INTRODUCTION

Klinefelter syndrome (KS) is one of the most prevalent sex chromosomal disorders with an incidence of about 1 to 2 per 1,000 male newborns (Bojesen, Juul, & Gravholt, 2003). It is based on an X-chromosomal polysomy, with X-disomy being the most common type of Klinefelter (47, XXX) (Jacobs & Strong, 1959; Linden, Bender, & Robinson, 1995). Interestingly, the diagnosis of KS is often made after a diagnostic workup on infertility in adults (Abramsky & Chapple, 1997), which might suggest that the real incidence might...
be even higher in the general population. About 90% of men with KS suffer from nonobstructive azoospermia (NOA) and about 10% from subfertility due to severe oligospermia (Lanfranco, Kamischke, Zitzmann, & Nieschlag, 2004). The development of intracytoplasmic sperm injection (ICSI) in combination with testicular sperm extraction (TESE) makes it possible for men with KS to father of their own biological children. However, only in 30%-50% of men with KS undergoing TESE, spermatozoa can be retrieved from the testis (Akslagae, Skakkebæk, Almstrup, & Juul, 2011). The aetiology for the presence or absence of spermatogenesis in Klinefelter patients is still unclear as no pathophysiological mechanism determining the absence or presence of spermatozoa has yet been revealed. Except for age and hormonal values of LH and testosterone, no prognostic markers or parameters have been found that could predict spermatogenesis in these males (Frank et al., 2016; Rohayem et al., 2015). However, both parameters are only very weak predictors.

Searching the possible mechanisms in predicting spermatogenesis to avoid performing unnecessary biopsies in males with a compromised testicular volume and testosterone production, we hypothesised that the origin of the additional X-chromosome might be a clinical marker for spermatogenesis. Phenotypic differences have already been described in the literature for males carrying an additional paternal X-chromosome (Stemkens et al., 2006). Therefore, it might be of diagnostic value to determine whether the extra X-chromosome is inherited paternally or maternally before starting fertility treatment. As about 10% of the genes located on the X-chromosome are expressed in the testis (Ross et al., 2005), these X-chromosomal genes are likely to influence spermatogenesis by over- or under-expression of testicular gene products, which might impair testicular physiology and the production of germ cells.

The objective of this study was to explore whether the inheritance of an additional maternal or paternal X-chromosome in Klinefelter men can predict the presence of any level of spermatogenesis and/or successful sperm retrieval after TESE.

2 | METHODS

2.1 | Study participants

A total of 35 adult males with nonmosaic KS (47,XXY) and NOA who underwent a diagnostic TESE procedure at the Radboud University Medical Centre between 2010 and 2016 were eligible for inclusion in the X-chromosomal inheritance study. Before 2010, no TESE could be performed in chromosomal abnormal patients due to a national law. An additional inclusion criterion for the genetic analysis was the ability of at least one biological parent to donate saliva samples for X-inheritance testing. Exclusion criteria for participation in this study were as follows: a language barrier to explain the procedure; no informed consent signed; impossibility to obtain a saliva sample from at least one parent.

All patients gave written informed consent to the publication of their case details. Serum testosterone (nmol/L), FSH (U/L), LH (U/L) and inhibin B (U/L) were measured prior to TESE in all participants using enzyme-linked immunosorbent assay (ELISA).

A short questionnaire including age, BMI and personal information about smoking, alcohol consumption and drug abuse was filled in by all participants. The questions asked included “Do you currently smoke cigarettes regularly? If yes, how many cigarettes per day?”, “How much units of alcohol do you drink per week?” and “Do you use substances such as marijuana or other illegal drugs?”. One unit of alcohol was defined as 0.2 L of beer or wine or 2 cl of strong alcohol. A consumption of up to 14 units of alcohol per week was defined as moderate alcohol consumption.

2.2 | DNA isolation and analysis

A DNA sample was collected from each participant using a sterile cotton swab for a mucosa sample. Subsequently, DNA was isolated from the swabs using QIAamp DNA Mini Kit. The isolated DNA was amplified by QF-PCR using fluorescent-labelled Anuefash™ V3 primerset S1, S2 and MXY. The fluorescent DNA fragments were subsequently analysed (GeneScan) using Applied Biosystems DNA Analyzer 3730. The data from the gene array were analysed using SoftGenetics GeneMarker (IG), a genescan analysis software from the sequence facility (Seqfac). Genotyping and analysis of the markers (AMXY, DXY2567, D21S1414, D21S1446, D21S1442, SRY, X22, DXYS218, HPRT) was performed with the SoftGenetics GeneMarker (IG) software. The results of the analysis were evaluated by three researchers independently.

2.3 | Testicular sperm extraction, cytology and histology analysis

Testicular biopsies of at least 10 × 5 mm were obtained by our standard and immediately transported to the fertility laboratory (Hessel et al., 2015). Testicular biopsies were prepared and evaluated for the presence or absence of spermatozoa using wet mount preparation. The presence of at least one spermatozoon was considered a positive sperm retrieval (TESE+). Cytology evaluation by scoring the ratios between spermatozoa, pachytene spermatocytes and Sertoli cells was also performed on the TESE samples, using a technique recently introduced by Hessel et al. (2015). The cell suspension obtained was prepared on a glass slide, and at least 400 cells were evaluated per slide at a 1000 × magnification. A second observer randomly confirmed the cell counts and spermatogenic patterns for validation. The ratios between the counted Sertoli cells, meiotic (pachytene) spermatocytes and testicular spermatozoa were calculated as a measure for the productivity of the meiotic cells. Furthermore, we carried out a histopathological analysis of the spermatogenesis for each tissue sample collected by TESE and analysed the structural changes within the testicular tissue using Giemsa-stained smears.

2.4 | Statistical analysis

A descriptive statistical analysis was performed using IBM SPSS statistics version 22. Mean and standard deviation was calculated for continuous variables. Unpaired student t test and Pearson's
Chi-squared tests were performed to test for goodness of fit and comparison of the baseline characteristics including hormone levels.

### 2.5 Ethical approval

Ethical approval was given by the local ethics committee from the Radboudumc, Nijmegen, The Netherlands.

### 3 RESULTS

Nine of the 35 eligible participants took part in the study. The baseline characteristics of the nine included participants are shown in Table 1. No statistically significant difference was observed in the baseline characteristics between the TESE-positive and the TESE-negative participants.

A paternal origin of the extra X-chromosome was found in six of nine (67%), and a maternal origin was found in three of nine (33%) participants. From the three TESE-positive participants, a paternal origin of the extra X-chromosome was found in two males (67%) and a maternal origin was found in one male (33%). From the six TESE-negative participants, four (67%) had a paternal origin and two (33%) had a maternal origin of the extra X-chromosome. No evidence was found for an association between the maternal or paternal origin of the extra X-chromosome and the presence of spermatozoa retrieval by TESE or the presence of spermatogenesis ($p = .69$). The descriptions of X-chromosomal inheritance and spermatogenesis are shown in Table 2.

The results of our analysis on spermatogenesis were not influenced by the evaluation of a unilateral or bilateral biopsy. All participants without spermatozoa retrieval had a bilateral TESE. While in only three cases spermatozoa was obtained for ICSI, spermatogenesis at different maturation stages was observed in histological or cytological preparations. In five of nine (56%) participants, various stages of spermatogenesis were identified in the histological analysis (varying from the presence of spermatozoa in three patients to the presence of pachytene spermatocytes in another two patients). The results of our cytological analysis, as previously introduced by Hessel et al., showed very low ratios of spermatozoa/pachytene spermatocytes in two of nine (22%) participants (Hessel et al., 2015). Maturation arrest was present in one (11%) participant upon cytological analysis. No evidence for a difference in age between the two subgroups with or without the presence of spermatogenesis was observed ($p = .09$). Analysis of hormonal levels as measured prior to the TESE did not show any statistical difference between the TESE-positive and TESE-negative subgroups with regard to the mean levels of testosterone (11.5 ± 6.8 vs 8.9 ± 6.8, $p = .66$), LH (14.6 ± 0.8 vs 19.3 ± 3.1, $p = .08$) and FSH (26.6 ± 5.1 vs 39.0 ± 17.4, $p = .42$). In our study population, the thresholds for sperm production previously suggested by Rohayem et al. (2015) (serum testosterone >7.5 nmol/L and serum LH < 17.5 U/L) could only predict spermatozoa retrieval in two cases (67%, $p = .06$). Inhibin B was under the detection level in all patients who underwent TESE.

### 4 DISCUSSION

In our study, we investigated whether a paternal or maternal origin of the second X-chromosome was associated with presence or absence of spermatogenesis and the chance of finding spermatozoa either in the ejaculates or testis biopsies of KS males. Unfortunately, the results show that neither maternal nor paternal origin of the extra X-chromosome can predict the presence or absence of spermatogenesis. To our knowledge, this was the first study investigating X-chromosomal origin in combination with the presence of spermatozoa by TESE.
The possible impact of maternal or paternal X-chromosomal origin in KS was previously investigated in a prospective study including 14 nonmosaic boys with KS 10-14 years old by Wikström, Painter, Raivio, Aittomäki, and Dunkel (2006), who suggested a later onset of puberty in KS with a paternally inherited supernumerary X-chromosome. A study by Iitsuka et al. (2001) investigated the inheritance pattern of the extra X-chromosome in 17 men with KS and found a maternal origin in 10 of 17 (59%) participants and a paternal origin in seven of 17 (41%) participants. Furthermore, they found skewed X-chromosomal inactivation (same X-chromosome inactivated in >80% of cells) in three of 14 participants (21%) and a varying degree of X-chromosomal inactivation pattern in the other patients (ranging from 30% to 69%). Unfortunately, there was no further correlation with the phenotype or the presence of spermatogenesis of the participants in this study (Iitsuka et al., 2001). Another study by Stemkens et al. (2006) also investigated X-chromosomal origin and found a paternal origin in 26/61 (43%) of cases and found a maternal origin in 35/61 (57%) cases. They reported a higher incidence of speech and language problems and motor impairment of men with KS who had a paternally inherited extra X-chromosome (Stemkens et al., 2006). It has been shown that 20% of the genes on the second X-chromosome are randomly inactivated; another 15% escape inactivation and 65% are always inactivated (Carrell & Willard, 2005). The presence or absence of spermatogenesis might depend on a specific maternal or paternal expression or inactivation for each of the genes being expressed in the testis. However, to further investigate this hypothesis extensive genetic studies with sufficient participants would be necessary to detect a specific expression pattern within the cluster of these many genes. Nevertheless, inactivation of some key genes on either the maternal or paternal X-chromosome could be responsible for the presence of spermatogenesis, as about 10% of the genes on the X-chromosome are expressed in the testis (Ross et al., 2005). It has been shown that 20% of the genes on the second X-chromosome are randomly inactivated; another 15% escape inactivation and 65% are always inactivated (Carrell & Willard, 2005). The presence or absence of spermatogenesis might depend on a specific maternal or paternal expression or inactivation for each of the genes being expressed in the testis. However, to further investigate this hypothesis extensive genetic studies with sufficient participants would be necessary to detect a specific expression pattern within the cluster of these many genes.

The results of our analysis on spermatogenesis show that for some KS patients, immature germ cells can be found by histology or cytology, while the TESE was negative for spermatooza. Our finding is supported by several earlier studies, also reporting the presence of spermatogenesis and a successful repeated TESE in

| Participant | Extra X-chromosomal origin | TESEa (wet-prep) | Cytologyb | Histology |
|-------------|----------------------------|------------------|-----------|-----------|
| P2          | Paternal                   | Positive         | Hypo-sperm. | Abnormal testis parenchyma, but some tubuli seminiferi with low levels of spermatogenesis. |
| P10         | Paternal                   | Negative         | SCO       | Leydig cell hyperplasia and tubular hyalination, no spermatogenesis. |
| P15         | Maternal                   | Negative         | Not performed | Leydig cell hyperplasia, tubular hyalination and Sertoli cell only, no spermatogenesis. |
| P17         | Paternal                   | Positive         | SCO       | Leydig cell hyperplasia and tubular hyalination, but some tubuli with spermatogenesis (<10%) |
| P21         | Maternal                   | Negative         | SCO       | Leydig cell hyperplasia and tubular hyalination, no spermatogenesis. |
| P23         | Paternal                   | Negative         | MA        | Leydig cell hyperplasia, tubular hyalination and Sertoli cell only, no spermatogenesis. |
| P24         | Paternal                   | Negative         | SCO       | Leydig cell hyperplasia and tubular hyalination, but some tubuli with spermatogenesis (<5%) |
| P28         | Maternal                   | Negative         | SCO       | Leydig cell hyperplasia and tubular hyalination, no spermatogenesis. |
| P35         | Maternal                   | Positive         | Not performed | No histology done, not enough material. |

SCO, Sertoli cells only; MA, maturation arrest (presence of pachytene spermatocytes); hypo-sperm, hypospermatogenesis (presence of mature spermatooza next to pachytene spermatocytes).
aPositive sperm retrieval by TESE.
bCytology performed based on ratio of number of Sertoli cells/pachytene spermatocyte/spermatozoa. The outcome is given as: Sertoli cell only (SCO)/maturation arrest (MA) or hypospermatogenesis (Hypo-sperm).
men with KS who had a negative TESE at first (Haliloglu, Tangal, Gulpinar, Onal, & Pabuccu, 2014; Van Saen, Tournaye, & Goossens, 2012). This apparent discrepancy supports the hypothesis that there are small patches of functioning germ cells and support cells present in men with KS. Despite degeneration and hyalinisation of the testicular environment, there still seem to be reserves of functional germ cells present in some men with KS (Maiburg, Repping, & Giltay, 2012). Neither hormone levels nor age appeared to be a significant prognostic marker for spermatozoa retrieval or the presence of spermatogenesis in our study population.

5 | CONCLUSION

The maternal or paternal origin of the second X-chromosome in men with KS does not predict the presence of spermatogenesis.

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CONFLICT OF INTERESTS

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