Germ cell loss in Klinefelter syndrome – when and why?

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Abstract

Klinefelter syndrome (KS) is a quite common disorder with an incidence of 1-2 in 1000 newborn males. Most patients are diagnosed in the light of a clinical check-up when consulting a fertility clinic with an unfulfilled child wish. Infertility in KS patients is caused by a massive germ cell loss, leading to azoospermia in more than 90% of the adult patients. Most seminiferous tubules in the adult KS testis are degenerated or hyalinized and testicular fibrosis can be observed, starting from puberty. However, focal spermatogenesis can be found in the testis of some patients. This offers the opportunity to extract spermatozoa from the testis by testicular sperm extraction. However, TESE is only successful in about half of the KS adults seeking to father children. The reason for the germ cell loss remains unclear. To date, it is still debated whether the testicular tissue changes and the germ cell loss seen in KS is directly caused by an altered X-linked gene expression, the altered somatic environment or a deficiency in the germ cells. In this review, we provide an overview of the current knowledge about the germ cell loss in KS patients.

Keywords

Klinefelter syndrome – Germ cell loss – Infertility – Aneuploidy – X-linked gene-expression
Introduction

The Klinefelter syndrome (KS) was first documented in 1942 by Dr. Harry Klinefelter. He described men with gynecomastia and a very specific type of hypogonadism (Klinefelter, 1942). Seventeen years later, Jacobs and Strong revealed the presence of an extra X chromosome in men diagnosed with KS (Jacobs & Strong, 1959). Upon this discovery, KS was recognised as a genetic disorder. In most affected men, a karyotype of 47,XXY is identified, although in 20% of KS patients variants hereof, including mosaicism and structurally abnormal X chromosomes, exist. The most common variant includes a karyotype with more than one supplementary X chromosome (e.g. 48,XXXY). The number of additional X chromosomes positively relates to the severity of the KS phenotype, resulting in a less severe phenotype for KS patients with mosaicism (Maiburg, Repping, & Giltay, 2012). Nowadays, KS remains the most common sex chromosome related disorder with an incidence of 1 to 2 in 1000 new-born males (Lanfranco, Kamischke, Zitzmann, & Nieschlag, 2004). However, it is believed that KS is only diagnosed in about 25% of those affected, since a great variation in the KS phenotype exists (Groth, Skakkebaek, Host, Gravholt, & Bojesen, 2013). Few KS patients show all known anatomical features such as a tall, feminine posture, gynaecomastia and small testes simultaneously, making a diagnosis difficult (Radicioni et al., 2010).

KS can be diagnosed at any time in life but is most frequently diagnosed at adult age when patients seek help in a centre for reproductive medicine as most of KS men are unable to conceive naturally. When diagnosed earlier in life, the diagnosis of KS is usually made at three specific periods: prenatally, during childhood or following adolescence. During the prenatal period, KS can be diagnosed incidentally through amniocentesis or chorionic villus sampling when screening for other possible diseases such as trisomy 21 (Down syndrome). In prepubertal KS patients, cryptorchidism is the most prevalent clinical sign, even though KS boys account for only 1.3% of all cryptorchidism boys (Ferlin et al., 2008). At school age, boys with a developmental delay and/or learning disabilities are eligible for a chromosomal examination as well as young adults with similar problems and small testes, which is mostly noticed during a medical examination (Gravholt et al., 2018; Pacenza et al., 2012). However, less than 10% of KS diagnoses are made before puberty (Bojesen, Juul, & Gravholt, 2003). Research has discovered a lot of different abnormalities in KS patients, including unfavourable muscle/fat ratio (Aksglaede, Molgaard, Skakkebaek, & Juul, 2008), decreased bone mineral density (Bojesen, Birkebaek, et al., 2011) and a higher prevalence of taurodontism amongst others (Giambersio, Barile, & Giambersio, 2019). In addition, the morbidity and mortality rates of KS men are significantly increased relative to non-KS men due to multiple comorbidities associated with KS as well as poor socioeconomic parameters. KS men are at higher risk to develop cancer, metabolic syndromes such as type II diabetes, endocrine diseases, cardiac disorders, osteoporosis, venous thromboembolism and asthma. Altogether, this leads to a two to six years life expectancy reduction in KS men compared to 46,XY men. Some of the known comorbidities can be linked to the androgen deficiency but for most of them, the reason for the increased risk is still unknown (Belling et al., 2017; Bojesen & Gravholt, 2011; Bojesen, Host, & Gravholt, 2010; Bojesen et al., 2006; Bojesen, Stochholm, Juul, & Gravholt, 2011; Zoller, Ji, Sundquist, & Sundquist, 2016).

Different studies on the hormonal values in KS patients have been conducted, however, contradictory findings have been reported. Results on androgen deficiency in KS infants have shown both a decline in testosterone level (Lahlou, Fennoy, Carel, & Roger, 2004; J. L. Ross et al., 2005) as normal levels (Aksglaede, Petersen, Main, Skakkebaek, & Juul, 2007; Cabrol et al., 2011). In addition, follicle stimulating hormone (FSH) levels have been found to be increased (Lahlou, Fennoy, Ross, Bouvattier, & Roger, 2011) as well as within the normal range (Pacenza et al., 2012) in non-mosaic XXY infants. Hormonal evaluation of peripubertal KS patients classically show increased levels of gonadotrophins
and a decreased testosterone level. However, normal hormone levels have also been reported (Pacenza et al., 2012; Stagi et al., 2016). An increased level of FSH and subnormal levels of testosterone have been associated with KS since its first description (Klinefelter, 1942). Nevertheless, increased to normal gonadotrophins levels and normal to high androgen levels have been reported in KS adults over the years (Pacenza et al., 2011; Stagi et al., 2016; Pasquali et al., 2013). Currently, there is no curative treatment available for KS. One possible method of managing the disease is through a testosterone replacement therapy (TRT), since the testosterone level in KS patients is often declined. TRT influences different negative symptoms associated with KS. In adults, the therapy can lead to a better mood, decreased fatigue, increased concentration and a higher libido, while in KS boys, a positive effect on behaviour and working memory has been suggested (Samango-Sprouse et al., 2015; Tran, Samango-Sprouse, Sadeghin, Powell, & Gropman, 2019; Wang et al., 2004). Recently, TRT has been suggested to also lower the thrombotic risk among KS men (Chang et al., 2019). The therapy is most commonly initiated at adolescent age when the patient shows hypogonadism, increased gonadotropin levels and testosterone concentration (Zganjar, Nangia, Sokol, Ryabets, & Samplaski, 2019). However, the opinions concerning the start of the TRT varies, since some physicians prefer to start TRT as soon as puberty hits, meaning when LH and FSH levels start increasing (Groth et al., 2013), while other physicians are hesitant to start TRT at a young age, since insufficient research on the mental and physical health of KS boys after TRT has been conducted (Nieschlag et al., 2016).

One of the main features of KS is the occurrence of azoospermia in more than 90% of non-mosaic patients. When first described, KS men were considered infertile. This has been contradicted since sperm has been found in the ejaculate in about 8% of KS men (Kitamura et al., 2000). In some rare cases, KS men have been able to reproduce via natural conception (Laron, Dickerman, Zamir, & Galatzer, 1982). In addition, a proportion of patients with KS has been able to father children due to the presence of focal spermatogenesis, which allows collection of sperm cells in the testis by testicular sperm extraction (TESE) and the use of artificial reproductive techniques such as intra-cytoplasmatic sperm injection (ICSI) (Palermo et al., 1999; Staessen et al., 2003). If no spermatozoa can be found, couples in which the man is diagnosed with KS can opt for a sperm donor or adoption. Nevertheless, research has shown that repeating the TESE procedure after a failed previous one could still lead to the retrieval of spermatozoa (Haliloglu, Tangal, Gulpinar, Onal, & Pabuccu, 2014). Since ICSI is the only means to father genetically own children for most KS patients, TESE negative patients are in need for other fertility treatment options. Evaluation of testicular biopsies from adult KS men enabled to divide adult KS men into three groups according to the presence of germ cells. The first group consists of KS men from which mature spermatozoa can be retrieved through the TESE technique, a second group consists of KS men with no testicular spermatozoa, but in which SSC can still be detected in testicular biopsies, and a last group is formed of men in which no germ cells are present (Van Saen, Tournaye, & Goossens, 2012). It is thus clear that infertility in KS men is the result of germ cell loss, but germ cells are not completely lost in all KS patients. It remains unclear why some patients still have ongoing spermatogenesis, while others only have spermatogonia or no germ cells at all. New insights on when these germ cells are lost were gathered from fertility preservation programs which have been set up with the aim of cryopreserving spermatozoa or spermatogonia at a young age.

This review aims to provide an overview of the current knowledge on when and why germ cells are lost in KS patients.
Germ cell loss – when?

When exactly do KS patients lose their germ cells? Can fertility preservation techniques be used to ensure the reproduction chances of young KS boys as an adult? Research on the loss of spermatogonia and spermatozoa has been conducted in testicular tissue of different developmental stages, from fetal up till adult age, trying to answer these questions.

Fetal and childhood

Total germ cell loss has not been observed in KS fetal testicular tissue obtained during the second semester of pregnancy. All studies which included KS fetal patients until now, have revealed a 100% success-rate on finding spermatogonia. A decreased number of germ cells in KS fetal life has already been reported (Coerd, Rehder, Gausmann, Johannisson, & Gropp, 1985; Winge, Dalgaard, Jensen, et al., 2018), while this was not observed in other research of fetal KS tissue (Jequier & Bullimore, 1989; Rock, Rock, & Rary, 1982; Van Saen et al., 2018). In 1980, spermatogonia were observed in testicular biopsies from two KS prepubertal patients (Arce & Padron, 1980). Currently, only four KS boys have been included in the fertility preservation program at the centre of reproductive medicine of the UZ Brussel. In only three of these four patients (4-7 years old) spermatogonia could be found. In addition, the number of spermatogonia which were found was very low. The fertility preservation strategy for these boys consists of the collection of SSC through the cryopreservation of a testicular biopsy. The collected data suggest that, even though the testicular architecture is still intact, germ cell loss is already present at a very young age in KS boys (Van Saen et al., 2018). Theoretically, the presence of SSCs in prepubertal patients should be confirmed in a larger study population, but this is difficult to realize due to ethical considerations. Therefore, fertility preservation strategies focused on spermatogonia before puberty is questionable (Franik et al., 2016). Moreover, options to restore fertility from stored SSCs are also experimental. Autologous transplantation of SSCs to the testis has not yet been performed in a clinical setting and will most probably not be an option in adult KS patients due to the fibrotic appearance of the testis. In-vitro maturation of SSCs on the other hand is not feasible at this moment.

Adolescence

Different studies on the presence of germ cells in peripubertal patients have been performed, often with different outcomes. In a study from 1968, the biopsy of one 16-year-old KS boy was found histologically positive for both spermatozoa and spermatogonia (Gomez-Acebo, Parrilla, Abrisqueta, & Pozuelo, 1968). One year later, six KS boys, between the age of 15 and 17 years were all found to be negative for both spermatozoa and spermatogonia (N. E. Skakkebaek, 1969). Few years ago, the option to preserve fertility in young KS boys was suggested by the cryopreservation of testicular tissue as is offered to prepubertal patients before undergoing gonadotoxic treatments. The first aim was to collect and preserve spermatozoa by TESE at adolescent age. Overall, the chance of finding spermatozoa in peripubertal KS patients is low, with some exceptions (Gies et al., 2012; Heckmann et al., 2018; Van Saen, Gies, De Schepper, Tournaye, & Goossens, 2012; Van Saen et al., 2015; Wikstrom, Hoei-Hansen, Dunkel, & Rajpert-De Meyts, 2007). TESE procedures to collect mature spermatozoa in peripubertal patients have led to a success rate in four out of 26 patients in two different centres. Van Saen et al., reported the collection of spermatozoa in one out of 20 patients, while three out of six patients had a positive TESE in the study of Rives et al. (Rives et al., 2013; Van Saen et al., 2018). In most studies, a testicular biopsy was cryopreserved as back-up for the TESE procedure with the aim of preserving spermatogonia. The presence of a low number of spermatogonia was observed in the biopsies from the patients in the above mentioned studies with a general chance of finding spermatogonia in 42.68% of the patients (reviewed in (Deebel et al., 2020)). Spermatogonia were thus
present in some of these biopsies but the spermatogonial cell count was low (Van Saen et al., 2018). Therefore, testicular tissue preservation at adulthood is questionable since low spermatogonial stem cell counts as well as low efficiency with TESE are reported at that age. Generally, it is concluded that TESE is not recommended when the patient is younger than 16 years old because of lower retrieval rates compared to adolescents and adults between 16 and 30 years (Franik et al., 2016; Gies, Oates, De Schepper, & Tournaye, 2016). However, spermatozoa retrieval rates have been studied in KS adolescents between the age of 15 and 22, resulting in a success-rate of 52%, compared to a success-rate of 65.5% in adults (Plotton et al., 2015). Therefore, the best time point to do a spermatozoa retrieval procedure seems to be during early adulthood.

**Adult age**

KS is the most common genetic cause for infertility. The disorder accounts for 3 to 4% of all male infertility cases and 10 to 12% of all nonobstructive azoospermia (NOA) cases (Forti, Corona, Vignozzi, Krausz, & Maggi, 2010; Lanfranco et al., 2004). Nonetheless, in some adult KS men, focal spermatogenesis can be found. The presence of spermatozoa is considered patient-dependent since in some KS men only degenerated or no seminiferous tubules can be found in their testes, while in others, normal tubules with ongoing spermatogenesis are still present. Because of the focal spermatogenesis present in some KS patients and other nonobstructive azoospermia patients, microdissection TESE (m-TESE) was developed to allow for selective biopsy. Colpi et al. demonstrated through a randomized controlled study that this technique is significantly more effective than the conventional TESE procedure in retrieving spermatozoa from NOA patients. In addition, several other studies have shown an increase in sperm retrieval through the m-TESE technique compared to the conventional TESE in men with NOA (Bernie, Mata, Ramasamy, & Schlegel, 2015; Mehta & Paduch, 2012). Furthermore, a new approach to retrieve spermatozoa from KS testes was studied, namely a testicular sperm sampling by subcapsular orchiectomy. This technique was considered easy and quick. Nevertheless, when comparing the success-rate of this technique to the successes obtained with the m-TESE, it showed that the m-TESE was the best option to retrieve spermatozoa from KS patients (Fedder et al., 2015). For adult KS patients, different success-rates of TESE ranging from zero to 65% for finding spermatozoa have been reported (Arce & Padrón, 1980; Corona et al., 2019; Foresta et al., 1999; Franik et al., 2018; Gordon, Krmpotic, Thomas, Gandy, & Paulsen, 1972; Plotton et al., 2015; Sciurano et al., 2009; Tournaye et al., 1996; Van Saen, Tournaye, et al., 2012; Van Saen et al., 2018; Yamamoto et al., 2002). Overall, the chance of finding spermatozoa in an adult KS testicular biopsy equals 48.53%. Nevertheless, of all adult KS patients who were negative for spermatozoa, 24.3% were found positive for spermatogonia (reviewed in (Deebel et al., 2020)).

**Predictive values for the presence of spermatozoa or spermatogonia?**

In men with nonobstructive azoospermia, a higher testicular volume, a decreased FSH level and an increased serum inhibit B level have been considered as useful markers for the presence of sperm. A younger age was also considered predictive for the time of sperm retrieval through TESE (Gnassi et al., 2018; Ziaee et al., 2006). However, since varied hormonal levels have been reported in KS patients, finding a predictive tool for TESE success-rates based on hormone concentrations, is not evident. Recently, Deebel et al. analysed the correlation between hormonal levels of KS patients at the time of biopsy and the outcome of the TESE procedure in order to find predictive values for the presence of spermatogonia/spermatozoa in KS patients. Contradictory results on the prediction of finding spermatogonia in KS patients with a low FSH level were already reported (Damani, Mittal, & Oates, 2001; Lin, Huang, Lin, & Kuo, 2004; Rives et al., 2013; Van Saen, Tournaye, et al., 2012) and Deebel et al. also suggested that the FSH level is not a reliable predictive tool for finding spermatogonia/spermatozoa in KS patients, even though it was shown that spermatogonia negative
patients tend to show increased FSH levels. Furthermore, significant elevated LH and testosterone levels were reported for spermatogonia negative patients compared to spermatogonia positive patients. In contrast, no significant differences in inhibin B values were reported between the two groups (Deebel et al., 2020). In 2015, Rohayem et al reported that the age of the patient at the moment of the microsurgical sperm retrieval procedure and markers of Leydig cell function were predictive values for the sperm retrieval in KS adolescents and adults (Rohayem et al., 2015). In contrast, other research groups have reported that age is not a predictive factor for the sperm retrieval rate in KS patients (Corona et al., 2017; Van Saen et al., 2018). The results of the study of Deebel et al. showed a significantly lower chance of finding spermatogonia in the biopsy of patients older than 18 years old when their biopsy was already negative for spermatozoa (Deebel et al., 2020). Overall, more research should be performed regarding this topic, since the retrieval of spermatozoa is an invasive procedure and KS testicular biopsies are often found to be negative for spermatogonia/spermatozoa.

In conclusion, germ cell loss is very likely to start at a young age in KS patients, eventually leading to infertility. Since the presence of spermatogonia is limited in prepubertal and adolescent KS samples, the best fertility preservation option for KS patients remains TESE at adulthood. An overview of spermatogonia/spermatozoa retrieval rates from fetal up till adult age is provided in table I. However, more studies with a bigger sample size should be conducted to verify this statement. An overview of all fertility preservation strategies for KS patients from childhood up to adulthood is depicted in figure 1. Depending on the age of diagnosis, different strategies might be followed. When diagnosis occurs before puberty, a testicular biopsy could be cryopreserved in order to preserve the SSCs for later use. Whether this procedure compromises the chances of finding spermatozoa at later age is not clear yet. When diagnosis occurs during adolescence, before the age of 17, preservation of SSCs or spermatozoa could be attempted but is not recommended due to low efficiency rates. The collection of spermatozoa through vibrostimulation or electroejaculation was also evaluated, but sperm cells could not be collected from the obtained sperm samples (Gies et al., 2012; Van Saen et al., 2018). Chances to collect spermatozoa increase when patients are older than 16 years. Therefore, early diagnosed patients can postpone fertility preservation until early adulthood with the aim of collecting sperm cells for later use when they have an active child wish.

If the presence of spermatogonia is already compromised during childhood, fertility preservation focussed on germ cells will not suffice to ensure fertility treatment in later stages of life. New fertility strategies should be developed for these patients. Nevertheless, this is difficult since the exact mechanisms of the germ cell loss and the reason why some patients still have focal spermatogenesis, remains unknown. Over the years, several possible mechanisms for the germ cell loss have been researched, with focus on the germ cell, the testicular environment and the gene dosage.

Germ cell loss - why?

- Is it the germ cell?

The presence of focal spermatogenesis in KS patients proofs that not all germ cells are lost. What makes certain germ cells able to survive in the testicular environment from KS patients and give rise to fully developed spermatozoa? This is a question that researchers have been trying to answer for more than two decades. Can the occurrence of testicular mosaicism explain the presence of spermatogenesis? If this is the case, it would mean that there is a mixture of spermatogonia having the normal and abnormal karyotype with the normal spermatogonia being the founder cells of spermatogenesis. It is indeed known that KS patients with 46,XY/47,XXY mosaicism (diagnosed from
lymphocytes karyotyping) have a better fertility prognosis compared to the non-mosaic patients since more than 90% of non-mosaic KS patients have azoospermia, while mosaic KS patients have more chance of having spermatozoa present in their semen (Samplaski et al., 2014). Does this mean that only spermatogonia with the normal karyotype can proceed through meiosis and give rise to spermatozoa? This hypothesis was initially contradicted by Foresta et al. They showed, through histological evaluation of chromosome patterns by fluorescent in-situ hybridisation, that a 47,XXY karyotype was present in spermatogonia and primary spermatocytes in KS patients. The distribution pattern of hyperhaploidy in spermatozoa also reflected their origin from 47,XXY spermatogonia since there was an increase in 24,XY spermatozoa but no increase in 22,0 hypohaploid spermatozoa (Foresta et al., 1999). Several studies report a higher occurrence of hyperhaploid sperm cells in non-mosaic KS patients compared to fertile controls and mosaic KS patients. Their increased frequency cannot be explained by meiotic nondisjunction in normal 46,XY cells that might be present in the testis (Cozzi et al., 1994; Estop et al., 1998; Guttenbach, Michelmann, Hinney, Engel, & Schmid, 1997). Therefore, it was concluded that 47,XXY spermatogonia are able to complete meiosis. Embryo’s generated with sperm cells from non-mosaic KS patients showed an increased risk of both sex (13.2% versus 3.1%) and autosomal (7.0 % versus 2.4%) chromosomal abnormalities compared to age-matched controls when subjected to preimplantation genetic diagnosis (Staessen et al., 2003). However, an increased aneuploidy rate was also reported when testicular sperm was used for fertilisation compared to epididymal and ejaculated sperm (Palermo, Colombero, Hariprashad, Schlegel, & Rosenwaks, 2002). Therefore, it is still not clear whether the higher incidence of abnormalities in embryos from KS patients are due to the 47,XXY karyotype or the use of testicular sperm.

The alternative hypothesis claims that only normal germ cell clones can enter meiosis (Bergere et al., 2002; Blanco, Egozcue, & Vidal, 2001; Sciurano et al., 2009). In this case, the increased sperm abnormalities are attributed to the abnormal environment which induces meiotic I non-disjunctions. The presence of spermatogonia with a normal karyotype within the KS testis and thus testicular mosaicism would explain the occurrence of focal spermatogenesis. The question remains: when do these clones arise and what happens with the 47,XXY spermatogonia? Are they already lost early during testicular development? An experimental fertility preservation program for adolescent and later on prepubertal KS boys revealed the presence of only few spermatogonia in these young KS boys (Gies et al., 2012; Van Saen, Gies, et al., 2012; Van Saen et al., 2018; Wikstrom et al., 2004). Findings on germ cell numbers in fetal KS samples are contradictory. There are several reports where no difference in germ cell number between KS and control samples was observed (Jequier & Bullimore, 1989; Rock et al., 1982; Van Saen et al., 2018), whereas others claim that the germ cell loss in KS is already present at fetal age (Coerdt et al., 1985; Winge, Dalggaard, Jensen, et al., 2018). In a recent report, a lack of differentiation of gonocytes into prespermatogonia was hypothesized based on the decreased expression of OCT3/4 without increase in expression of MAGE-A4 as was observed in age-matched control samples (Winge, Dalggaard, Jensen, et al., 2018). The low number of germ cells present in testicular samples from fetal and young KS patients seems to indicate that only a limited number of germ cells is able to survive in the KS testis supporting the hypothesis that only the germ cells with the normal karyotype resist. Recently, it was shown that germ cells in KS patients have a normal transcriptome, indicating that the few remaining germ cells in the KS testis are similar to germ cells in samples with normal spermatogenesis. These germ cells showed a normal DNA methylation profile in selected germ cell specific markers, but a variation in imprinted genes (Laurentino et al., 2019).
- **Is it the testicular environment?**

If it is assumed that only spermatogonia with the normal karyotype can proceed through meiosis to produce mature spermatozoa, the testicular environment is believed to be responsible for the higher aneuploidy rate in sperm in KS patients. However, during childhood, a normal testicular architecture is conserved, whereas degeneration and testicular fibrosis can be observed starting from puberty. Histological images of an adult KS testis show significant architectural changes such as fibrosis, Leydig cell hyperplasia, high numbers of degenerated and hyalinized tubules and absence of germ cells (Aksglaede et al., 2006). The testicular histology is patient dependent since tubular degeneration and fibrosis can be severe in some patients while in others, normal tubules can still be observed (Van Saen et al., 2018). Since the changes to the testicular histology are positively associated with germ cell loss, it has been suggested that the altered testicular environment leads to germ cell loss.

Leydig cells are known to be essential for male reproduction, since these cells produce testosterone. Very large clusters of Leydig cells have been discovered in the testes of infertile male, including KS patients (Wikstrom & Dunkel, 2011). Research has shown that even though the Leydig cells in KS appear to be morphologically normal, these cells are often functionally deficient. The Leydig cell hyperplasia seen in KS patients could be an attempt to compensate for the loss in testosterone production. Micronodules of >15 Leydig cells can often be detected in KS testes, but the exact function of these clusters remains unknown (Holm, Rajpert-De Meyts, Andersson, & Skakkebaek, 2003; Wistuba et al., 2010). Lottrup et al. investigated the differentiation stages of Leydig cells in KS testicular tissue, which showed that some of the Leydig cells present in the micronodules were arrested in differentiation e.g. immature. This led to the suggestion that the Leydig cells in KS tissue do not differentiate into testosterone-producing cells (Lottrup et al., 2014). However, an increased intratesticular testosterone level has been measured in adult KS patients in contrast to the low circulating testosterone level. For this reason, it has been suggested that the Leydig cells produce a normal level of testosterone but through a disturbed vascularisation within the KS testicular tissue, the testosterone release to the blood flow is restricted (Foresta et al., 2012; Tuttelmann et al., 2014b). Since defects from the somatic compartment may be involved in the testicular degeneration seen in KS testes, the blood-testis-barrier (BTB) has recently been studied, showing a reduction of BTB proteins and a deviant distribution pattern (Giudice, Vermeulen, & Wyns, 2019). In KS patients, the seminiferous tubules typically show tremendous thickening of the basement membrane and a loss of epithelial cells. These so called ‘hyalinized’ tubules eventually completely disintegrate, resulting in fibrotic tissue (McLachlan, Rajpert-De Meyts, Hoei-Hansen, de Kretser, & Skakkebaek, 2007). The walls of the seminiferous tubules consist of several layers of flat cells and extracellular matrix (ECM) proteins. The cells of these walls, the peritubular myoid cells (PTMCs), are myofibroblastic cells which are known to transport immotile spermatozoa. In men with impaired spermatogenesis, the composition of the tubular wall is frequently altered, suggesting an important role for PTMC in male fertility (Mayerhofer, 2013). Therefore, the PTMCs and ECM were recently studied in KS testicular tissue of different developmental stages. Loss of ACTA2, which is a contractile marker of PTMC was observed in the adult KS testis. It has been suggested that the PTMCs change their phenotype as a consequence of the changes in the testicular environment. Furthermore, altered expression patterns for the ECM proteins collagen I and IV were shown (Van Saen et al., in press). Overall, the mechanisms responsible for the architectural changes of the KS testes and the association with germ cell loss require more investigation.
Is it the gene dosage?

It is obvious to assume that the additional X chromosome is at least partially responsible for the phenotypical symptoms in KS patients. Several genetic aspects of the X chromosome and the regulation of altered gene expression in these patients might have an effect on the phenotype. Dosage compensation from the sex-linked genes between males and females is regulated by X inactivation in females early in development. X inactivation is established by the expression of the XIST gene which functions as a non-coding RNA and coats the inactive X (Brown & Robinson, 2000). X inactivation in KS patients occurs in a similar way as in females (Kleinheinz & Schulze, 1994).

The expression profile of X-linked genes can be biased by altered X chromosome inactivation. X chromosome inactivation normally occurs at random during the early blastocyst stage with an equal chance for the maternally and paternally derived X chromosome to be active. However, X inactivation ratios can be skewed when the inactivation of one X chromosome is favoured over the other and could be involved in the high phenotypical differences in KS patients. Theoretically, skewing could result in the expression of only paternal genes in 47,XmXpY patients when the maternal derived X chromosome is skewed (Iitsuka et al., 2001). The inactivation pattern of the X chromosomes in KS has been studied but resulted in contradictory results. While some studies revealed the presence of skewed X inactivation in KS patients (Iitsuka et al., 2001; Mehta et al., 2012; Zitzmann, Depenbusch, Gromoll, & Nieschlag, 2004), other reports observed that X inactivation followed the distribution pattern from healthy young women (Bojesen, Hertz, & Gravholt, 2011; Kinjo et al., 2020). The androgen receptor (AR) gene is X encoded and is thus subject to X inactivation. It is encoded by a highly polymorphic trinucleotide (CAGn) repeat with a normal length between 9 and 37 repeats. Zitzmann et al. reported a preferential inactivation for the shorter allele in KS patients meaning that the longer allele is active. The authors observed that the higher length and bone density are associated with the length of the CAGn repeat. Gynaecomastia and smaller testes were also more observed in patients with higher repeats. On the other hand, shorter repeats were associated with higher educational levels and stable relationships. Since the response to TRT is also influenced by the CAGn repeat, it has been suggested to determine the length of the CAG polymorphism on the AR allele of each KS patient to administer the optimal testosterone dose per patient (Zitzmann et al., 2004). The influence of the CAGn repeat in the AR on the phenotypic variations in KS patients was confirmed by another study, but the influence of X inactivation was not (Bojesen, Hertz, et al., 2011). In adolescent boys, the length of the CAGn repeat was positively correlated with a later increase in LH and testosterone, a slower progression of puberty and a later onset of testicular degeneration (Wikstrom, Painter, Raivio, Aittomaki, & Dunkel, 2006).

Maternally derived X chromosomes can arise during the first or second meiotic division or during early postzygotic mitotic divisions, while the paternal origin arises during meiosis I only (Tuttelmann & Gromoll, 2010). Errors occurring during maternal meiosis II or in mitosis result in the presence of two identical X chromosomes (Lanfranco et al., 2004). However, X chromosome isodisomy or heterodisomy did not seem to influence the phenotype in KS patients (Wikstrom et al., 2006). The distribution between maternal (51-59%) and paternal (41-49%) derived supernumerary X chromosome seemed similar between different studies. An association between paternal origin from the X chromosome and higher incidence of speech and language problems and motor impairment was observed by Stemkens et al, while no influence on the phenotype was observed in other studies (Iitsuka et al., 2001; A. Skakkebaek et al., 2014; Stemkens et al., 2006). The parental origin of the additional X chromosome did also not affect the chance of spermatozoa retrieval during TESE or the presence of spermatogenesis in adult KS patients (Franik et al., 2018). However, a delay in pubertal onset and a slower progression of puberty was correlated with the paternal origin of the extra X in
adolescent KS boys. Testicular degeneration also occurred later in the same study population (Wikstrom et al., 2006). It must be noted that adolescent KS patients might not represent the adult population since these boys were diagnosed at early age and might thus represent the more affected group within KS.

In general, it seems that the parental origin is equally distributed and X inactivation occurs randomly. Nevertheless, gene dosage might still explain some of the phenotypical aspects in KS patients. It is described that about 15% of X linked genes are known to escape X inactivation and an additional 10% show variable patterns of inactivation in females (Carrel & Willard, 2005). The same event in KS patients will lead to overexpression of X linked genes compared to normal men. The transcriptomic profile from KS patients was compared to normal males and females (Belling et al., 2017; Huang et al., 2015; A. Skakkebaek et al., 2018; Vawter, Harvey, & DeLisi, 2007; Winge, Dalgaard, Belling, et al., 2018; Winge, Dalgaard, Jensen, et al., 2018; Zitzmann et al., 2015). In several studies, RNA was extracted from blood samples, since these are relatively easy to get (Belling et al., 2017; Huang et al., 2015; A. Skakkebaek et al., 2018; Vawter et al., 2007; Zitzmann et al., 2015). The number of differentially expressed transcripts (DETs) varied substantially between the different studies. DETs were not only encoded from the X chromosome, but deregulated expression was observed genome wide, including all autosomal chromosomes showing that not only X related genes are deregulated. A unique genetic landscape was identified in KS patients compared to normal men. Gene set enrichment analysis revealed associations with known comorbidities, such as obesity, cardiovascular abnormalities, obesity, verbal and cognitive dysfunction (Table II). A recurrent finding represents the increased expression of XIST in KS males indicating the occurrence of X inactivation. Several escape genes (KDM5C, EIF1AX, KDM6A, DDX3X, TXLNG, PRKX, EIF2S3, ZFX) showed an altered expression in KS patients (Belling et al., 2017; A. Skakkebaek et al., 2018; Zitzmann et al., 2015). Several of these genes were common between different studies, but when the Y chromosome homologues were also considered, differences disappeared for most of them except for TXLNG, PRKX an EIF2S3. EIF2S3 is involved in hypogonadism and spermatogenesis (A. Skakkebaek et al., 2018) and illustrates that altered X expression could be involved in testicular dysfunction.

Since a large proportion of genes on the X chromosome is involved in spermatogenesis (M. T. Ross et al., 2005), it is likely that a different gene dosage in KS men might have an impact on their fertility problems. Therefore, the transcriptome of testicular tissue in KS patients is also of high interest. Several studies focused on the differential expression in testicular biopsies from KS patients. The difficulty in these studies is to define and select the control group to account for the cellular differences between the different study groups. On the one hand, testicular biopsies from KS, without germ cells present, were compared with samples from azoospermic patients with conserved spermatogenesis (D’Aurora et al., 2015). On the other hand, KS samples with focal spermatogenesis were compared with samples with ongoing spermatogenesis (D’Aurora et al., 2017). Down regulation of genes involved in the meiotic process and male germ cell movement was observed in KS patients with hypospermatogenesis. While genes involved in germ cell differentiation were mainly down regulated, up-regulated genes were expressed in the somatic compartment (D’Aurora et al., 2017). In samples without germ cells, main differences were observed in the somatic cells. Of the 903 DETs, only 247 were down regulated from which only 24 transcripts were expressed in the germinal epithelium. Only one upregulated transcript was located on the X chromosome. Dysregulated genes clustered to pathways involved in apoptotic and inflammatory processes and the regulation of the blood-testis barrier (D’Aurora et al., 2015). This suggests a strong involvement of the somatic compartment in the testicular pathophysiology in KS patients. However, results can be biased by the cellular composition between samples with hypospermatogenesis and normal spermatogenesis. Therefore, cellularity-matched matched samples, which had Sertoli-cell-only syndrome pattern and
Leydig cell hyperplasia present, were chosen as controls in another study. In this study, also no significant enrichment of X linked upregulated transcripts was observed in adult KS samples (Winge, Dalgaard, Belling, et al., 2018). However, an enrichment in non-coding RNAs was observed, indicating that the supernumerary X chromosome rather has an indirect effect (Winge, Dalgaard, Belling, et al., 2018). When the testicular transcriptome of fetal, prepubertal and adult KS samples were compared, the three different groups clustered separately according to their developmental timepoint instead of their karyotype. Seven transcripts were identified to overlap between the three developmental timepoints and only XIST was shared at all time points. Nevertheless, none of these overlapping DETs were X linked genes (Winge, Dalgaard, Belling, et al., 2018). A significant enrichment of upregulated X chromosomal transcripts was however found in fetal KS samples (Winge, Dalgaard, Jensen, et al., 2018). This led to the hypothesis that the effect of an extra X chromosome is less direct in adult compared to fetal life and thus, it might be assumed that the disturbed testicular architecture at adult age is already caused by disturbed fetal gonadal development. Overexpression of X linked genes during fetal life might lead to the initiation of germ cell loss and maturation failure of the somatic cells in the KS testis (Winge, Dalgaard, Belling, et al., 2018).

The overlap between DETs in different transcriptome studies of KS samples is limited. Only 12 transcripts of the 235 DETs reported in Winge et al. were also differentially expressed in other studies. Three of them were found in blood samples from KS patients, while the other nine were identified in adult testicular biopsies. However, different control samples and methodology (arrays versus RNA-sequencing) can also account for these differences. A recurrent finding in these studies reflects the involvement of dysregulated maturation in the somatic compartment in KS biopsies (D’Aurora et al., 2015; Winge, Dalgaard, Belling, et al., 2018). DACH2 is an X linked transcript which was upregulated in KS samples and expression in KS samples was observed in immature Sertoli cells. The same finding was observed in immature Leydig cells for another upregulated transcript, FAM9A (Winge, Dalgaard, Belling, et al., 2018). Another immature Leydig cell marker, DLK1, was found to be differentially expressed in two studies, suggesting that a large proportion of the Leydig cells are immature ((D’Aurora et al., 2017; Winge, Dalgaard, Belling, et al., 2018). A distinct expression of DLK1 was observed in testicular biopsies from 5 KS patients. Large clusters of Leydig cells either expressed DLK1 or a marker for more mature Leydig cells, INSL3, but the proportion of DLK1 positive Leydig cells was found to be higher (Lottrup et al., 2014). Different studies thus suggest the immaturity of the somatic cell compartment in KS patients. Next to genes related to the somatic compartment, genes involved in apoptotic processes and inflammation were also commonly found to be dysregulated in these studies (D’Aurora et al., 2017; Winge, Dalgaard, Belling, et al., 2018). Table II represents an overview of these studies in which differential gene expression was analyzed in KS patients.

Lessons from animal models

Two mouse models with a similar phenotype to KS have been described. Both are generated through a staggered breeding scheme starting from a male mouse line with a mutated Y chromosome (B6Ei.Lt-Y*) and wild-type females. One of the possible offspring from this breeding model represents the first model, 41,XXY* (Lewejohann et al., 2009). In this model, the Y chromosome is not completely separated from the X chromosome, but in close association with one of them. These mice had smaller testes, seminiferous tubules were devoid of germ cells and a higher number of Leydig cells was present at adulthood. Both LH and FSH serum levels were significantly increased, while testosterone was reduced but not significantly (Wistuba et al., 2010). The second model, 41,XY mice, can be generated by a four-generation breeding scheme, as described by Hunt and Eicher, starting from the same breeding couples as reported above (Hunt & Eicher, 1991). These mice had lower levels of
testosterone and FSH, but the level of LH was not significantly lower compared to control mice. Testicular weight was significantly lower starting from the age of 7 days. At adult age, small seminiferous tubules with mostly degenerating Sertoli cells were observed but in some tubules ongoing spermatogenesis was observed (Lue et al., 2005). Both mouse models were found to be a reliable model for KS. Next to the mouse model, which is bred to obtain mice with an extra X chromosome, there have been several reports of the detection of spontaneous occurrence of the KS karyotype in different animal species, like cats, pigs and cattle (Makin, Andersson, & Nikunen, 1998; Pedersen, Berg, Almstrup, & Thomsen, 2014; Slota, Kozubksa-Sobocińska, Koscielny, Danielak-Czech, & Rejduch, 2003).

Germ cell loss in the neonatal period was characterized in both models. In 41,XX<sup>y</sup> mice, spermatogonia were present until 10dpp, but were lost by 21dpp, while a significant decrease in the number of gonocytes was already noticed at day 3 in 41,XXY mice, followed by a continued progressive loss with only few detectable spermatogonia at 10dpp (Lue et al., 2005). However, in a later study the germ cell loss in the 41,XX<sup>y</sup> mice was studied again with a significant reduction in gonocyte number already detected at 1dpp. These findings suggest that germ cell loss already occurs early during lifetime and thus way before onset of meiosis (Werler et al., 2014). This finding confirms the low number of germ cells in prepubertal KS patients (Van Saen et al., 2018). A loss of LIN28A and PGP9.5 was observed in the early developmental period which indicated an early loss of the stem cell potential of the SSC population in KS mice. Although low germ cell numbers, from 1dpp until 10dpp, were detected, the numbers remained stable meaning the germ cells did not go in apoptosis. However, germ cell proliferation was disturbed in XXY germ cells probably indicating a problem in mitotic proliferation (Werler et al., 2014).

The somatic environment in the KS testis is capable of supporting spermatogenesis since 46,XY spermatogonia were able to complete differentiation when transplanted to the testes of XXY mice (Lue et al., 2010). The presence of donor cells in testicular biopsies and the ejaculate was observed after transplantation of SSCs with normal karyotype to a Klinefelter bull testis. However, sperm cells were never present in the ejaculate until nine months after germ cell transfer. The observed donor cells in the ejaculate were morphologically identified as epithelial cells and germ cells (Joerg et al., 2003). Histological evaluation was not performed, but the low success of germ cell transplantation can be expected since degeneration of the seminiferous tubules hampers donor cell colonization. Leydig cell hyperplasia and a higher number of Sertoli cells per testicular volume were observed in the KS mouse testis (Werler et al., 2014; Wistuba et al., 2010). Intratesticular testosterone levels were normal, indicating normal Leydig cell function. Leydig cells were even found to be hyperactive since they produced significantly more testosterone in vitro when stimulated with human chorionic gonadotropin (Wistuba et al., 2010). The authors hypothesized a disturbed transportation of LH via the blood supply caused by altered vasculature leading to a disturbed hormone supply and transport of testosterone out of the testes. The reduced blood vessel/testis surface ratio in this mouse models seems to support this hypothesis. In the same study, significantly increased intratesticular testosterone concentrations were observed in KS patients, despite the presence of reduced serum testosterone levels. The alternative hypothesis of higher binding capacity in the testis due to increased levels of sex hormone-binding globulin was not supported in human KS testis (Tuttelmann et al., 2014a).

X inactivation in the 41,XX<sup>y</sup> model resembles the situation in female mice indicating proper inactivation of the extra X chromosome (Wistuba et al., 2010). The expression of four genes, known to escape X inactivation in females, was evaluated in brain, kidney and liver. The expression level of a
known X inactivated gene was similar between male, female and KS mice. A large variation between genes and tissue was observed in the escapee genes. A statistical difference between KS and male mice was only observed for one gene in liver and kidney. The other genes showed a trend towards a female expression pattern but did not reach statistical difference. In contrast, escaped genes were expressed higher in the brain of KS mice, indicating that these altered expression levels might be involved in the memory recognition deficits observed in KS mice (Werler, Poplinski, Gromoll, & Wistuba, 2011). Testis-specific genes were not investigated in this study.

**Conclusion**

In this review, we provided an overview of the time span in which germ cell loss occurs in KS patients as well as an overview of the possible causes. To conclude, germ cell loss in KS patients is very likely to start very early during childhood, or even already during fetal development. Fertility preservation at early age, focussing on spermatogonia in these patients is thus questionable. Since no spermatozoa can be retrieved before puberty and the success-rate of finding sperm in adolescent patients is not as high compared to adult patients, the recommended fertility preservation strategy for KS patients outside research programs should be focussed on TESE during adulthood. However, for KS adults in whom no spermatozoa can be retrieved by TESE, other strategies should be developed. Future possible strategies based on in vitro spermatogenesis could provide a solution for infertile KS patients with spermatogonia present. On the other hand, unravelling the mechanisms behind the massive germ cell loss could also provide novel therapies. Nevertheless, it remains unclear whether the germ cell loss in KS patients occurs due to the altered germ cell karyotype, the disturbed testicular environment or the supplemental X chromosome. Most probably, a combination of these factors is causing the phenotypical symptoms in KS patients.

**Conflict of interest**

No conflict of interest is declared by any of the authors.

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**Figure legends**

**Figure 1:** Overview of the different fertility preservation options for KS patients according to the age of diagnosis. When diagnosed before puberty, fertility preservation can be delayed until spermatozoa collection is possible (green arrows), since the fertility preservation treatment for KS patients younger than 16 years, the collection of SSC’s for later use, is still experimental. When SSC preservation is considered, this should be performed at a young age. Adolescent patients can provide a semen sample, either by masturbation or by vibrostimulation or electroejaculation. When a collected sperm sample from KS patients (adolescent/adult) is positive for spermatozoa, the sperm sample can be cryopreserved for later use, if necessary. When no spermatozoa can be identified in the sperm sample, a testicular sperm extraction can be considered. However, this option is not recommended for patients younger than 16 years old. Spermatozoa can be collected through a conventional TESE or a micro-TESE. Another option includes sperm sampling by subcapsular orchietomy, but this is rarely performed. Micro-TESE has rendered the best success-rate in retrieving spermatozoa, making it the designated fertility preservation technique for KS azoospermic patients.

SSC = spermatogonial stem cells; TESE = testicular sperm extraction
Cryopreservation of testicular biopsy

Age at diagnosis

- **Prepubertal** (< 11y)
  - Collection of SSC (experimental)

- **Adolescence** (11-18y)
  - 11-16y
    - Able to produce sperm sample?
      - **NO**
        - Electro-ejaculation
        - Vibrostimulation
      - **YES**
        - Conventional TESE
  - > 16y
    - Presence of spermatozoa in sperm sample?
      - **YES**
        - Micro-TESE
      - **NO**
        - Collection of mature spermatozoa through testicular sperm extraction

- **Adulthood** (> 18y)

- Low efficiency for patients <16y

Collection of sperm cells
Possibility to cryopreserve sperm sample for later use
Sperm sampling by subcapsular orchietomy
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**Number of studies**

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**Positive for spermatogonia**

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**Positive for spermatozoa**

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