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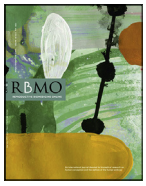

ARTICLE

Testicular biopsy for fertility preservation in early-diagnosed Klinefelter patients: patient characteristics and long-term follow-up

BIOGRAPHY

Aude Braye is a PhD student at the Biology of the Testis research group, Vrije Universiteit Brussel, Brussels, Belgium. Her research interests include fertility preservation in prepubertal and pubertal boys.

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KEY MESSAGE

Early-diagnosed Klinefelter patients presented with behavioural, cognitive and/or psychological problems or typical clinical features of Klinefelter syndrome. Only psychological problems seemed to influence the decision towards fertility preservation. Long-term follow-up did not show evidence of altered gonadal development in Klinefelter patients who underwent a testicular biopsy for fertility preservation. Studies characterizing young Klinefelter syndrome patients who are offered experimental testicular tissue banking for fertility preservation and investigating the long-term effects related to the testicular biopsy procedure are lacking.

ABSTRACT

Research question: Which early-diagnosed Klinefelter syndrome patients have been offered cryopreservation of testicular tissue as part of fertility preservation before spermatogonial stem cell (SSC) loss? Do these Klinefelter syndrome patients present with behavioural, cognitive and/or psychological problems? Does a testicular biopsy procedure have long-term effects on the gonadal development of Klinefelter syndrome patients?

Design: Early-diagnosed Klinefelter syndrome patients followed between 2009 and 2020 and offered testicular tissue banking in an experimental context at the Universitair Ziekenhuis Brussel were included. The prevalence of behavioural, cognitive and/or psychological problems was determined. Changes in testicular volume and in gonadal function (LH, FSH, testosterone and inhibin B [INHB]) were studied.

Results: Of the 48 Klinefelter syndrome patients included, 22 had testicular tissue removed (biopsy group) and 26 had no surgical intervention (control group). The need for specialized education was significantly higher in prenatally ($P = 0.0159$) and prepubertally ($P = 0.0002$) diagnosed Klinefelter syndrome patients. Psychological problems were significantly more prevalent in Klinefelter syndrome patients who did not opt for fertility preservation ($P = 0.0447$). In the first 4.2 (1.9–9.1) years after testicular biopsy, no difference in testicular volume was observed between the biopsied and the contralateral non-biopsied testes ($P > 0.9999$). After pubertal onset, no differences in LH, FSH, testosterone and INHB were found between the biopsy and the control groups ($P = 0.1324$ for LH, $P > 0.9999$ for FSH, $P = 0.5433$ for testosterone, $P > 0.9999$ for INHB).

Conclusion: Early-diagnosed Klinefelter syndrome patients presented with behavioural, cognitive and/or psychological problems. Only psychological problems seemed to influence the decision towards fertility preservation. Follow-up data confirm that harvesting testicular tissue does not have a long-term impact on the gonadal development of Klinefelter syndrome patients.

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KEYWORDS

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INTRODUCTION

Klinefelter syndrome (*Klinefelter et al., 1942*) remains the most prevalent sex-chromosomal aberration found in men, occurring with an estimated frequency of 1–2 in 1000 newborn males. However, Klinefelter syndrome is highly under-diagnosed as most patients develop only few or discrete symptoms. Roughly 25% of all cases are diagnosed, with less than 10% before puberty (*Bojesen et al., 2003*). Most Klinefelter syndrome diagnoses are made during adulthood due to difficulties in conceiving naturally. Earlier diagnosis during childhood is usually made because of cognitive and/or behavioural problems, as these are known to be increased in Klinefelter syndrome patients compared with the general population (*Boada et al., 2009*; *Cederlöf et al., 2014*; *Skakkebaek et al., 2020*). Emotional and/or psychosocial problems like depression, social anxiety and withdrawal, timidity, inappropriate social behaviour and impulsivity are also common for Klinefelter syndrome patients (*Bender et al., 1995*; *Skakkebaek et al., 2020*; *van Rijn et al., 2008*). Adolescent Klinefelter syndrome diagnosis is made through clinical signs such as small testes and/or symptomatic hypogonadism. Indeed, the testicular tissue of Klinefelter syndrome patients shows germ cell depletion before puberty and hyalinization of the seminiferous tubules, Leydig cell hyperplasia and extensive fibrosis during puberty. This progressive testicular degeneration results in regression of testicular volume and increasing serum FSH concentrations combined with decreasing serum inhibin B (INHB) and anti-Müllerian hormone (AMH) concentrations around mid-puberty (*Bastida et al., 2007*; *Van Saen et al., 2018*). The exact mechanism(s) explaining the histological and hormonal changes remain unclear.

Given the testicular damage and subsequent infertility, fertility preservation and restoration options for Klinefelter syndrome patients have been widely studied. The finding of focal spermatogenesis in the testes of adult 47,XXY men by *Tournaye et al. (1996)* enabled the collection of testicular spermatozoa by testicular sperm extraction (TESE). Testicular spermatozoa can be found in up to 50% of TESE procedures and used for intracytoplasmic sperm injection (ICSI) (*Corona et al., 2017*; *Sciurano et al., 2009*; *Vloeberghs et al., 2018*). As an alternative and because germ cell depletion starts before puberty, the cryopreservation of spermatogonial stem cells (SSC) has been initiated in research settings. This option requires a testicular biopsy procedure at a young age. Reports on TESE and/or testicular biopsy for banking in adolescent Klinefelter syndrome patients has reported no surgical complications during or after the testicular biopsy procedure (*Damani et al., 2001*; *Rives et al., 2013*; *Van Saen et al., 2012*). However, both anatomical and functional testicular damage following testicular biopsy could potentially cause long-term effects. Follow-up studies on reproductive health following gonadotoxic treatment in boys have shown subnormal testicular size and impaired reproductive hormone concentrations. These complications were more as a result of the oncological treatment than the testicular biopsy procedure (*Borgström et al., 2020*; *Kanbar et al., 2021*). Harvesting testicular tissue in young cancer patients also had no impact on testicular growth and development (*Uijldert et al., 2017*), but the evidence is very limited.

There is currently a lack of longitudinal studies investigating the safety of testicular biopsy for fertility preservation in young Klinefelter syndrome patients. The present study aimed to evaluate the gonadal development of Klinefelter syndrome patients after testicular biopsy to identify potential effects related to the testicular biopsy procedure. Therefore, changes in testicular volume and in the reproductive hormones LH, FSH, testosterone and INHB, retrospectively collected between 2009 and 2020, were analysed and compared with the observations in Klinefelter syndrome patients who did not undergo testicular biopsy. This study also aimed to characterize the young Klinefelter syndrome patients who were offered testicular tissue banking by studying the prevalence of behavioural, cognitive and/or psychological problems. These characteristics were analysed in relation to the timing of Klinefelter syndrome diagnosis and in relation to the decision concerning fertility preservation.

MATERIALS AND METHODS

Ethical approval

The Institutional Review Board of the UZ Brussel approved this retrospective cohort study (BUN 143201731260, approval date 8 March 2017). Patient data were retrospectively collected from the patient's medical files and anonymized for further analysis.

Patients

Initially only pubertal Klinefelter syndrome patients showing biological (increasing serum FSH concentrations >10 IU/l, decreasing serum INHB concentrations) and/or clinical (arrested testicular growth) signs of testicular failure, as well as azoospermia, were offered testicular tissue banking at the UZ Brussel. After severe SSC depletion was demonstrated in the testicular tissue of pubertal Klinefelter syndrome patients, testicular tissue banking was no longer offered to these patients (*Van Saen et al., 2012*, *2015*). From 2015 onwards, testicular tissue banking was only offered before puberty (*Van Saen et al., 2018*). Klinefelter syndrome patients carrying 47,XXY/46,XY mosaicism, needing testosterone replacement therapy prior to testicular tissue banking and/or diagnosed with cryptorchidism were not included in the present study as they are not eligible for banking at the UZ Brussel (*Cobellis et al., 2014*; *Cozzi et al., 1994*; *Goel et al., 2015*; *Kaplan et al., 1963*). The testicular biopsy procedure was performed after informed consent was obtained from the parents and the patient himself (if ≥12 years old). The testicular tissue was surgically removed from the lower pole of the largest testis under general anaesthesia. The amount of testicular tissue biopsied depended on the testicular volume at time of biopsy and the wishes of the patient and/or his parents (*Van Saen et al., 2012*).

Klinefelter syndrome patients were assigned to the biopsy group if they and/or their parents accepted testicular tissue banking or to the control group if not consenting to fertility preservation. Both groups were invited for yearly follow-up.

Follow-up

The Klinefelter Clinic of the UZ Brussel provides multidisciplinary, specialized annual follow-ups for young Klinefelter syndrome patients. The pubertal maturation stage was assessed according to the Marshall and Tanner method (*Marshall and Tanner, 1970*) and grouped into prepuberty (G1), early puberty (G2), mid-puberty (G3–G4) or late puberty (G5). The volume of both testes was measured using a Prader orchidometer. All

assessments were performed by the same two trained endocrinologists to minimize variability between follow-up assessments. Morning serum LH, FSH, testosterone and INHB concentrations were analysed annually. The serum concentrations were recorded as normal or abnormal (low/high) based on the patient's pubertal stage at time of measurement. All hormonal analyses were done using commercially available immunoassays: Elecsys LH, FSH and testosterone assay on the Cobas e immune analyser (Roche Diagnostics, Machelen, Belgium) and Inhibin B Gen II enzyme-linked immunosorbent assay kit (Diagnostics Systems Laboratories, Webster, TX, USA). To investigate the long-term impact of the testicular biopsy procedure on the gonadal development of early-diagnosed Klinefelter syndrome patients, the mean volumes of the biopsied and the contralateral non-biopsied testes were compared in relation to the pubertal stage after testicular biopsy, and the serum concentrations measured after pubertal onset and testicular biopsy (biopsy group) were compared with those from the control group.

In addition, the prevalence of autism, attention deficit hyperactivity disorder (ADHD), psychological problems and the need for specialized education were assessed at diagnosis and during follow-up. The relation between these characteristics and the timing of Klinefelter syndrome diagnosis, as well as the acceptance rate for fertility preservation, was investigated by comparing prenatally, prepubertally and pubertally diagnosed Klinefelter syndrome patients, as well as comparing characteristics of Klinefelter syndrome patients who accepted testicular biopsy (biopsy group) with those who refused it (control group).

Statistical analysis

Patient age at diagnosis and at testicular biopsy, the follow-up period for testicular volume and reproductive hormones are presented as median (range). Testicular volumes (ml) are expressed as mean ± SD. The Wilcoxon matched-pairs signed-rank test was used to compare the biopsied with the non-biopsied testicular volumes. For reproductive hormones and patient characteristics, Fisher's exact test was used to compare the biopsy with the control group. Statistical analysis was performed with GraphPad Prism, Version 9.1.2 (La Jolla, CA, USA). *P*-values of less than 0.05 were considered statistically significant.

RESULTS

Patients

In total, 48 early-diagnosed Klinefelter syndrome patients were followed during the study period (2009–2020): 44 47,XXY patients and four patients carrying higher-grade aneuploidies (HGA) (FIGURE 1). Thirteen 47,XXY patients and one 48,XXXY patient (29%)

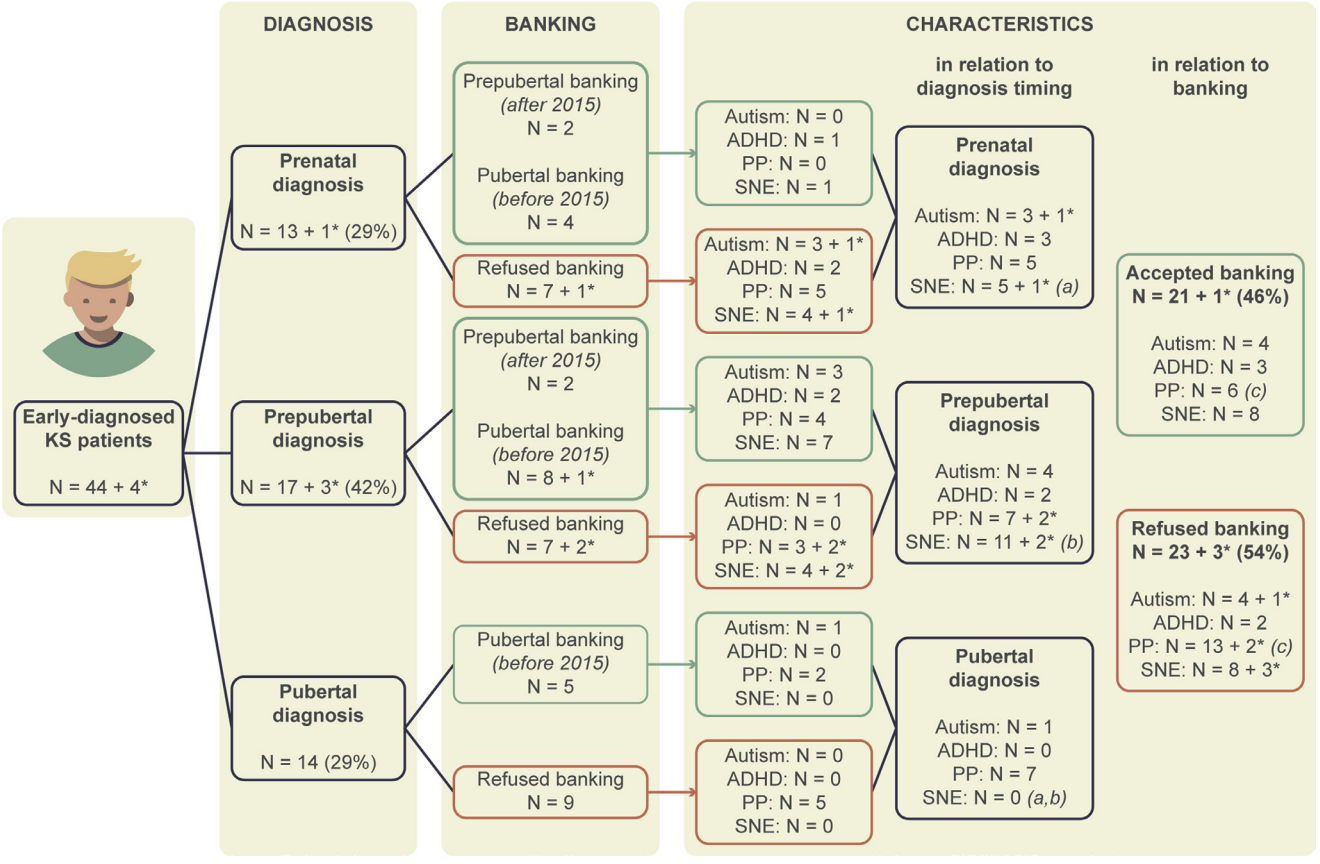

FIGURE 1 Testicular tissue banking and characteristics of early-diagnosed Klinefelter patients. A testicular biopsy was removed for fertility preservation in 17 47,XXY patients and one 48,XXYY patient after puberty (before 2015) and in four 47,XXY patients before puberty (after 2015). The need for specialized education was significantly more prevalent in prenatally (a, $P = 0.0159$) and prepubertally (b, $P = 0.0002$) diagnosed Klinefelter patients. Psychological problems were significantly more prevalent in Klinefelter patients without surgical intervention for fertility preservation (c, $P = 0.0447$). *Patients carrying higher-grade aneuploidies. ADHD = attention deficit hyperactivity disorder; KS = Klinefelter syndrome; PP = psychological problems; SNE = special needs education.

were diagnosed prenatally through karyotyping for risk pregnancies or advanced maternal age. Seventeen 47,XXY patients and three patients carrying a 48,XXYY (n = 2) or 49,XXXXY (n = 1) karyotype were diagnosed prepubertally (42%), mostly due to learning difficulties and/or unspecified behavioural problems. Fourteen 47,XXY patients were diagnosed pubertally (29%) through hypogonadism and/or small firm testes.

Before 2015, 17 47,XXY patients (n = 4 prenatally, n = 8 prepubertally, n = 5 pubertally diagnosed) and one prepubertally diagnosed 48,XXYY patient had testicular tissue removed and frozen for fertility preservation after puberty at a median age of 14.3 (12.4–18.4) years. From 2015 onwards, the testicular tissue of four 47,XXY patients (n = 2 prenatally, n = 2 prepubertally diagnosed) was harvested before puberty at a median age of 5.8 (4.8–7.6) years (FIGURE 1). Almost all 47,XXY patients and the 48,XXYY patient had one or multiple smaller biopsies removed (91%) and about one-third (35%) of these smaller biopsies were bilateral. After puberty, only smaller biopsies were removed. One of the prepubertally biopsied 47,XXY patients had one entire testis removed (4.5%) and another had one-half of one testis removed (4.5%) because of a very small testicular volume. No intra- or post-surgical complications occurred and none of the Klinefelter syndrome patients needed a second intervention. The parents of 23 47,XXY patients (n = 7 prenatally, n = 7 prepubertally, n = 9 pubertally diagnosed) and three HGA patients (n = 1 prenatally, n = 2 prepubertally diagnosed) declined testicular tissue banking (FIGURE 1).

Patient characteristics

Psychological problems (44%), need for specialized education (40%), autism (19%) and ADHD (10%) were observed in early-diagnosed Klinefelter syndrome patients (FIGURE 1). Patients needing specialized education were significantly more prevalent in the prenatally (32%, P = 0.0159) and prepubertally (68%, P = 0.0002) diagnosed group. None of the pubertally diagnosed Klinefelter syndrome patients needed specialized education or showed signs of ADHD during the study period. Three out of four HGA patients needed specialized education and their parents refused testicular tissue banking. The fourth HGA (48,XXYY) patient who banked testicular tissue did not show signs of autism, ADHD or psychological problems or needed specialized education. Psychological problems were significantly more prevalent in Klinefelter syndrome patients who did not undergo testicular biopsy (P = 0.0447). Twenty-one patients presented psychological problems, of which six (29%) were in the biopsy group and 15 (71%) in the control group.

Testicular volume

For 34 Klinefelter syndrome patients (n = 13 in the biopsy group and n = 21 in the control group), testicular volume was measured once a year over a median period of 4.2 (1.9–9.1) years after testicular biopsy (biopsy group) and 5.0 (1.1–9.7) years (control group). After testicular biopsy, the mean volumes of the biopsied and the contralateral non-biopsied testes were both 3 ± 1 ml at G1, both 6 ± 0 ml at G2, 8 ± 1 ml and 8 ± 2 ml, respectively at G3–G4 and both 7 ± 2 ml at G5 (FIGURE 2). No significant difference was observed between the biopsied and the non-biopsied testicular volumes for each pubertal stage (P > 0.9999). The testicular volumes in the biopsy group were comparable to the ones in the control group: 2 ± 1 ml at G1, 6 ± 2 ml at G2, 8 ± 2 ml at G3–G4 and 8 ± 4 ml at G5. Testicular volumes of HGA patients were in line with those of 47,XXY patients.

Reproductive hormones

For 28 Klinefelter syndrome patients (n = 11 in the biopsy group and n = 17 in the control group), serum concentrations were analysed once a year over a median period of 6.0 (3.5–9.0) years after testicular biopsy (biopsy group) and 5.7 (1.4–11.0) years (control group). After testicular biopsy , all recorded serum concentrations for LH and INHB and almost all for FSH (91%) were abnormal (high for LH and FSH and low for INHB). Similarly, in the control group, mainly abnormal serum concentrations for LH (76%), FSH (82%) and INHB (94%) were observed. Most of the recorded testosterone serum concentrations were normal after testicular biopsy (82%) and in the control group (94%) (FIGURE 2). No significant differences in serum concentrations were found between the biopsy and the control group after puberty (P = 0.1324 for LH, P > 0.9999 for FSH, P = 0.5433 for testosterone, P > 0.9999 for INHB). Hypergonadotrophic hypogonadism (increasing LH and FSH serum concentrations and decreasing INHB serum concentrations) was observed starting from G2. Reproductive hormone concentrations of HGA patients were in line with those of 47,XXY patients.

DISCUSSION

In this clinical and hormonal observational cohort of 22 early-diagnosed Klinefelter syndrome patients undergoing experimental testicular biopsy before (n = 4) or after (n = 18) puberty, no significant differences in testicular growth and hormonal gonadal function were found compared with a control group consisting of 26 early-diagnosed Klinefelter syndrome patients.

Klinefelter syndrome patients are usually diagnosed at adulthood because of fertility problems and/or sexual dysfunction. Less frequently, earlier Klinefelter syndrome diagnosis occurs during childhood due to learning difficulties, developmental delay and/or behavioural problems or at adolescence due to symptomatic hypogonadism, small firm testes and/or gynaecomastia. In this patient cohort, the Klinefelter syndrome diagnosis occurred during prenatal screening (29%), prepubertally (42%) due to unspecified behavioural problems and/or learning difficulties mostly requiring specialized education, or pubertally (29%) through clinical signs like small testes and/or hypogonadism. All Klinefelter syndrome patients needing specialized education were diagnosed prenatally (32%) or prepubertally (68%). None of the pubertally diagnosed Klinefelter syndrome patients followed special needs education or were diagnosed with ADHD during the study period and only a small percentage showed autism characteristics (7%). Because of important cognitive problems in three out of four HGA patients, fertility preservation was declined by the parents. Previous studies have described an increased risk of behavioural, cognitive and/or psychological problems in young Klinefelter syndrome patients (*Bruining et al., 2009*; *Cederlöf et al., 2014*), but with a high variability in prevalence among the different studies. In the present study, psychological problems were observed in 21 prenatally (n = 5), prepubertally (n = 9) or pubertally (n = 7) diagnosed Klinefelter syndrome patients (44%) and were significantly more prevalent in patients who did not opt for fertility preservation. The presence

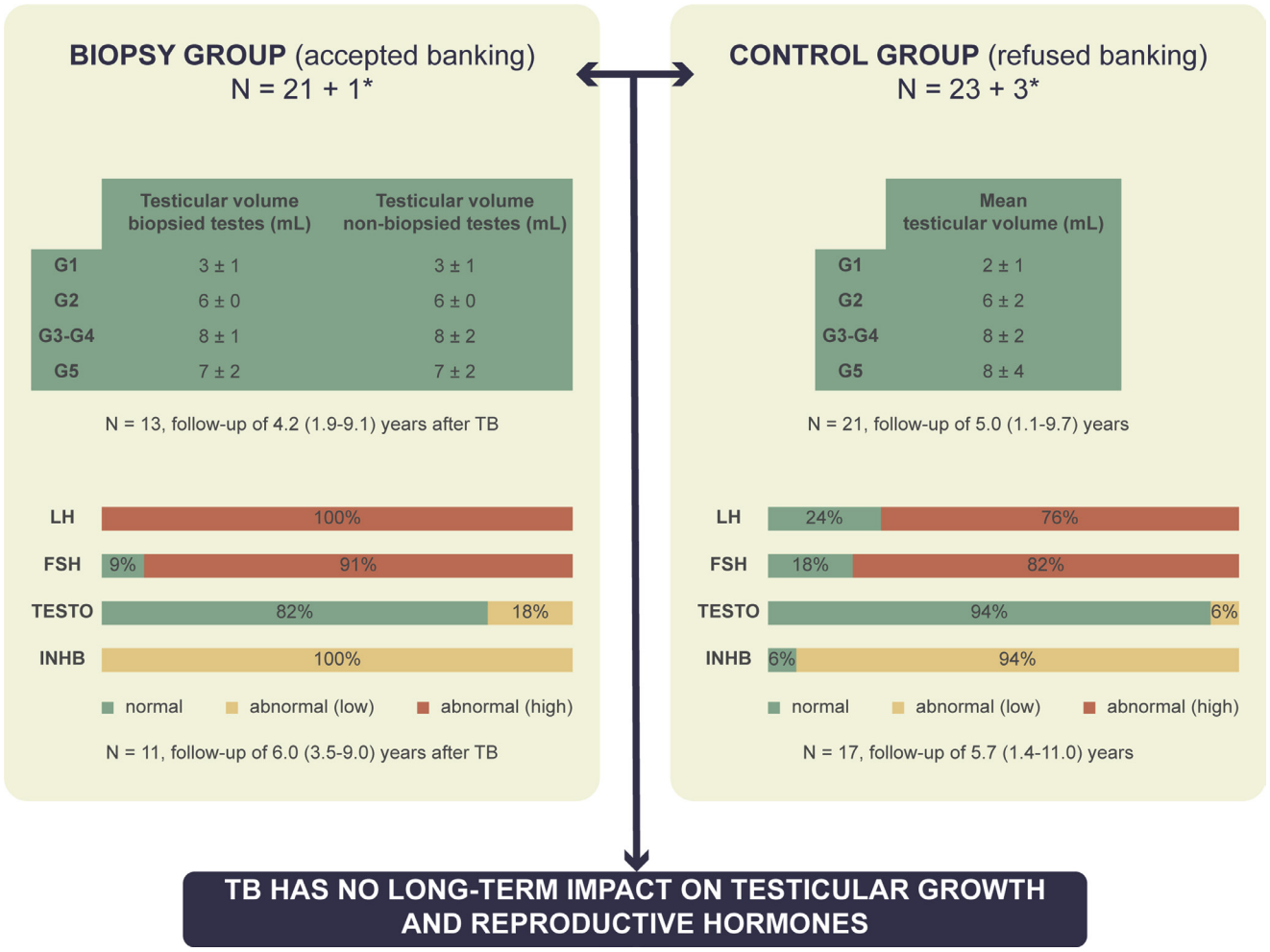

FIGURE 2 Follow-up of testicular volume and reproductive hormones in early-diagnosed Klinefelter patients. Testicular volumes of the biopsied and the contralateral non-biopsied testes are not significantly different ($P > 0.9999$) and comparable to the testicular volumes in the control group. After pubertal onset, the reproductive hormones LH, FSH, testosterone and INHB are not significantly different between the biopsy and the control group ($P = 0.1324$ for LH, $P > 0.9999$ for FSH, $P = 0.5433$ for testosterone, $P > 0.9999$ for INHB). *Patients carrying higher-grade aneuploidies. INHB = inhibin B; TB = testicular biopsy; TESTO = testosterone.

of autism, ADHD and/or the need for specialized education appeared not to influence the decision by the parents and/or patient about fertility preservation.

At the Klinefelter Clinic of the UZ Brussel, young Klinefelter syndrome patients have annual clinical and biological examinations, allowing investigation of the long-term effects of a testicular biopsy on gonadal function. The testicular volumes of the biopsied and the contralateral non-biopsied testes were not significantly different up to 4.2 (1.9–9.1) years after testicular biopsy. This is consistent with the findings of a cohort study on testicular growth up to 1 year after fertility preservation in prepubertal cancer patients (*Uijldert et al., 2017*). The mean testicular volumes measured after testicular biopsy were similar to the those in the control group, although testicular growth arrest and/or regression were observed in both groups. Arrested testicular growth or regression of testicular volume is characteristic for Klinefelter syndrome patients from mid-puberty onwards (*Bastida et al., 2007*; *Ratcliffe, 1982*;*Topper et al., 1982*). The present study reports normal testicular volumes at G1 and G2 and testicular growth arrest and/or regression at G3–G4 and G5 compared with the testicular volumes in non-Klinefelter syndrome boys: <4 ml at G1, 4–8 ml at G2, 9–20 ml at G3–G4 and >20 ml at G5.

During the 5.8 (1.4–11.0) years of hormonal follow-up, no significant differences in the serum concentrations of LH, FSH, testosterone and INHB were found when comparing the biopsy with the control group from puberty onwards. Longer follow-up is needed to investigate whether these concentrations recover to baseline levels, as has been reported for testosterone in Klinefelter syndrome men undergoing TESE (*Eliveld et al., 2018*). The serum concentrations of the reproductive hormones were within normal ranges before puberty in both groups (data not shown) but started to deteriorate around puberty. LH and FSH increased gradually, testosterone remained at a low to normal range and INHB decreased rapidly during puberty. These hormonal changes occurring during mid- to late puberty are characteristic for Klinefelter syndrome patients (*Bastida et al., 2007*).

The future use of the banked testicular tissue is still unclear as fertility restoration methods are not yet available for humans. Autotransplantation into the

patient's testes is not an option for Klinefelter syndrome patients because of the testicular degeneration that starts around mid-puberty. However, *Fayomi et al. (2019)* reported successful autotransplantation under the back or scrotal skin. Another alternative to restore fertility in Klinefelter syndrome patients is the in-vitro maturation of frozen–thawed SSC, but this method is not yet clinically applicable.

In recent years, the question has emerged as to whether testicular tissue banking should still be recommended for young Klinefelter syndrome patients (*Bhasin and Oates, 2020*; *Gies et al., 2016*; *Pook et al., 2021*). The benefit of fertility preservation in young Klinefelter syndrome patients is questionable, because SSC are already lost at a very young age (<4 years) (*Deebel et al., 2020*; *Van Saen et al., 2018*). In addition, TESE outcomes performed in Klinefelter syndrome adolescents are no different compared with Klinefelter syndrome adults (*Van Saen et al., 2018*) and no clinical or hormonal biomarkers to predict positive TESE outcomes exist (*Gies et al., 2012*; *Vernaeve et al., 2004*). For these reasons, testicular tissue banking is no longer proposed to young Klinefelter syndrome patients at the study centre (*Braye et al., 2019*).

Young Klinefelter syndrome patients presented with behavioural, cognitive and/or psychological problems or typical clinical features of Klinefelter syndrome. Except for psychological problems, these characteristics did not influence the decision-making process towards fertility preservation. Klinefelter syndrome patients with psychological problems and/or their parents seem less likely to accept testicular tissue banking for fertility preservation.

This follow-up study shows that the testicular biopsy procedure has no long-term impact on the gonadal development of Klinefelter syndrome patients. Nevertheless, given the retrospective design and the limited number of Klinefelter syndrome patients included, further and regular clinical follow-up remains necessary until adulthood.

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