrome (KS). Biopt +, presence of spermatogonia.

Figure 2 Serum inhibin B concentration in adolescents with KS. Biopt +, presence of spermatogonia.
Spermatids and spermatozoa were not observed in any of the biopsies. As shown in Table II, in all but one of the patients with KS with spermatogonia present, inhibin B was measurable in the serum, whereas in all but one with undetectable inhibin B, no spermatogonia were observed at biopsy.

**Discussion**

Our data demonstrate that in non-mosaic 47,XXY patients with KS who were assessed during puberty, despite the occurrence of a normal initial increase in testicular volume, massive hyalinization of seminiferous tubuli was present when a drop in serum inhibin B was documented by 4 monthly measurements. While spermatura could not be observed, spermatogonia were found in biopsy specimens of those with a still measurable serum inhibin B, i.e. three out of seven adolescents. We observed a normal testicular growth accompanied by a normal increase of serum inhibin B in the youngest three adolescents with KS, while in those older than 14.5 years and/or presenting a Tanner stage 3, a decline in Sertoli-cell function was observed based on hormone levels.

Today, information in the medical literature on the tubular testicular function during early puberty in KS is rather scarce. Bastida et al. (2007) found normal testicular volumes and serum inhibin B and FSH values in three patients with KS, Tanner stage 2, while serum inhibin B concentrations were normal in four patients assessed during early puberty by Wikström et al. (2004). Furthermore, Christiansen et al. (2003) reported a gradual decline in serum inhibin B from the onset of puberty in five patients with KS who were assessed every 3–6 months over 2 years. Akselrud et al. (2011) found normal to low testicular volumes in a series of 79 patients with KS followed during childhood and adolescence and also documented normal serum inhibin B values during childhood and early adolescence but found a dramatic decline after puberty. Interpretation of testicular function is hampered in these studies as they also included patients with KS with cryptorchidism, and Tanner staging was not always taken into account. In our study, serum inhibin B levels were abnormally low or undetectable from Stage 3 of pubertal development onwards. It is important to underline that all our study patients had a normal testicular descent and normal penile development. From our findings and the reports of other investigators, it can be concluded

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (year)</th>
<th>Tanner stage</th>
<th>Testes volume (ml)</th>
<th>Serum FSH (IU/l)</th>
<th>Serum inhibin B (ng/l)</th>
<th>Serum testosterone (µg/l)</th>
<th>SPTG*</th>
<th>Degree of fibrosis</th>
<th>Tubular diameter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15.3</td>
<td>A2P3G4</td>
<td>8/10</td>
<td>12.6</td>
<td>21.7</td>
<td>N.D.</td>
<td>Yes</td>
<td>+++</td>
<td>85.3 ± 14.5</td>
</tr>
<tr>
<td>2</td>
<td>14.2</td>
<td>A2P5G5</td>
<td>12/12</td>
<td>30</td>
<td>&lt;10</td>
<td>4.95</td>
<td>No</td>
<td>+++</td>
<td>120.5 ± 21.3</td>
</tr>
<tr>
<td>3</td>
<td>13.3</td>
<td>A1P3G3</td>
<td>4/4</td>
<td>1.1</td>
<td>146.5</td>
<td>0.17</td>
<td>Yes</td>
<td>(+)</td>
<td>65.2 ± 8.6</td>
</tr>
<tr>
<td>4</td>
<td>15.3</td>
<td>A1P3G4</td>
<td>8/8</td>
<td>11.9</td>
<td>&lt;10</td>
<td>0.3</td>
<td>Yes</td>
<td>+++</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>A3P5G5</td>
<td>4/4</td>
<td>33.7</td>
<td>&lt;10</td>
<td>6.63</td>
<td>No</td>
<td>+++</td>
<td>118.2 ± 23.2</td>
</tr>
<tr>
<td>6</td>
<td>15.9</td>
<td>A1P4G4</td>
<td>6/6</td>
<td>16.4</td>
<td>&lt;10</td>
<td>3.28</td>
<td>No</td>
<td>+++</td>
<td>91.5 ± 7.8</td>
</tr>
<tr>
<td>7</td>
<td>15.9</td>
<td>A2P3G5</td>
<td>6/6</td>
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<td>28.3</td>
<td>2.23</td>
<td>Yes</td>
<td>++</td>
<td>80.5 ± 12.5</td>
</tr>
</tbody>
</table>

*SPG, spermatogonia documented by staining for melanoma-associated antigen 4.

**Figure 3** Spermatogonia cells positive for melanoma-associated antigen 4, brown coloured, were observed in tubules with normal architecture from boys with KS. Magnification ×200.

**Figure 4** One spermatogonium (brown coloured) was detected in hyalinized tissue from a boy with KS. Magnification ×200.
that excretory gonadal failure is not detected hormonally before the age of 12 years or Stage 3 of pubertal development, since before puberty serum inhibin B is only Sertoli-cell dependent, while during puberty serum inhibin B reflects both Sertoli and germ cell function (Andersson et al., 1998).

We speculated that sperm production and thus spermaturia might be present in young adolescents with KS, since spermarche is a relatively early event in pubertal development. Testicular volume increases at a normal age and continues to increase for several years in most patients with KS who were followed from the onset of puberty (Aksglaede et al., 2011). Also, serum inhibin B values are reported to be normal at the onset of puberty in most patients (Nielsen et al., 1986; Bastida et al., 2007). Spermaturia can be detected in normal boys from the age of 12 year; however, a high false-negative rate has been reported owing to its intermittent nature, making repetitive measurements necessary (Hirsch et al., 1985; Kulin et al., 1989). We did not observe spermaturia despite the fact that all subjects with KS were older than 12 years at the first urine collection and they all performed at least one urine collection, over a 5-day consecutive morning sampling period. Also, Ratcliffe et al. (1982) could not find spermatozoa in the morning urine in a group of 12 pubertal boys with KS, but in their study only one urine collection was done and all their patients were older than 16 years. In early puberty, spermaturia in first-voided morning samples may result from predominantly spontaneous and continuous nocturnal sperm flow to the urinary tract, while in late adolescence intermittent masturbatory ejaculations are thought to be responsible for the finding of more variable spermaturia. Masturbation behaviour was confirmed by the three oldest boys with KS, of whom only one agreed to deliver a semen sample, showing azoospermia at standard examination. No data on masturbation behaviour or oigarche of adolescents with KS are available in the literature. In Belgian boys, interviewed in the early 1980s, mean age at oigarche was 13.1 years, in general when a testicular volume of 10 ml was reached (Carlier and Steeno, 1985).

In agreement with the absence of spermaturia, no meiotic spermatogenic cells were observed in the testicular biopsies of our seven adolescents with KS. These findings corroborate the results of Wikström et al. (2004) in their study population of 14 patients with KS, 8 of whom were pubertal. Even taking into account that none of ours and their adolescents with KS had received testosterone injections, which may block spermatogenesis, active spermatogenesis is thus a rare phenomenon in adolescents with KS.

Our previous experience of histology in 50 adult men with KS undergoing TESE showed a nearly complete tubular sclerosis and atrophy in 38 (76%) patients (Vernaeev et al., 2004). In this study of seven adolescents with KS, tubular sclerosis was present in all patients, even in the youngest, but in four of them spermatogonia could be observed.

Experience with testicular tissue sampling in teenagers with KS is very limited (Damani et al., 2001; Wikström et al., 2004). In 14 adolescents who had a testicular biopsy between the age of 10.1 and 14 years, Wikström et al. (2004) found spermatogonia in testicular biopsies of seven boys who were all younger than 12 years of age and still had prepubertal-sized testes volumes and normal serum inhibin B and FSH concentrations. The absence of cryptorchidism might be critical, since Müller et al. (1995) found few or no germ cells at testicular biopsy in the majority of 11 prepubertal boys with KS who had been or were cryptorchid. In our study and the study by Wikström et al. (2004), testes were always in a scrotal position and none of our patients with KS were prepubertal. In accordance with Wikström et al. (2004), measurable serum inhibin B did not fully correlate with the presence of germ cells in the testis. Whereas none of the four older adolescents with unmeasurable or very low serum inhibin B concentrations in the Finnish study (Wikström et al., 2004) had spermatogonia at biopsy, one of the four older patients in our study had spermatogonia in his biopsy. Damani et al. (2001) also reported spermatogonial stem cells in the testicular tissue of a 15-year-old boy with KS with Tanner stages 4–5 (testicular volume of 10 ml) and presenting with an elevated FSH concentration (39 IU/l). Aksglaede et al. (2011) described the loss of germ cells from the age of 10 years in an observational retrospective study with 29 testicular cell biopsies from variable ages. It is of importance to note that in our study and in the Finnish study, only a single biopsy was examined and that in the older boy reported by Damani et al. (2001) several samples were examined.

Our combined histological and hormonal approach suggests that the tubular hyalinization process starts at the onset of puberty and is followed, rather than preceded, by a decline in serum inhibin B. While in other conditions, such as cancer patients, changes in serum inhibin B and FSH can be used as indicators of declining spermatogenesis, it is becoming clear that in the case of an extremely poor or rapidly declining spermatogenic potential, as in KS, changes in serum markers may not at all be indicative of a decrease in spermatogenesis, since the process in the testes of these patients is so globally disrupted. Testicular tissue preservation should thus preferentially be proposed before any decline in serum inhibin B is observed whenever optimal preservation of spermatogonial stem cells is anticipated. On the other hand, it is unknown whether in adolescents with KS, in whom spermatogonia are detected, focal spermatogenesis might persist until adulthood. It is tempting to speculate that the potential of maintaining spermatogonial stem cells and focal spermatogenesis is programmed in utero. This would explain the finding of spermatogonial stem cells in half of patients with KS at different ages. In fetuses with KS at 19–22 weeks of gestation, spermatogonia were found to be reduced to approximately half of the number observed in normal XY fetuses (Coerd et al., 1985). It is striking that while spermatogonia are found in half of the adolescent patients in whom a biopsy was performed, successful recovery of spermatozoa by TESE in adults is also around 50% (Tournaye et al., 1996; Vernaeev et al., 2004). However, given the limited number of adolescents with KS investigated to date and the lack of reliable longitudinal historical data, we cannot exclude the possibility that in some of these adolescents the hyalinization process might progress very rapidly, making it unlikely to find residual spermatozoa when sampling is performed in adulthood. Certainly, cryopreservation of semen samples that contain even minuscule numbers of spermatozoa could be offered to all adolescents with KS who are interested in their future fertility. However, at present we cannot recommend cryopreservation of testicular tissue in adolescents with KS other than for study purposes, because at the moment spermatogonial stem cell banking in these patients should be considered as experimental. Furthermore, in these patients, spermatogonial stem cell transplantation or tissue grafting might not be feasible in adulthood because of the ongoing deterioration of the tubular and interstitial architecture. Hunt et al.
(1998) studied the proliferative potential of XXY-mice germ cells in vitro and found no significant changes. It has been reported that adult patients with KS having spermatozoa recovered after TESE harbour euploid spermatogonial stem cells (Sciurano et al., 2009). Thus, as in vitro maturation techniques may evolve and become feasible in the future, the banking of spermatogonial stem cells at a prepubertal age in boys with KS may become a strategy of clinical importance, by the generation of mature gametes in vitro.

Our results should be interpreted with caution. First of all, only a small number of adolescents with KS were studied. Also, our adolescents may not be representative for the general population with KS as none of them had been referred for cryptorchidism or small genitalia, although these abnormalities may be a regular finding in young patients. Furthermore, in our patients, low-grade mosaicism in peripheral lymphocytes was not excluded by interphase fluorescence in situ hybridization. Spermaturia was assessed in only five consecutive urine samples, whereas a 10 day repetitive morning collections might have been more reliable, but less feasible on the long-term.

In conclusion, we did not observe spermaturia in adolescents with KS and found serum inhibin B levels to decrease at variable age but never before 12 years. Serum inhibin B and testicular volume did not fully correlate with the presence of spermatogonia, which were observed in the testis biopsies in half of the studied adolescents with KS. More data are certainly needed to make reliable recommendations on fertility preservation in peripubertal patients with KS by testicular tissue sampling. Until more data are available, cryopreservation of testes tissue from adolescents with KS should not be recommended other than for study purposes.

Authors’ roles
Participation in study design and execution was made by I.G., J.D.S., D.V.S., E.G., E.A. and H.T. Testicular tissue biopsy was performed by H.T. Histological examinations were carried out by E.G. Analysis of the data and manuscript drafting and discussion was performed by all authors.

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Conflict of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

References
Andersson AM, Muller J, Skakkebaek NE. Different roles of prepubertal and postpubertal germ cells and Sertoli cells in the regulation of serum inhibin B levels. J Clin Endocrinol Metab 1998;83:4451–4458.


