Hidden mosaicism in Klinefelter's syndrome patients: implications for genetic reproductive counselling

Running title: Mosaicism in Klinefelter syndrome

L. Garcia-Quevedo[1], J. Blanco[1], Z. Sarrate[1], V. Català[2], L. Bassas[3] and F. Vidal[1,*]

[1]Unitat de Biologia Cel·lular. Universitat Autònoma de Barcelona. 08193 Cerdanyola del Vallès (Barcelona) SPAIN

[2]Prenatal Genetics, SL. 08036 Barcelona. SPAIN

[3]Laboratori de Seminologia i Embriologia, Servei d'Andrologia, Fundació Puigvert. 08025 Barcelona. SPAIN

*** Corresponding author:** francesca.vidal@uab.cat.

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Abstract

BACKGROUND: Klinefelter syndrome (KS) is the most common chromosome abnormality, affecting 9%-11% of azoospermic patients. Genetic diagnosis is usually ascertained by lymphocyte karyotyping, but the incorporation of complementary cytogenetic studies for better accuracy in the detection of mosaicism is under discussion. Although most KS individuals are azoospermic, residual foci of spermatogenesis have been observed in some patients, and they are frequent candidates for testicular sperm extraction (TESE). However, no consistent predictive factors for success have been established and mosaicism degree could be a factor to be investigated. Furthermore, the controversy over the safety of KS individuals using their own gametes for assisted reproduction is still an open question. The aims of the present study were to assess the degree of mosaicism in somatic and germinal tissues in KS patients previously diagnosed as being non-mosaic. The meiotic competence of the 47,XXY germ cells and the aneuploidy rate of post-reductional germ cells have also been analysed. **METHODS:** Five azoospermic KS patients previously diagnosed as pure 47,XXY have been studied. The chromosome constitution of three tissues: blood lymphocytes, buccal mucosa and testicular tissue, was assessed by interphase FISH for chromosomes X, Y and 18. In the meiotic figures, sex-chromosome number and pairing was confirmed and subsequently a sequential protocol using whole-chromosome painting probes for chromosomes X and Y was applied. **RESULTS:** The presence of mosaicism, with 46,XY cell lines, was evident in all patients and tissues analysed. The degree differed among the tissues, the lowest being in lymphocytes (4.8% ± 2.5%) and the highest in Sertoli cells (37.9% ± 11.1%). Meiotic figures were found in three cases (KS1, KS2, KS5), all of the figures showed an XY complement, and cells with the XXY complement were not observed in any of the cases. Hyperhaploid post-meiotic cells have been observed in all patients (range: 0.7% - 37.2%). **CONCLUSIONS:** Homogeneous diagnosis in KS should be contrasted in other tissues. Mucosa cells could help to better approximate the degree of germ cell mosaicism, however, further studies would be of interest to evaluate the predictable value of the degree of

mosaicism for successful sperm retrieval. Our results support the hypothesis that 47,XXY germ cells are not meiotically competent. The increased post-reductional aneuploidy rate in KS is related to meiotic errors in 46,XY cell-lines, thus KS individuals should be considered as being patients with a reproductive risk, and appropriate genetic counselling is recommended.

Key words: aneuploidy, cytogenetics, Klinefelter syndrome, meiosis, mosaicism.

Introduction

Klinefelter syndrome (KS) is the most common chromosome abnormality, with an incidence of 3% in infertile males, and it represents 9%-11% of azoospermic patients (De Braekeleer and Dao, 1991; Mau-Holzmann, 2005). Genetic diagnosis is usually ascertained by cytogenetic studies from blood lymphocytes through the evaluation of 20-50 metaphases (Hook, 1977). More than two-thirds of all cases are reported as homogeneous (47,XXY), and the remaining as mosaic (46,XY/47,XXY) or higher-grade chromosome aneuploidies (reviewed by Lanfranco et al., 2004). Because mosaicism can be the source of the well-known clinical variations reported in KS individuals, and as karyotype analysis can overlook low mosaicism degree, several authors recommend incorporating interphase FISH analysis as a complement to conventional cytogenetics for a more specific and sensitive detection of mosaicism (Okada et al., 2001; Abdelmoula et al., 2004; Lenz et al., 2005). Moreover, the study of mucosa cells has also been proposed as a quick and reliable screening test for a better ascertainment of the chromosome constitution of these patients (Kruse et al., 1998; Westlander et al., 2001).

Klinefelter individuals are traditionally described as being infertile, however, residual foci of spermatogenesis have been observed in both non-mosaic and mosaic individuals (reviewed by Lanfranco et al., 2004). The reported presence of spermatozoa in testicular tissue of KS patients has lead to offering testicular sperm extraction (TESE), coupled with ICSI, to give to KS patients the opportunity to father children (Tournaye et al., 1996; reviewed by Ramasamy et al., 2009). Successful spermatozoa recovery in adult KS men ranges from 16% to 60% (reviewed by Fullerton et al., 2010). Patient age seems to correlate with the presence of spermatozoa (Ferhi et al., 2009; Ramasamy et al., 2009), however, no other consistent predictive factors of successful testicular sperm recovery have been reported (Radicioni et al., 2010).

Concerns and controversy over the safety of KS individuals using their own gametes for assisted reproduction have been raised, and several studies have focused on the analysis of the chromosome constitution of these patients' germ cells (reviewed by Templado et al., 2011). All

reports published to-date are coincident in describing increases in sex chromosome abnormalities and hyperhaploidies in the post-reductional germ cells from 47,XXY and 46,XY/47,XXY individuals (Estop et al., 1998; Foresta et al., 1998; Kruse et al., 1998; Foresta et al., 1999; Bielanska et al., 2000; Levron et al., 2000; Rives et al., 2000; Blanco et al., 2001; Hennebicq et al., 2001; Bergere et al., 2002; Yamamoto et al., 2002). The origin of these abnormalities has been related to the possible meiotic progress of the 47,XXY germ lines (Skakkebaek et al., 1969). This hypothesis has been supported by some authors (Foresta et al., 1998; Ferlin et al., 2005) based upon the deviation in the X/Y sperm ratio (in favour of X-bearing spermatozoa) and the presence of equivalent percentages of XX and XY sperm hyperhaploidies. However, most studies do not agree with these results, but rather sustain that in KS individuals the only cells that progress through meiosis are 46,XY cells (Blanco et al., 2001; Bergere et al., 2002; Sciurano et al., 2009), thus indicating that patients with spermatogenic patches are individuals whose testicular tissue is mosaic. Thus, the cytogenetic abnormalities observed in post-reductional germ cells must result from the abnormal meiotic progression of 46,XY spermatocytes in the compromised testicular environment, which is distinctive in KS patients (Mroz et al., 1999).

The alteration of the testicular environment has been related to abnormal hormonal levels and to the dysfunction of testicular somatic cells (reviewed by Radicioni et al., 2010). It has been suggested that the nursing function of XXY Sertoli cells may not be as effective as those of XY cells (Wikstrom et al., 2007; Sciurano et al., 2009). Thus, the mosaicism level in testicular tissue, not only concerning germ cells but also for somatic cells, could be of relevance for the final outcome of spermatogenesis in KS and could have practical implications for the clinical management of these individuals.

The aims of the present study were: a) to assess the occurrence of mosaicism in different tissues and cell-types (somatic and germinal) in KS individuals previously diagnosed as being pure 47,XXY; b) to evaluate if any predictive value for successful testicular sperm retrieval can be

1 inferred from mosaicism data; c) to analyse the meiotic competence of 47,XXY spermatocytes,
2 and d) to evaluate the genetic reproductive risk in this patients.
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Materials and methods

Study populations

Five azoospermic individuals (KS1-KS5) aged between 29 and 35, diagnosed as homogeneous 47,XXY by standard cytogenetic procedures (analysis of 20 G-banded metaphases from a peripheral blood lymphocyte culture) were the subjects of our study. Hormonal values were: 13 IU/L to 47.7 IU/L for plasma FSH levels, 6.7 IU/L to 32.8 IU/L for plasma LH levels and 4.5 nmol/L to 16.2 nmol/L for Testosterone. All patients had small testis volume (5 ml to 10 ml) and were enrolled as candidates for TESE. For every patient a new sample of peripheral blood, a buccal swab and a sample of the testicular tissue obtained for sperm retrieval were analyzed. Four cell-types have been assessed: lymphocytes from peripheral blood, epithelial cells from buccal mucosa and Sertoli and germ cells (pre- and post-reductional cells and meiotic figures) obtained from testicular biopsies.

Four testicular samples from adult organ donors aged between 20 and 33 have been used as the control population. Furthermore, control samples from buccal mucosa and lymphocytes were obtained from four healthy men (mean age 31 ± 9.5).

Informed consents were obtained and protocols were approved by the ethics committees of the centres involved.

Lymphocyte and buccal mucosa cells

Heparinised peripheral blood from patients KS1 to KS4 was obtained and cultured in 4% phytohaemagglutinin-supplemented medium (PHA M; GibCo, Invitrogen; Paisley, UK) at 37°C for up to 72h. Cytogenetic preparations were obtained following standard procedures after 25 min Colcemid incubation (0.16µg/ml; GibCo, Invitrogen), hypotonic treatment (KCl 0.075M) for 20 min at 37°C and Carnoy's fixation.

Oral smears were obtained by scraping the inner cheek and were processed for cytogenetic analysis. Buccal mucosa cells were incubated with hypotonic solution (KCl 0.035M) for 30 min at 37°C. Cells were washed twice with Carnoy's fixative solution before spreading. Slides were treated with acetic acid solution (50% in H_2O) for at least 30 min at 40°C to permeabilise the cells prior to FISH processing.

A triple-colour Fluorescent In Situ Hybridisation (FISH) was performed in both lymphocytes and buccal mucosa cell with centromeric DNA probes for chromosomes X, Y and 18 (CEP Y, Spectrum Orange; CEP X, Spectrum Green; CEP 18, Spectrum Aqua) as described by the manufacturer (Aneuvyison Assay Kit, Abbott Molecular; Abbot Park, IL, USA).

Control samples were processed following the same protocols described for KS individuals.

Testicular tissue

Testicular biopsies were obtained under local anaesthesia with the aim of freezing tissue for further ICSI. All testicular samples were sent to the laboratory at 4°C in isotonic solution and were processed for cytogenetic studies with the conventional method of Evans et al. (1964).

Previous to FISH, samples were stained with Leishman (20%) for 8–10 min. Prophases I, metaphases I, and metaphases II figures were captured and coordinates were recorded to facilitate location and analysis after FISH. Preparations were de-stained in an ethanol series (70%, 80%, 90%) for one minute each before FISH. The chromosome constitution of Sertoli cells, pre- and post-reductional germ cells, and meiotic figures were analysed for chromosomes X, Y and 18 (Aneuvyison Assay Kit, Abbott Molecular).

A sequential FISH with Whole-Chromosome Painting probes (WCP) for the sex chromosomes (X-XCP 23-FITC, Y-XCP 24-TexasRed; MethaSystems GmbH; Altlussheim, Germany) was performed to evaluate the sex chromosome pairing at pachytenes and metaphases I, and to confirm the sex chromosome constitution of metaphases II.

Control testicular samples were processed following the same protocols described above.

Microscope analysis and evaluation criteria

All evaluations were carried out using an Olympus BX-60 fluorescent microscope (Olympus Barcelona; Spain) equipped with specific filters for FITC, Cy3, Aqua and a multiband pass filter (DAPI/FITC/Texas Red).

A minimum of 500 lymphocytes (except for KS5), 200 buccal mucosa cells and 1000 interphase nuclei (germ cells and Sertoli cells), and all of the meiotic figures observed were analysed for each individual.

FISH analysis in interphase cells was performed in accordance to the criteria described by Blanco et al. (2001). Briefly, germ cells were classified according to the number of sex chromosomes hybridisation signals, and chromosome 18 was used as ploidy control. Strict scoring criteria for signal evaluation were used: signals must be of the same size and intensity, and the distance must be at least the same as the diameter of the signal. Overlapped nuclei were discarded from the analysis.

Sertoli cells exhibit a characteristic morphology, clearly distinct from interphase germ cells, showing a large fusiform nucleus with a prominent nucleolus and diffuse chromatin.

Meiotic figures were classified taking into account the distribution of hybridization signals from the two rounds of FISH. In pachytene cells, the presence of sex chromosomes-specific centromeric signals plus one single WCP domain for the X and Y chromosomes (configurating the sex vesicle), and a single centromeric signal for chromosome 18 (assuming pairing) was considered as being normal in 46,XY cells.

A cut-off-level for false negative-FISH results was established by the analysis of 45,X and 45,Y interphase nuclei in testicular cells (Sertoli cells and pre-meiotic cells), buccal mucosa cells and lymphocytes from control samples.

Statistical analysis

The X-bearing/Y-bearing ratio was performed with McNeman test (SPSS 15.0.1 for windows). The rest of comparisons were performed with Fisher's exact test (GraphPad InStat, version 3.05, 32bit for Windows 95/NT).

Results

Successful FISH results were obtained in the three tissues analysed (Fig. 1). Control preparations were used to establish de cut-off-levels for possible FISH-false negatives in the different cell types analyzed. Values for 1952 lymphocyte nuclei, 663 buccal mucosa cells, 620 Sertoli cells and 1113 pre-reductional cells were recorded. The mean (±SD) percentages of 45,X and 45,Y nuclei in the different cell types analyzed were: 0.5% (±0.9%) for lymphocytes, 0% for buccal mucosa and Sertoli cells and 0.8% (±0.5%) for pre-reductional germ cells. The results obtained were in accordance with the sensitivity and specificity expected for the DNA probes used and are consistent with the figures obtained in the standard internal controls for FISH studies currently performed in our laboratory.

Cells with the XY complement (Fig. 1, Fig. 2) were observed in all the five KS individuals at different percentages, among the tissues analysed (Fig. 3,Table I). Nuclei corresponding to Sertoli cells were clearly identified in the testicular cell suspension. (Fig. 2). The lowest percentage of 46,XY cells corresponds to the lymphocytes (4.8% ± 2.5%), while the highest corresponds to the Sertoli cells (37.9% ± 11.1%)(Table I) except KS3, who has a lower percentage of Sertoli XY cells than mucosa and post-meiotic germ cells (Fig. 3). Furthermore, the cytogenetic analysis of mucosa cells shows that the percentages observed in this tissue (22.2% ± 10.9%) are closer to the degree of mosaicism in testicular tissue in the five patients.

Table II summarize the number of mitotic and meiotic figures analysed. Eight spermatogonial metaphases corresponding to the 46,XY line were found. Meiotic figures were seen in three out of the five cases. All 758 prophases I observed had the XY complement (Table II). Concerning the pachytene cells, in all cases chromosomes X and Y were seen forming the sex vesicle (Fig. 4). Nine metaphases I, having the XY complement, and eleven metaphases II, with either the X chromosome or Y chromosome, were observed.

Post-reductional germ cells were observed in all patients (Table III). The ratio between X-bearing/Y-bearing post-reductional cells was equivalent to 1:1 for all patients. Cases K1-K4

showed a significant increase of aneuploidy rates (24,XY), with respect to controls (Table III). Increased percentages of 25,XXY cells were observed in four out of the five patients (KS2: 0.7%; KS3: 8.2%; KS4: 20.9%; KS5: 3.9%). Spermatozoa were not found in the samples studied.

Discussion

Karyotyping from lymphocyte culture of peripheral blood is the standard diagnostic technique used to determine chromosomal anomalies. However, there is a high heterogeneity in the methods to search, establish and confirm mosaicism. Although the study of 50 metaphases is recommended to exclude less than 10% of mosaicism with a 0.99 confidence level (Hook, 1977) most routine karyotyping is based on the study of 20 metaphases. The patients analysed in our study would be a clear example of undetected mosaicism, since all have previously been diagnosed as pure 47,XXY. But, upon amplifying the study by interphase-FISH, the presence of XY lymphocytes was seen with variable percentages (2.7% to 8%). These figures are clearly over the cut-off-level for FISH-false negatives established in our laboratory for control individuals (0.5% ± 0.9%). Thus, for the diagnosis of "pure KS", it is advisable to increase the number of cells analysed, either by karyotyping or by FISH in interphase nuclei.

The observation of different percentages of 46,XY cells in the analysed tissues indicates that the degree of mosaicism is not uniform. Our data demonstrate that even in cases of a low percentage of mosaicism in peripheral blood (<10%), the patients present degrees of germinal mosaicism superior to 35% (Table I). Our results confirm that the analysis of lymphocytes is not a good indicator of the testicular status, and are in good agreement with what other authors suggest (Bielanska et al., 2000; Lanfranco et al., 2004). It has been described that the analysis of buccal mucosa is a rapid method to confirm and increase the accuracy of the diagnosis of Klinefelter patients (Kamischke et al., 2003). In our study the percentages of observed buccal mucosa 46,XY cells (22.2% ± 10.9%) are closer to the mosaicism observed in the germinal line (34.6% ± 8.7%) although its predictive value of successful sperm recovery has not been able to be confirmed due to the fact that spermatozoa were not observed in any of the five patients.

Although the clinical significance of the mosaicism level in KS remains to be elucidated, the knowledge of the degree of mosaicism can help to understand the reason for the variable response that some KS patients show when faced with different therapies (treatments with

Aromatase inhibitors, hCG or Clomiphene). It has been described that 77% of the patients who, after treatment show concentrations equal or superior to 250ng/dl of Testosterone in blood, have successful sperm retrieval (Ramasamy et al., 2009). It could be hypothesised that the Sertoli 46,XY cells would be those which would respond to the Testosterone levels, recovering the functions of nursing. In fact, in our series, the two patients observed who do not show meiotic phases are those who have the lowest percentages of Sertoli 46,XY cells. Furthermore, patient KS5 with a high percentage of euploid Sertoli cells (46.5%), and despite having a low percentage of pre-meiotic XY cells (28.2%), has meiotic phases. Thus, all together suggests that the progression of spermatogenesis would depend on the percentage of Sertoli XY cells which colonise the seminiferous tubules. However, further studies would be of interest to evaluate the value of mosaicism to envisage successful sperm retrieval when pharmacologic therapeutic treatments are used.

The origin of mosaicism in germinal cells has been attributed to the occurrence of "correcting mitotic errors" associated with the mitotic proliferation of the primordial germinal cells in the foetal testicle (Levron et al., 2000) and also of the spermatogonia in adult tissue (Sciurano et al., 2009) giving rise to isolated zones of euploid cells. These corrective processes would also have occurred to the rest of cell types causing different degrees of mosaicism in each one. On the other hand, it has been described that the lack of inactivation of genes of the X supernumerary affects the germinal cells as well as somatic ones at different levels (Aksglaede et al., 2006). In the mouse model, Sertoli XXY cells show low levels of expression of Androgen Receptor (AR) (Lue et al., 2005). In human males it has been described a delay in disappearance of Anti-Müllerian Hormone (AMH) expression jointly with the upregulation of AR, and it is known that both are required for the last step of Sertoli maturation during puberty (Wikstrom et al., 2007). This, together with an altered activation pattern of apoptosis due to the hypergonadotrophic hypogonadism (Aksglaede et al., 2006), suggests that the degeneration of the Sertoli cells would preferentially affect the XXY cells, raising the relative presence of the XY cells. Thus, the

maintenance of the mosaicism or degeneration of the aneuploid line would be determined by the presence of two functional X chromosomes.

Mosaicism has been observed at the testicular level in all of the patients studied (mean of 34.6% ± 8.7%). All prophases and metaphases I (Table II) show the XY complement, even in the patient where the aneuploid line represents almost 55% of the cells (Table I). These results coincide with those previously reported by our group (Blanco et al., 2001) and with recent studies (reviewed by Tuttelmann and Gromoll, 2010) and confirm the meiotic incompetence of the 47,XXY spermatocytes. Furthermore, neither the deviation from the 1:1 ratio nor the equivalent proportions of hyperhaploidies (XX and XY) in post-meiotic cells described by other authors (Foresta et al., 1999; Yamamoto et al., 2002; Ferlin et al., 2005) have been observed in our study. What is more, the few spermatogonia metaphases observed were also 46,XY, indicating that this line would be more proliferative than that the 47,XXY germ line.

The meiotic incompetence of the 47,XXY line has been related, as in the mouse model (Hunt et al., 1998), to the presence of the second X chromosome and the insufficient inactivation of the genes associated with the extra chromosome, which could cause the degeneration of the 47,XXY germinal cells (Aksglaede et al., 2006). On the other hand, given that it has been described that anomalies in the sex vesicle formation lead to apoptotic processes (Burgoyne et al., 2009) it can be inferred that the anomalies of synapsis of the XXY complement lead to the cell degeneration of any 47,XXY spermatocytes. Thus, the results of our study are in agreement with the descriptions made in the mouse model (Hunt et al., 1998) and support the hypothesis that the spermatogenic patches are populated by 46,XY cells.

The occasional presence of spermatozoa in testicular tissue has been described in certain KS individuals (Foresta et al., 1999; Rives et al., 2000; Blanco et al., 2001; Bergere et al., 2002; Lanfranco et al., 2004; Sciurano et al., 2009; Fullerton et al., 2010) and confirms that the process of spermatogenesis can be accomplished in some of the spermatogenic patches.

Our findings, where a significant incidence of aneuploidies in post-meiotic cells was observed, clearly give support to the hypothesis that an altered testicular environment compromises the meiotic progression of the 46,XY cells (Mroz et al., 1999). Furthermore, the evidence from this study and others (reviewed by Templado et al., 2011) of increased chromosomal abnormalities, in mosaic KS patients as well as non-mosaic ones, deserve to be taken into account when considering offering assisted reproduction techniques to these individuals. The offer of TESE and ICSI in KS men has resulted in the reported births of more than one hundred children (Fullerton et al., 2010) and has made to put a "scientific question mark" on the infertile definition classically attributed to KS, nevertheless the label of genetic risk patients still merits being maintained and highlighted.

In conclusion: Homogeneous KS diagnosis should be contrasted in other tissues. FISH analysis on mucosa cells could help to ascertain the degree of germ cell mosaicism, however, further studies would be of interest to evaluate the predictable value of the degree of mosaicism in successful sperm retrieval. Spermatogenesis foci are populated by 46,XY cells, and our results support the hypothesis that 47,XXY germ cells are not meiotically competent. The post-reductional aneuploidy rate in KS is higher than in controls and is related to meiotic errors in 46,XY cell-lines. KS individuals should be considered as being patients with a reproductive risk, and appropriate genetic counselling is recommended.

Author's roles

L.GQ. was involved in experimental procedures, data collection and assembly, data analysis and interpretation, manuscript writing and final approval. J.B. was involved in study conception and design, data analysis and interpretation, manuscript writing and final approval. Z.S. was involved in data analysis and interpretation, and final approval of the manuscript. V.C. was involved in data collection and final approval of the manuscript. L.B. was the consultant Andrologist, contributed in clinical assessment, tissue sampling, data collection and final approval of the manuscript. F.V. was involved in study conception and design, data collection and assembly, data analysis and interpretation, manuscript writing and final approval.

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Tables

Table I: Percentage of 46,XY cells in the tissues analyzed.

Cases	Age	Blood	Buccal mucosa	Testis	
				Pre-reductional cells	Sertoli cells
KS1	31	14/527 (2.7%)[a]	37/203 (18.2%)[a]	95/210 (45.2%)[a]	5/11 (45.5%)[a]
KS2	29	16/530 (3%)[ab]	44/132 (33.3%)[b]	355/836 (42.5%)[a]	74/137 (54%)[a]
KS3	35	41/510 (8%)[c]	97/292 (33.2%)[b]	287/714 (40.2%)[a]	58/239 (24.3%)[b]
KS4	31	28/509 (5.5%)[bc]	31/192 (16.1%)[a]	225/866 (26%)[b]	24/58 (41.4%)[a]
KS5	34	-	18/206 (8.7%)[c]	235/834 (28.2%)[b]	40/86 (46.5%)[a]
Mean ± SD	**32 ± 2.4**	**4.8% ± 2.5%**	**22.2% ± 10.9%**	**34.6% ± 8.7%**	**37.9% ± 11.1%**

[a,b,c]; different superscripts indicate significant differences in columns ($p > 0.05$).

Table II: Mitotic and meiotic figures analyzed

	Spermatogonial metaphases	Prophases			Metaphase I	Metaphase II	Total
		Leptotene	Zygotene	Pachytene			
KS1	-	48	32	27	-	-	107
KS2	5	60	188	166	7	10	436
KS5	3	29	122	86	2	1	243
Total	8	137	342	279	9	11	786

Table III: Chromosome constitution of post-reductional cells

	23,X	23,Y	24,XY	25,XXY	Others
KS1	6/14 (41.8%)	4/14 (27.9%)	4/14 (30.3%)*	-	-
KS2	14/34 (41.1%)	9/34 (26.4%)	7/34 (20%)*	1/34 (0.7%)*	4/34 (11.7%)
KS3	12/38 (31.6%)	12/38 (31.6%)	11/38 (28.6%)*	3/38 (8.2%) *	-
KS4	9/33 (27%)	5/33 (15%)	12/33 (37.2%)*	7/33 (20.9%) *	-
KS5	27/61 (44.2%)	27/61 (44.2%)	5/61 (7.6%)	2/61 (3.9%) *	-
Control mean	667/1454 (45.9%)	687/1454 (47.3%)	87/1454 (6%)	1/1454 (0.1%)	12/1454 (0.8%)

*; indicate significant differences versus controls.

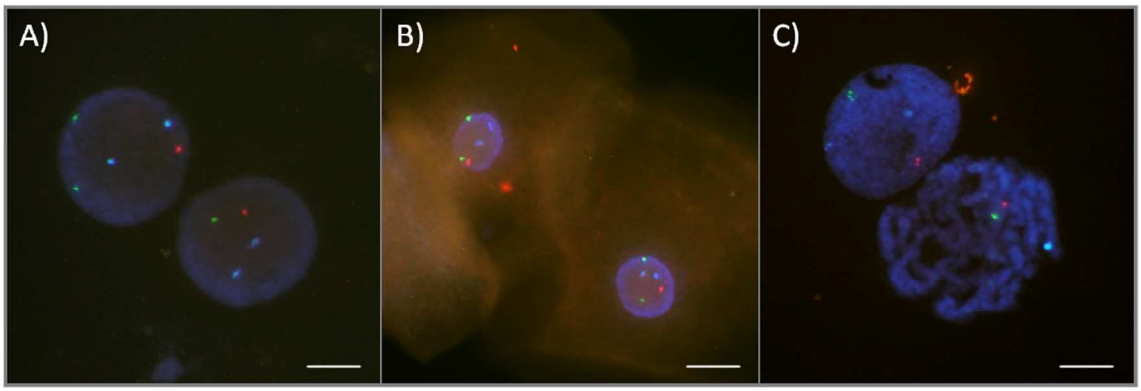
Figure legends

Figure 1: Triple-colour FISH with centromeric probes for chromosome X (green), Y (red) and 18 (blue). A) Peripheral blood lymphocytes; 46,XY cell on the right and a 47,XXY cell on the left. B) Buccal mucosa cells, both showing an XXY complement. C) Testicular tissue cells. On the left, a 46,XY Sertoli cell nucleus, and on the right, a pachytene nucleus with a single 18-chromosome signal (homologue chromosomes paired) and the sex vesicle showing a single signal for both X and Y chromosomes.

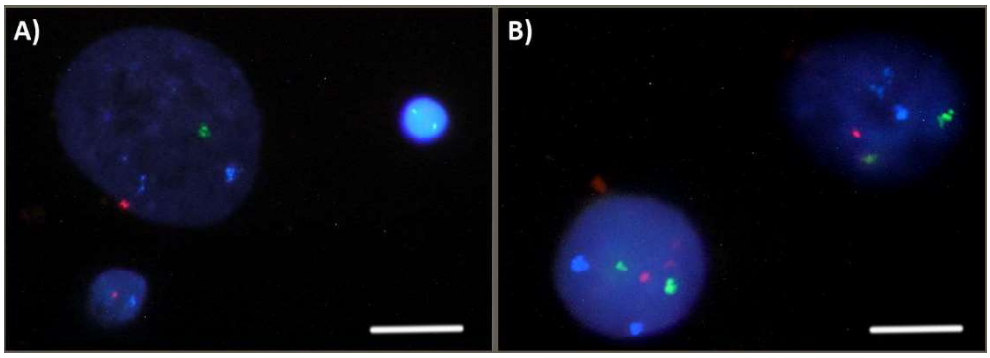
Figure 2: Triple colour-FISH images of Sertoli cells nuclei and germ cells nuclei. A) Top left: Sertoli cell with an XY complement; bottom left: Y-bearing secondary spermatocyte; top right: X-bearing round spermatid. B) Bottom left: XXY pre-meiotic germ cell; top right: XXY Sertoli cell

Figure 3: Percentage of 46,XY cells.

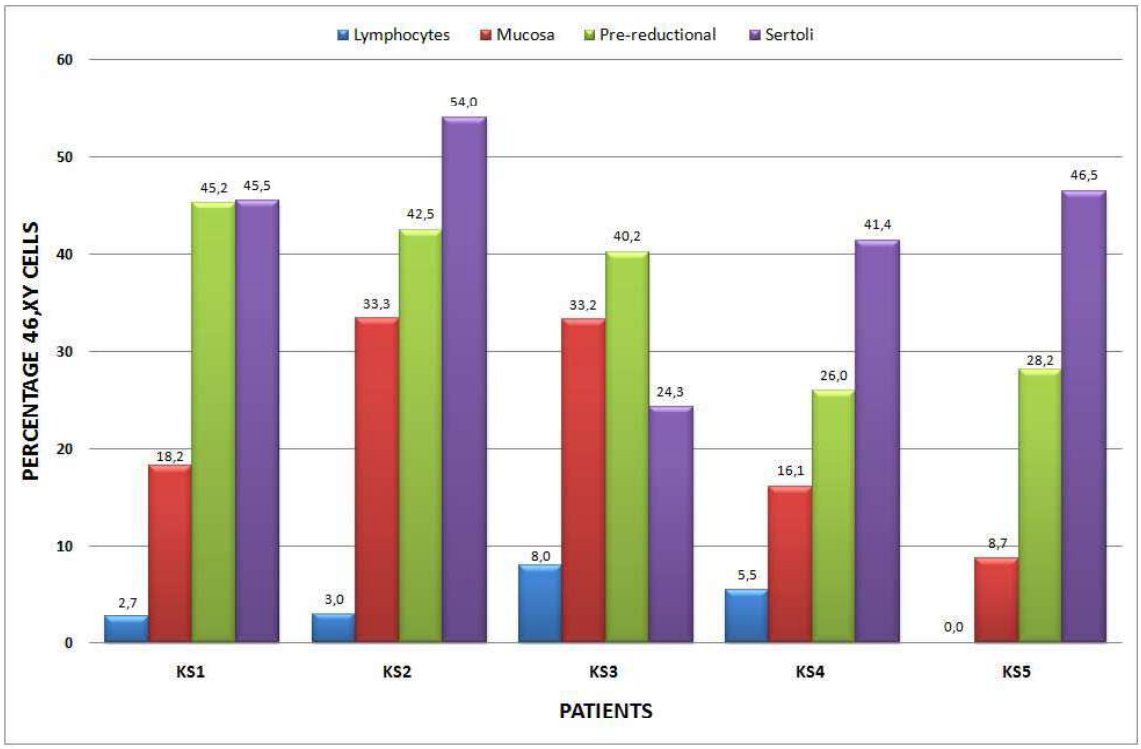
Figure 4: Sequential cytogenetic analysis in pachytene-stage spermatocyte. **A)** Leishman stain. Arrows point to the sex vesicle. **B)** Triple-colour FISH with centromeric DNA probes for chromosomes X (green), Y (red) and 18 (blue). A single signal for each sex chromosome (XY) is identified inside the sex vesicle. **C)** Whole-Chromosome Painting (WCP) for the sex chromosomes (X-green and Y-red) confirming the presence of a single X chromosome plus a Y chromosome forming the sex vesicle.


A)
B)
C)

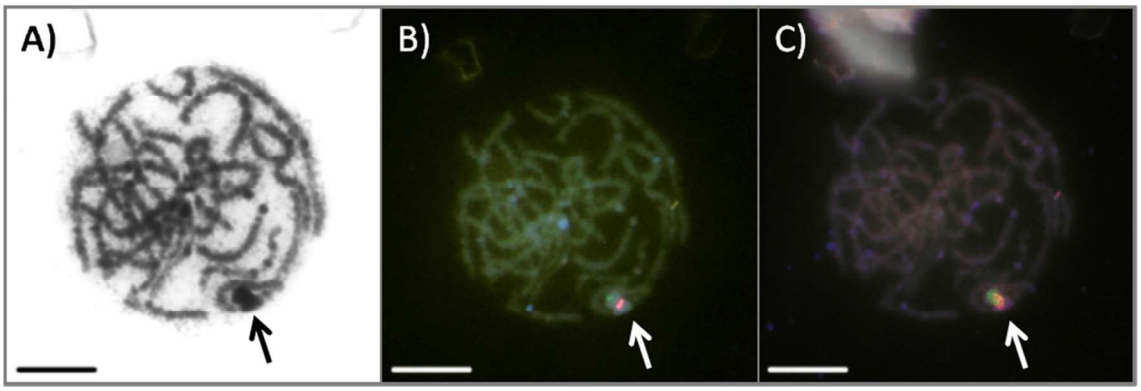
1


A)
B)

2


Lymphocytes
Mucosa
Pre-reductional
Sertoli
PERCENTAGE 46,XY CELLS
60
50
40
30
20
10
0
2,7
18,2
45,2
45,5
3,0
33,3
42,5
54,0
8,0
33,2
40,2
24,3
5,5
16,1
26,0
41,4
0,0
8,7
28,2
46,5
KS1
KS2
KS3
KS4
KS5
PATIENTS

3


A)
B)
C)