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CASE REPORT *Reproductive genetics*

Selective advantage of euploid spermatocytes I in an azoospermic 47,XYY man with gonadal mosaicism

R.B. Sciurano[1,2,3,*], I. M. Rahn[1,2], B. González Arias[1], G. Rey Valzacchi[4], R. Benavente[3], and A.J. Solari[1,2]

[1]2nd. A.U. Cell Biology, Histology, Embryology and Genetics, School of Medicine, University of Buenos Aires [2]CONICET, Procrearte/Servicio de Urología, Hospital Italiano [3]Department of Cell and Developmental Biology, Biocenter, University of Würzburg, Germany [4]Procrearte/Hosp. Italiano, Buenos Aires, Argentina

*Correspondence address. 2nd. A.U. Cell Biology, Histology, Embryology and Genetics, School of Medicine, University of Buenos Aires. Paraguay 2155, Piso 10. (C1121ABG) Ciudad Autónoma de Buenos Aires, Argentina. Tel.: +541149618703. E-mail: roberta_sciur@hotmail.com

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ABSTRACT: Although most XYY men have normal sperm counts and are fertile (supposedly due to the loss of the extra Y before meiosis), there is a minority who are infertile. In these cases, the XYY spermatocytes are able to enter meiosis and form different synaptic configurations. With regard to mosaics, there is scarce well-defined information on the presence of the second Y and its meiotic behaviour. In this study, the chromosome constitution and the synaptic behaviour of pachytene spermatocytes from an azoospermic man with testicular hypotrophy and non-mosaic 47,XYY karyotype were analysed. Furthermore, we determined the chromosome constitution of the somatic Sertoli cells. Five karyotypically normal men with obstructive azoospermia, but having complete spermatogenesis, were included as controls. Immuno-FISH using specific protein markers of synapsis and recombination (SYCP3, SYCP1, BRCA1, MLH1, CREST) and a specific Yq12 DNA probe were used. In addition, we used the newly developed Super-Resolution Structured Illumination Microscopy (SR-SIM) to clearly define the synaptic configurations. FISH analysis was also performed on Sertoli cells. The histopathological analysis showed variable degrees of spermatogenesis development in the testicular tissue of the propositus. Immuno-FISH analysis showed that most of the primary spermatocytes were euploid 46, XY. The use of SR-SIM confirmed the existence of this euploidy. Only a few pachytene spermatocytes showed an aneuploid X + YY constitution. Sertoli cells showed two different populations with one or two Y chromosomes, in similar proportions. Thus an abnormal niche of sex-trisomic Sertoli cells should be also considered when searching for the origin of spermatogenesis failure in XYY men.

Key words: XYY syndrome / human infertility / pachytene spermatocytes / immuno-FISH / structured illumination microscopy

Introduction

Among live male births, the 47,XYY syndrome and the 47,XXY (or Klinefelter) syndrome are the most common chromosome aneuploidies (Ross *et al.*, 2009). Although most XYY men have normal sperm counts and are fertile (likely due to the loss of the extra Y before meiosis), there is a minority of XYY men who are infertile and show severe oligospermic or azoospermic phenotypes. Previous studies on spreads of synaptonemal complexes (SCs) by electron microscopy have shown that the Y bivalent plus an X univalent (YY + X) is the most frequent synaptic configuration in pachytene nuclei of non-mosaic 47,XYY men (Speed *et al.*, 1991; Solari and Rey Valzacchi, 1997). The remaining primary spermatocytes could form an XYY trivalent, three different sex chromosome univalents (X + Y + Y) or an XY pair plus a Y univalent (XY + Y). The persistence of the extra chromosome has been suggested to be detrimental to the normal progression of meiosis (Rives *et al.*, 2005). Only the trivalent configuration with their intact recombination regions might bypass the pachytene checkpoint, as suggested for XYY mice by Rodriguez and Burgoyne (2000). It has been postulated that the mechanism by which the meiotic (or premeiotic) cells could reach an euploid condition might be due to the random loss of the additional Y from a primitive germ cell or a spermatogonium, followed by a proliferative advantage of 46,XY germ cells over the 47,XYY germ cells (Evans *et al.*, 1970).

Scarce well-defined information has been reported regarding the presence of the extra Y and its meiotic behaviour in mosaic and non-mosaic 47,XYY men. Even less is known about the chromosome constitution of the somatic cells that support those meiocytes during the spermatogenic process. The present work analyses the chromosome constitution and the synaptic behaviour of pachytene spermatocytes in

an azoospermic man with testicular hypotrophy and a non-mosaic 47, XYY karyotype (as ascertained in lymphocyte cultures). Moreover, we define whether there is a gonadal, somatic mosaicism in Sertoli cells.

Materials and Methods

Patients

The propositus is a 35-year-old man consulting for primary infertility. Physical examination showed a tall man with a BMI 27.7 (1.92 mts tall, weight 102 kg) having two descended hypotrophic testes (testicular volume: left,10 ml; right,12 ml). He had azoospermia with a semen volume of 1.1 ml. The hormone profile showed an elevated FSH (15.6 mU/ml) and a low total testosterone level (2.8 ng/ml). The patient underwent bilateral surgery of an inguinal hernia. Blood lymphocyte metaphases show an additional Y chromosome in all cells examined, determining a non-mosaic 47, XYY karyotype.

A testicular biopsy was indicated by the andrologist for histopathological diagnosis and for the recovery of germ cells for infertility treatment through testicular sperm extraction (TESE). After fertilisation, preimplantation genetic testing for aneuploidy (PGT-A) on Day 3 blastomeres was carried out by fluorescent *in situ* hybridisation (FISH) for chromosomes 13, 15, 16, 18, 21, 22, X and Y in two embryos. One embryo showed a normal karyotype and the other embryo had multiple chromosomal aneuploidies (identified as monosomy for chromosomes 15, 16, 18, 22 and X). Pregnancy was not achieved.

The testicular tissue was processed as previously described (Sciurano and Solari, 2014). Too few spermatozoa were available in spreads to assess sperm aneuploidy levels by FISH in this patient. Five karyotypically normal men, between 30 and 44 years old, with obstructive azoospermia but having complete spermatogenesis were included in this study as controls.

All the research procedures on the tissues from patients were submitted to and accepted by the Ethics Committee of the School of Medicine (CIEI, Facultad de Medicina, UBA, Buenos Aires, Argentina). Informed consent was obtained from the analysed patients.

Immunofluorescence of specific meiotic markers

Fluorescence immunolocalisation was done as previously described (Sciurano and Solari, 2014). The following primary antibodies were incubated overnight at 4°C with: guinea pig anti-SYCP3-Nt (a home-made designed antibody against aa 1–48 from N-terminus of human origin and generated by Seqlab) at 1:100 in PBT (1% BSA, 0.01% Tween 20, 1X PBS); rabbit anti-SYCP1 at 1:100 (Abcam Ltd., UK); mouse anti-SYCP1 at 1:100 (P.J. Moens and B. Spyropoulus, York University, Canada); mouse anti-MLH1 at 1:10 (BD Pharmingen, USA); CREST serum at 1:30 (Laboratorios IFI, Argentina) and rabbit anti-BRCA1 at 1:10 (C20, Santa Cruz Biotechnology, USA).

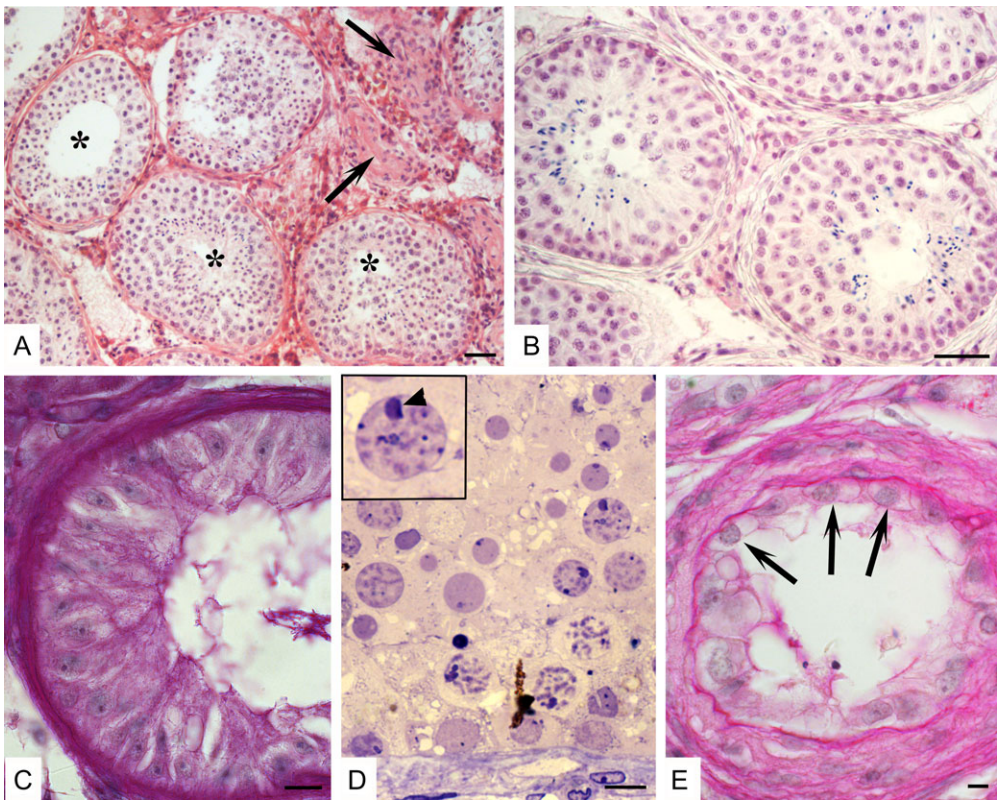

Figure 1 Testicular histopathology. Testicular tissue of the XYY patient showed a variable degree of spermatogenesis development ranging from total hyaline atrophy (arrows, **A**) to complete spermatogenesis (asterisks, A). (**B**) Complete spermatogenesis in a karyotypically normal patient (Control 3). (**C**) Periodic acid–Schiff stain of seminiferous tubule showing only Sertoli cells. (**D**) Primary spermatocytes having an apparently normal XY body (arrowhead, inset). (**E**) Periodic acid–Schiff stain showing spermatogonial arrest in a seminiferous tubule with only spermatogonia (arrows). Bars scale: A–B, 40 μm; C–E, 10 μm.

After examining and photographing the spreads of SCs that had been previously subjected to immunolocalisation of BRCA1, SYCP1 and CREST, the same spermatocyte nuclei were subjected to a FISH using a DNA probe that binds specifically to a biotinylated Chromosome Y band q12 (Invitrogen's SPoT-Light®, 84–1800 Invitrogen Corp., CA, USA).

Structured illumination microscopy

For super-resolution imaging (Schücker *et al.*, 2018) of spermatocyte nuclei, labelled by SYCP3, SYCP1 and CREST, an Elyra S.1 SIM microscope (Zeiss, Germany) equipped with 405, 408 and 642 nm lasers for excitation was used. The images were taken using a Plan Apochromat 63×/1.4 DIC M27 oil immersion objetive lens (Zeiss, Germany) and sCMOS, Edge 5.5 cameras (PCO, Germany).

The images were acquired using 10 z-stacks (total depth = 0.90 µm) and three rotating phase gratings. The structured illumination microscopy (SIM) reconstruction process added pixels to result in a 2430 × 2430 × 10 image (9231.52 pixels per μm^3). To further process our data, we used the processing software Zen 2012 SP1 (Black Edition, Zeiss).

The chromatic alignment was established using 1:10 000 dilution of 100 nm TetraSpeck fluorescent multispectral microspheres (T7279, Invitrogen, USA) seeded in each slide.

All SIM images were processed using the FIJI image software (Image J 1.51W, 2010–2018, NIH, USA, Schindelin *et al.*, 2012).

Results

Testicular histopathology

Half of the analysed seminiferous tubules showed variable degrees of spermatogenesis compared to controls (Fig. 1A vs. Fig.1B; Table I). While most tubules had complete spermatogenesis (32.8%) (Fig. 1A, asterisks), other tubules showed only primary spermatocytes (14.3%) or spermatogonia (1.7%, Fig. 1E) as the most advanced stages. Primary spermatocytes showed an XY body of apparently normal size and structure (Fig. 1D, inset, arrowhead) and a large, round main nucleolus. The remaining testicular tissue presents as either severe tubular hyalinisation (39.5%, Fig. 1A, arrows; Table I) or seminiferous tubules with Sertoli cells only (5.9% vs. 0% in controls, Fig. 1C and Table I). Thus, the histological pattern is consistent with a focal spermatogenesis having a variable degree of development in 54.6% of the testicular biopsy and a severe hyaline tubular atrophy or a Sertoli-cell-only pattern in the remaining tissue. The interstitium shows Leydig cells which are grouped in large clumps of hypertrophic cells.

Meiotic behaviour of pachytene spermatocytes

In most of the analysed pachytene spermatocytes (189/191, Fig. 2A) of the propositus, BRCA1 was restricted to the asynaptic, differential axes of the X and Y chromosomes, and the SYCP1 marker labelled 22 autosomal bivalents and a synaptic segment corresponding to the pseudoautosomal region (PAR) between one X and one Y chromosome (Fig. 3A), as observed in normal pachytene nuclei. The analysis of the same spermatocyte nuclei that were previously immunolabeled, using a specific DNA probe from the region q12 of the Y chromosome, revealed that they have only one Y chromosome (Fig. 3A). Thus, almost all the examined spermatocyte nuclei are euploid 46,XY (98%, Fig. 2A). The observation of the synaptic behaviour of the sex chromosomes at the super-resolution level, using a structured illumination microscope (SR-SIM), confirmed the euploid condition (Fig. 3C). The normal XY pair was also seen at late pachytene substages (Fig. 3D), showing the typical tangled configuration of the axes in human spermatocytes (Solari, 1980). Furthermore, the late recombination protein marker, MLH1, was consistently observed as one focus

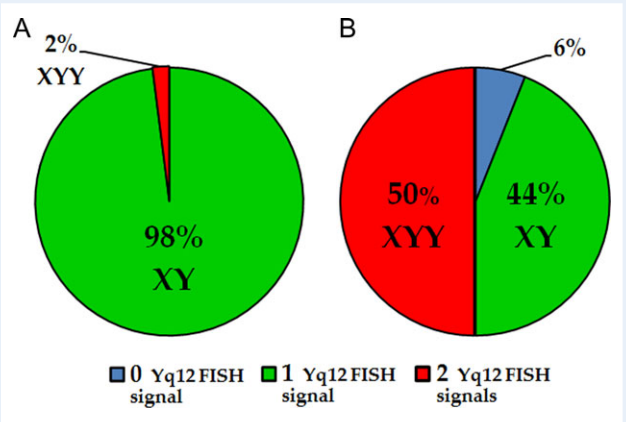

Figure 2 Percentages of primary spermatocytes (A) and Sertoli cells (B) having one (green area), two (red area) or no (blue area) Yq12-FISH signals.

Table I Quantification of the number of analysed tubules having hyalinosis, Sertoli-cell-only and complete or partial spermatogenesis in the XYY patient and in five men having normal karyotype and obstructive azoospermia. Among those tubuli with germ cells, the most advanced germ cell type found was indicated.

Patient	XYY	Control 1	Control 2	Control 3	Control 4	Control 5
Hyalinosis	94 (39.5%)	0	0	0	0	0
Sertoli cell only	14 (5.9%)	0	0	0	0	0
Complete spermatogenesis	78 (32.8%)	384 (95%)	118 (95.9%)	111 (96.5%)	205 (97.6%)	234 (94.4%)
Most advanced germ cell type						
Spermatogonium stage	4 (1.7%)	2 (0.5%)	1 (0.8%)	0	0	0
Spermatocyte stage	34 (14.3%)	12 (3%)	2 (1.6%)	2 (1.7)	5 (2.4%)	9 (3.6%)
Round spermatid stage	14 (5.9%)	6 (1.5%)	2 (1.6%)	2 (1.7)	0	5 (2%)
Total number of analysed tubules	238	404	123	115	210	248

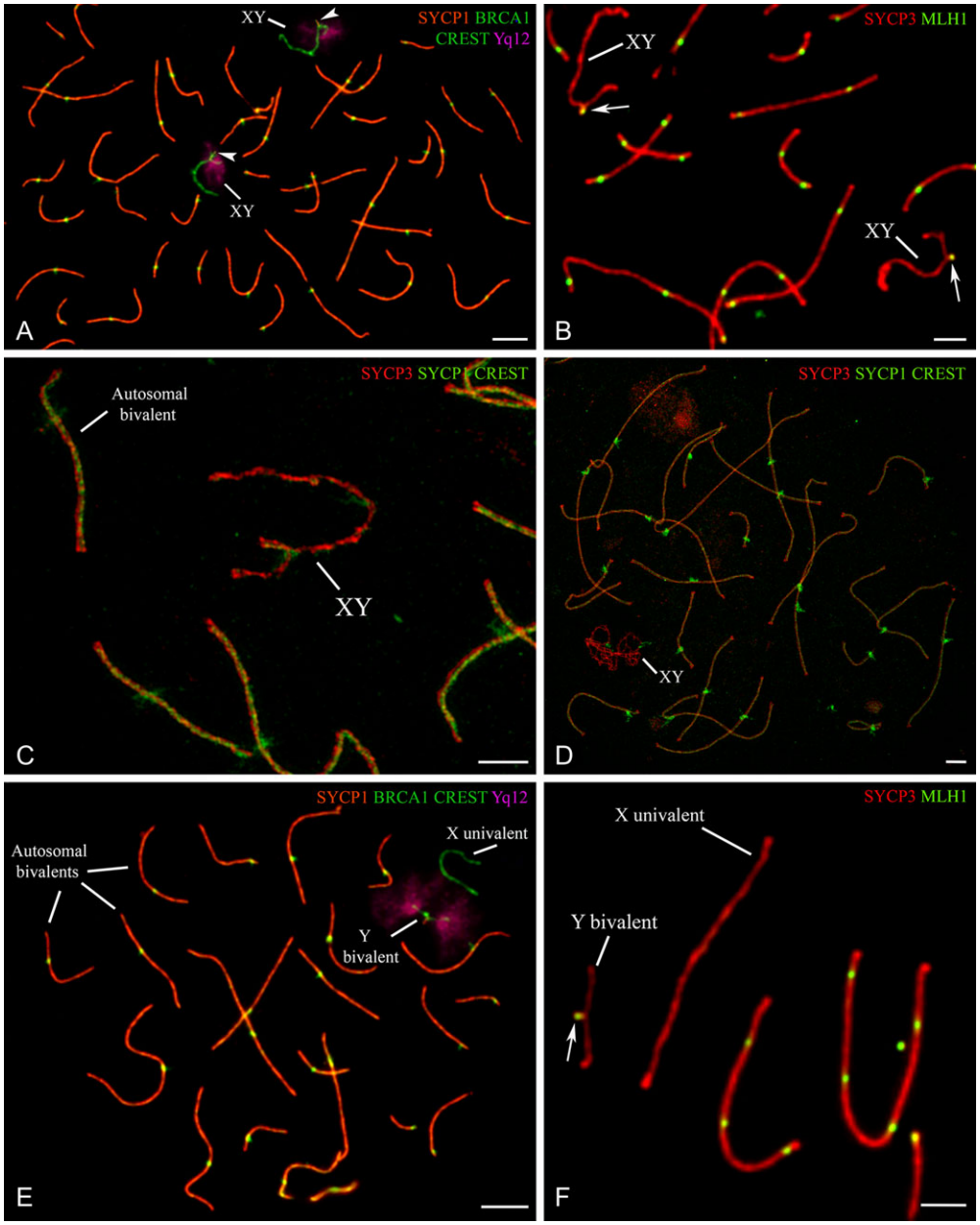

Figure 3 Chromosomal constitution of primary spermatocytes in the azoospermic patient having XYY karyotype. (**A**) Immuno-FISH using a combination of primary antibodies against BRCA1 (green axes) and SYCP1 (red axes) proteins, and an Yq12-specific DNA probe (purple cloud) allowed us to define the euploid constitution (22 bivalents plus XY pair) of pachytene spermatocytes. The protein BRCA1 decorated the differential X and Y axes. SYCP1 is located along the synapsed regions of autosomes and the pseudoautosomal region (PAR, arrowheads) from the XY pair. (**B**) Double immunolocalization of SYCP3 (red axes) and MLH1 (green foci) on pachytene spermatocytes showed that meiotic recombination in the XY pair occurs as expected (arrows). The analysis of early (**C**) and late (**D**) substages of pachytene spermatocytes by immunolocalization of SYCP3 (red axes) and SYCP1 (green axes) and structured illumination microscopy (SIM) confirmed that only one Y chromosome was partially synapsed with the X chromosome as seen in karyotypically normal patients. (**E**) Only very few spermatocytes I revealed an XYY constitution. BRCA1 (green axes) decorated the asynaptic regions of the Y bivalent and the entire axis of the X univalent. SYCP1 was present along the autosomal bivalents and the partially synapsed segment of the Y bivalent. (**F**) This Y bivalent showed only one MLH1-focus (arrow). Kinetochores were labelled with CREST serum (green foci, A, C and E). Bars scale: A, 5 μm; B–D, 2 μm; E, 5 μm; F, 2 μm.

on the distal region of the PAR (Fig. 3B), meaning that recombination between the X and Y chromosomes is not altered in most of the spermatocyte nuclei.

Only 2 out of 191 pachytene spermatocytes showed an X + YY configuration (Fig. 2A). BRCA1 labelled the asynaptic segments of the Y bivalent and the single axis of the univalent X chromosome (Fig. 3E).

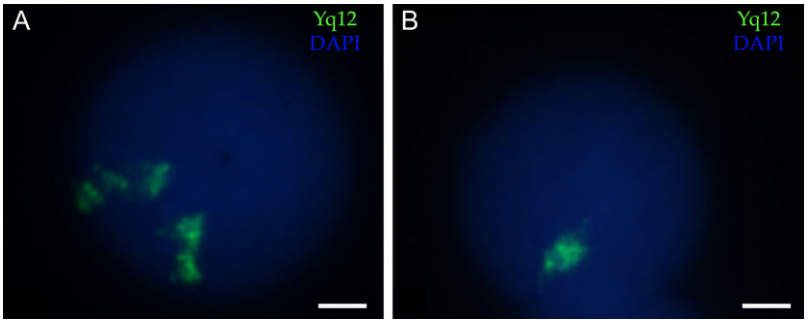

Figure 4 Determination of Y chromosome constitution of Sertoli cells using a specific Yq12 DNA probe. Two different subpopulations of Sertoli cells are present in the seminiferous tubules: one carrying two Y chromosomes (**A**, two FISH signals) and the other having only one Y chromosome (**B**, one FISH signal). Bars scale: A–B, 5 μm.

In those few pachytene spermatocytes with a Y bivalent, only one recombination focus was observed (Fig. 3F).

Prevalence of the additional Y chromosome in Sertoli cells

The analysis of Sertoli cells by FISH showed that 75 out of 149 Sertoli cells give two signals of the Y chromosome DNA probe, while 65 of them have only one signal. In those Sertoli cells where two labels are seen, both of them are clearly separated one from each other. Thus, there are two different subpopulations of Sertoli cells: those carrying two Y chromosomes (XYY Sertoli cells, Fig. 4A), and the euploid cells which have a single Y chromosome (XY Sertoli cells, Fig. 4B). As seen in the pie chart (Fig. 2B), both sex chromosome constitutions are present at similar proportions in the seminiferous epithelium.

Discussion

Although the 47,XYY condition is one of the most frequent chromosomal aneuploidies in human males, scarce analytic results have been reported in the literature on the presence of the extra Y chromosome and its meiotic behaviour (reviewed in Kim *et al.*, 2013). Recently, Wu *et al.* (2016) reported that all of the 71 analysed spermatocytes from testicular tissue were 47,XYY, with the X + YY configuration being the most frequent one, as previously described by other authors (Speed *et al.*, 1991; Solari and Rey Valzacchi, 1997).

Using immuno-FISH and SIM techniques, the present work clearly showed that the majority of pachytene spermatocytes of our non-mosaic 47,XYY patient are euploid, having 22 autosomal bivalents, one X chromosome and only one Y chromosome. These results are consistent with those reported by Chandley *et al.* (1976) on air-dried preparations of meiocytes from two 47,XYY patients with normal meiosis. Furthermore, our results show that only very few pachytene spermatocytes are actually XYY trisomies, all of them having the most frequent configuration (X + YY) and a single MLH1 focus in the Y bivalent. The presence of this single focus is consistent with the formation of a single chiasma in the short arm of the Y bivalent at MI, as observed by Hultén and Pearson (1971).

The present results on the chromosomal constitution of the Sertoli cells, using a specific DNA probe of the Yq12 region reveals, for the first time, a somatic mosaicism apparently restricted to the testicular tissue; these cells show almost equal proportions of the presence of one and two Y chromosomes. Thus, the permanence of the three sex chromosomes follows different paths in different cell types; the most reluctant cell line to support the three gonosomes is the germ line, while lymphocytes are the most supportive, and Sertoli cells fall in between those two cell types. This observation leads to new ways of looking to the sex chromosome–autosome balance.

It also agrees with the previous suggestion that the loss of the additional sex chromosome from the human germ line occurs randomly in a germ cell precursor, and that this is followed by a selective development of euploid clones over the aneuploid germ cells (Evans *et al.*, 1970). Our results enlarge this suggestion, showing the existence of different evolutionary paths of the aneuploid Sertoli cells as compared with germ cells and lymphocytes.

Another question raised by the present results is the possible role of trisomic Sertoli cells in the disruption of spermatogenesis. Trisomic Sertoli cells may be unable to build a healthy 'niche' for meiotic germ cells, leading them to cell degeneration. In the present case, the extremely low proportion of trisomic spermatocytes and the lack of the previously observed path of spermatocyte loss (Solari and Rey Valzacchi, 1997), as well as the strong preponderance of euploid spermatocytes, leads us to discard the presumptive origin of spermatogenesis failure from a meiotic hindrance *per se*. Thus, the origin of the failure in this case might be attributed to a hypothetical abnormality in the germ cell 'niche' made by Sertoli cells.

The recent success of trisomic rescue in sterile XXY and XYY mice (Hirota *et al.*, 2017), during the reprogramming of induced pluripotent stem cells from fibroblasts into functional sperm, giving rise to fertile offspring, may be a promising procedure in the future to overcome infertility in human patients with aneuploid karyotypes. However, the incidence of the aneuploid testicular microenvironment and the high risk of teratoma formation arising through the autotransplantation of these reprogrammed germ cells should be seriously considered.

Finally, based on the heterogeneous spectrum of the reported analyses (including the present results) of testicular tissue in non-mosaic 47,XYY infertile men, a detailed meiotic analysis in these patients is warranted. Furthermore, given the increased risk of passing the extra chromosome to the offspring, it is recommended that these patients should be offered genetic counselling and PGT-A as a part of any assisted reproductive treatment.

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Authors' roles

R.B.S. contributed to the conception of the study, the experimental design and execution, the analysis of data and the draft of the article. I.M.R. participated in experimental procedures and FISH procedures and contributed to draft. B.G.A. participated in the acquisition and analysis of histochemical data. G.R.V. performed the present clinical study, testicular biopsy and TESE/ ICSI treatment. R.B. participated in the SR-SIM analysis. A.J.S. participated in the conception of the study, and collaborated with design of the study and the draft of the article. R.B.S., G.R.V., R.B. and A.J.S. contributed to the critical discussion of the draft. All authors critically reviewed and approved the manuscript.

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Conflict of interest

None of the authors have any conflicting interests to declare.

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