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Sperm chromosomal abnormalities and their contribution to human embryo aneuploidy

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Abstract

In this work we reviewed 18 years of experience using fluorescence in situ hybridization (FISH) for sperm aneuploidy testing. We evaluated parameters associated with increased numerical sperm chromosome abnormalities and determined the male contribution to embryo aneploidies in terms of reproductive outcome by increased sperm aneuploidy. This retrospective study analyzed data from 2008 sperm samples of infertile males undergoing FISH analysis because of clinical history of repetitive implantation failure, recurrent miscarriage, impaired sperm parameters, or mixed causes. Sperm concentration was the only sperm parameter associated with FISH results—we observed a gradual increase of abnormal sperm FISH results in males with decreasing sperm concentration. However, a great proportion of normozoospermic males also showed increased sperm aneuploidies, suggesting that sperm parameters alone do not enable identification of a substantial proportion of infertile males at risk of sperm aneuploidies. Regarding reproductive outcomes, couples with normal sperm FISH results for the male had similar outcomes regardless of conventional in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), or preimplantation genetic testing for aneuploidies (PGT-A). However, couples with abnormal sperm FISH results for the male showed better clinical outcomes after PGT-A, suggesting a potential contribution of sperm to embryo aneuploidy. Moreover, PGT-A cycles showed better clinical outcomes when 24 chromosomes were analyzed by array comparative genome hybridization (aCGH) or next-generation sequencing (NGS) instead of only nine chromosomes analyzed by FISH. In conclusion, sperm FISH analysis offers clinical prognostic value to evaluate reproductive possibilities in infertile couples. Therefore, couples with abnormal sperm FISH results should be offered genetic counseling and presented with clinical options such as PGT-A.

Summary Sentence

Fluorescent in situ hybridization analysis of sperm is a good clinical tool to identify males at risk of having sperm aneuploidies, which can be translated into clinical consequences such us infertility problems or genetic risk for offspring.

Key words: sperm, aneuploidy, male infertility, preimplantation embryo, blastocyst, in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), implantation, pregnancy

Introduction

Embryo aneuploidies can originate from meiotic and mitotic errors. Male contribution to meiotic-origin embryo aneuploidies occurs when an aneuploid sperm fertilizes a euploid oocyte [1]. Infertile men frequently show cytogenetic anomalies—some can be detected by karyotype, but those resulting from impaired meiosis are confined to the germ cell line [2–4]. Synapsis, recombination, and DNA repair errors can produce abnormal segregation of homologous chromosomes in meiosis I or sister chromatids in meiosis II and can generate spermatozoa with numerical chromosome abnormalities, such as aneuploidy or diploidy [5–7].

Fluorescence in situ hybridization (FISH) has been used the most to analyze sperm aneuploidies in the past 15 years, and most publications have shown higher incidence of sperm aneuploides in infertile males compared to the fertile population [8–15]. Although clinical indications for FISH analysis of sperm are not clearly defined, it has been applied mainly to patients with impaired sperm parameters and to couples with a clinical history of recurrent miscarriage (RM) or repetitive implantation failure (RIF) [16–21]. Here, we evaluated the impact of sperm concentration, motility, morphology, and other indications for sperm FISH analysis on the incidence of numerical sperm abnormalities. Clinical outcome according to the incidence of sperm aneuploidy was also evaluated.

Study design

This restrospective study was approved by the Institutional Review Board (IRB) of the Instituto Valenciano de Infertilidad (IVI). The study collected data from clinical studies on sperm and embryos in 1999–2017. The study was divided into two phases (Figure 1).

Phase I

From 1999–2009, a total of 2008 couples from IVI clinics who underwent sperm FISH analysis were selected. Male mean age (±SD) was 37.5 ± 5.1 years and all men had normal 46,XY karyotypes. Indications to perform sperm FISH analysis were (1) ≥2 RIF after assisted reproduction technique ($n = 594$); (2) ≥2 RM ($n = 391$); (3) male factor infertility (MF) due to impaired sperm parameters following World Health Organization criteria [22] and without reproductive background of RIF or RM ($n = 748$); and (4) mixed causes (MixC), such as previous trisomic pregnancy, chemotherapy/radiotherapy, or poor embryo quality ($n = 275$). The effect of sperm parameters and the indications for sperm FISH analysis were evaluated in this group of infertile males.

These 2008 couples underwent assisted reproduction treatments on the advice of their physicians. According to sperm FISH results and the specific treatment indicated, the 2008 patients were divided in four groups:

(1) Normal sperm FISH result and conventional in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI): 3841 cycles performed in 1385 patients.
(2) Normal sperm FISH result and preimplantation genetic testing for aneuploidies (PGT-A): 779 cycles performed in 329 patients.
(3) Abnormal sperm FISH result and IVF/ICSI: 302 cycles in 142 patients.
(4) Abnormal sperm FISH result and PGT-A: 318 cycles in 152 patients.

For conventional IVF/ICSI cycles, embryos were selected for transfer in day 5/6 of development following morphological criteria. For PGT-A cycles, embryos were biopsied at day 3 and aneuploidy screening was performed on 9 chromosomes by FISH. Euploid embryos were selected for transfer in day 5/6 of development, in the morula or blastocyst stages.

Reproductive outcomes, including clinical pregnancy rate per transfer (CPR/T, visible embryonic sac), implantation rate (IR), miscarriage rate (MR), and live birth rate per cycle (LBR/C), were compared between patients with normal and abnormal sperm FISH results in IVF/ICSI and PGT-A cycles.

Phase II

From 2010–2017, only assisted reproduction treatments performed on couples with female ≤ 37 years old and with abnormal sperm FISH results were evaluated in four groups:

(1) A subgroup of 276 conventional IVF/ICSI cycles from 127 patients included in Phase I, in which embryos were selected for transfer in day 5/6 of development by morphological criteria.
(2) A subgroup of 280 PGT-A cycles from 133 patients included in Phase I, in which embryos were biopsied in day 3 and 9 chromosomes were screened for aneuploidy by FISH.
(3) A group of 175 PGT-A cycles from 155 patients in which 24 chromosomes were screened for aneuploidy in single-cell day 3 embryo biopsies by array comparative genome hybridization (aCGH) or next-generation sequencing (NGS).
(4) A group of 34 PGT-A cycles from 33 patients in which 24 chromosomes were screened for aneuploidy in trophectoderm day 5/6 biopsies by aCGH or NGS.

For PGT-A cycles, euploid embryos were transferred in day 5/6 of development.

Reproductive outcomes, including CPR/T, IR, MR, and LBR/C, were compared among the four groups.

Materials and methods

FISH in sperm

FISH analysis of sperm from 2008 infertile males included in Phase I was done at the IVI clinic, and Phase II analysis was done by the same technicians at the Igenomix laboratory. A single sperm ejaculate was analyzed for each male. Ejaculated sperm samples were processed to analyze numerical abnormalities for chromosomes 13, 18, 21, X, and Y as previously described [16,20]. Briefly, after sperm centrifugation

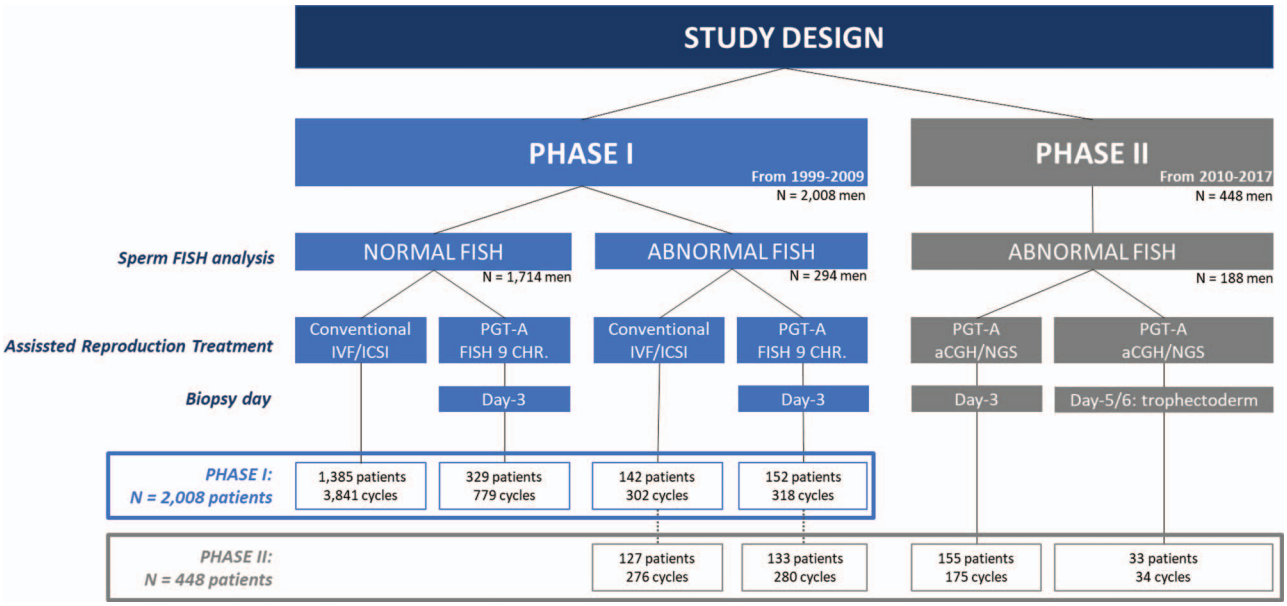

Figure 1. Schematic of the overall study design. In Phase I, a total of 2008 patients were included without considering female age. In Phase II, only cycles with female age ≤37 years were included, which represent a total of 448 patients, 260 patients were also included in Phase I and 208 were only included in Phase II.

with sperm culture media, the pellet was fixed in methanol:acetic acid (3:1) and sperm nuclei were decondensed by slide incubation in 5 mmol/l dithiothreitol (DTT) and 1% Triton X-100. Triple hybridization with centromeric DNA probes for chromosome 18 (locus D18Z1, centromeric probes (CEP) 18 Spectrum Aqua; Vysis Inc. Downers Grove, IL, USA), chromosome X (locus DXZ1, CEP X Spectrum Green; Vysis Inc.) and chromosome Y (locus DYZ1, CEP Y Spectrum Orange; Vysis Inc.) and dual hybridization with locus-specific DNA probes for chromosome 13 (locus RB, locus-specific probes (LSI) 13 Spectrum Green; Vysis Inc.) and chromosome 21 (loci D21S259, D21S341, D21S342, LSI 21 Spectrum Orange; Vysis Inc.) were performed following the manufacturer's protocol.

Analysis was carried out using an epifluorescence microscope equipped with a triple-band pass filter for 4′6-diamino-2-phenylindole (DAPI)/Texas Red/fluorescein isothiocyanate (FITC) and single-band pass filters for FITC, Texas Red, and Aqua Blue. Hybridization efficiency was > 95% in all the samples evaluated. Sperm nuclei were scored as follows: *normal haploid sperm* with one hybridization signal for each of the chromosomes evaluated; *abnormal disomic sperm* with two hybridization signals for this specific chromosome and one signal for the other chromosomes evaluated; and *abnormal diploid sperm* with two hybridization signals for all the chromosomes evaluated (Figure 2). Because of the difficulty of discriminating between nullisomic spermatozoa and hybridization failures, nullisomies were not scored. Whenever possible, 2000 sperm cells per sample were scored for each hybridization with this number being less for some oligozoospermic sperm samples.

Individual FISH results of each male were compared to those of an internal control group of 10 normozoospermic donors (disomy rate for chromosome 13 = 0.07%; disomy rate for chromosome 18 = 0.03%; disomy rate for chromosome 21 = 0.12%; disomy rate for sex chromosomes = 0.21%; diploidy rate = 0.10%) [23]. An abnormal FISH result was considered when the sample showed a statistical increased incidence of diploid sperm or spermatozoa with disomy for any evaluated chromosome when compared to controls. For each sperm sample, total aneuploidy rate was considered as the total disomy rate for chromosomes 13, 18, 21, X, and Y. Total abnormality rate was considered as the diploidy rate plus the total aneuploidy rate.

Conventional IVF/ICSI

Conventional IVF or ICSI treatments were performed at IVI clinics following standardized protocols [24]. Sperm preparation was performed by density gradient centrifugation. For ICSI cases, selection was always based on morphology and motility.

Preimplantation genetic testing for aneuploidies

From 1999–2005, embryo biopsies and PGT-A were done at IVI clinics. Embryo biopsy for these PGT-A cycles was performed on day 3 developing embryos with ≥5 nucleated blastomeres and ≤25% fragmentation degree. Biopsied cells were analyzed by FISH for chromosomes 13, 15, 16, 18, 21, 22, X, and Y using a protocol described by Rubio and coauthors [25]. Starting in 2005, chromosome 17 was also included in the analysis with a 4CC probe kit and Multivysion PB kit (Vysis Inc., Downers Grove, IL, USA).

From January 2011–December 2015, single-cell embryo biopsies on day 3 or trophectoderm biopsies on day 5/6 of development were done at IVI clinics, and biopsies were sent to the Igenomix laboratory for genetic analysis. After DNA amplification, aCGH was used (24sure kit, BlueGnome, Cambridge, UK) to screen 24 chromosomes for aneuploidy. From January 2016–November 2017, NGS was used to analyze 24 chromosomes (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis

Chi-square test with Yate correction was used in Phase I to compare individual FISH results (disomy and diploidy) in each male with the control group for normal/abnormal sperm FISH result classification. Chi-square test with Yate correction or Bonferroni correction and Fisher exact test were used to compare reproductive outcome

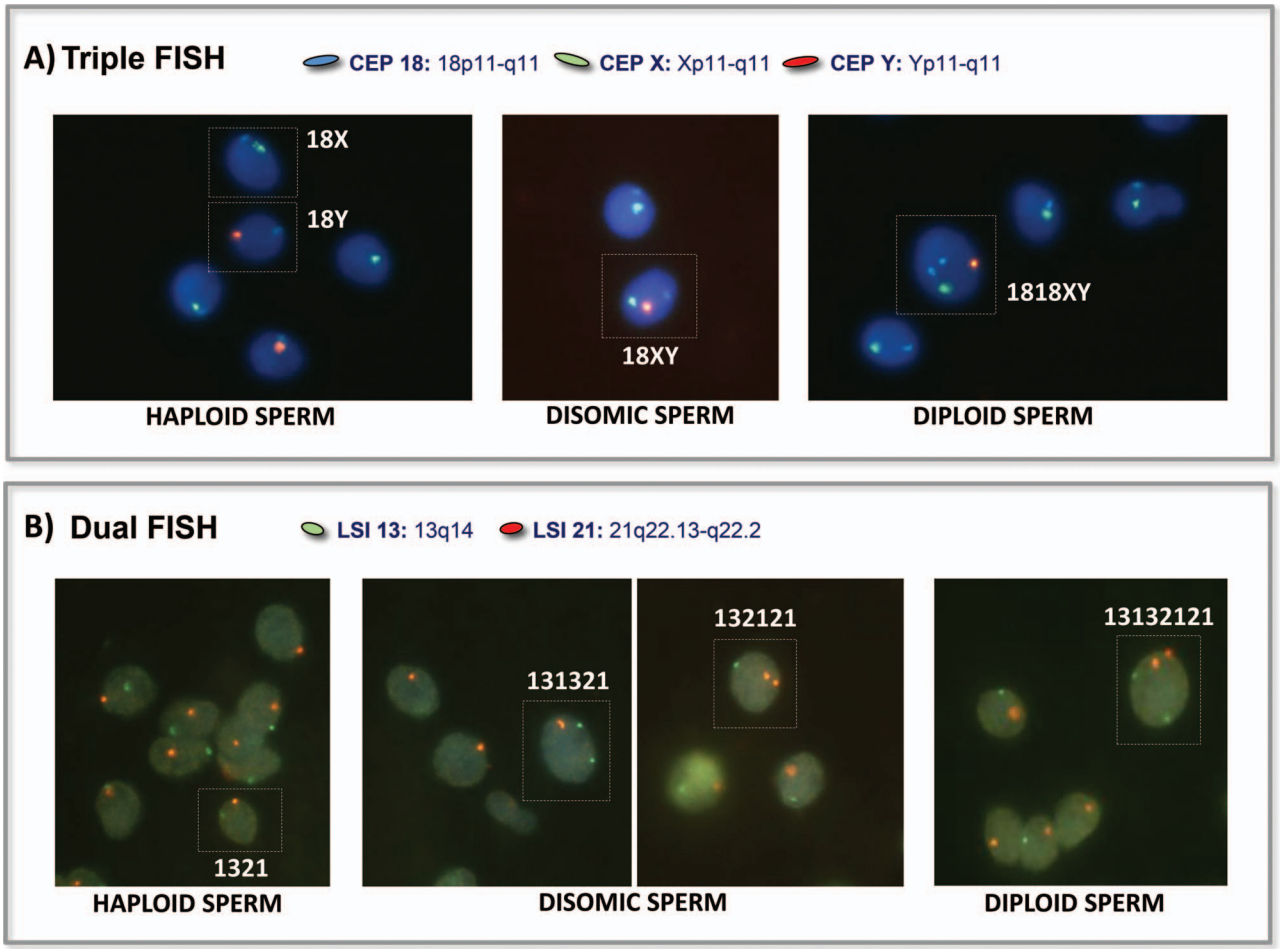

Figure 2. Evaluation of FISH signals using epifluorescence microscopy. (A) Triple FISH spermatozoa with CEP for chromosomes 18, X, and Y. Spermatozoa with one signal for each of the autosomes and one signal for the sex chromosomes (X or Y) were classified as *haploid sperm*; spermatozoa with two signals for chromosome 18 and one signal for sex chromosomes or one signal for chromosome 18 and two signals for sex chromosomes were classified as *disomic sperm*; spermatozoa with two signals for chromosome 18 and two signals for sex chromosomes were classified as diploid sperm. (B) Dual FISH with locus-specific probes (LSI) for chromosomes 13 and 21. Spermatozoa with one signal for each of the autosomes were classified as *haploid sperm*; spermatozoa with two signals for chromosome 13 or 21 and one signal for the other chromosome were classified as *disomic sperm*; spermatozoa with two signals for chromosomes 13 and 21 were classified as diploid sperm.

between groups in Phases I and II. Logistic regression, analysis of variance (one-way ANOVA), or analysis of contingency were used in Phase I to determine (1) the effect of sperm parameters and indication for FISH analysis on sperm FISH results and sperm chromosomal abnormality; and (2) the effect of sperm FISH results on reproductive outcomes. $P < 0.05$ was considered statistically significant (SPSS Inc., Chicago, IL, USA).

Results

Phase I

Sperm FISH analysis. A total of 294 out of 2008 sperm samples (14.6%) analyzed in Phase I had abnormal sperm FISH results, and the remaining 1714 (85.4%) had normal results when compared to controls.

Effect of sperm parameters. Males with abnormal sperm FISH results had significantly lower sperm concentration (21.3 $\times$ 10^6 sperm/mL vs. 37.0 $\times$ 10^6 sperm/mL, $P < 0.0001$) and progressive sperm motility (34.3 vs. 39.4%, $P < 0.0001$) compared to those with normal sperm FISH results. Logistic regression analysis showed a negative association between sperm concentration and an abnormal sperm FISH result, with every 1 $\times$ 10^6 decrease of sperm concentration increasing 0.988-fold the probability of an abnormal FISH result (Supplementary Table 1).

Sperm samples were distributed into deciles of sperm concentration to assess the effect on FISH results (Figure 3A). Contingency tables showed a gradual decrease in the percentage of males with abnormal FISH results from the lowest decile of sperm concentration (D1, $\leq$1 $\times$ 10^6 sperm/mL) to the highest decile (D10, $\geq$80.1 $\times$ 10^6 sperm/mL). The incidence of males with abnormal FISH results was significantly higher in the lowest sperm concentration decile (D1: 31.4%) compared to other deciles ($P < 0.0001$).

To assess the effect of sperm concentration on the different types of sperm chromosomal abnormalities (sex chromosome disomy rate; diploidy rate; total aneuploidy rate; and total abnormality rate), samples were distributed into quartiles of sperm concentration, and

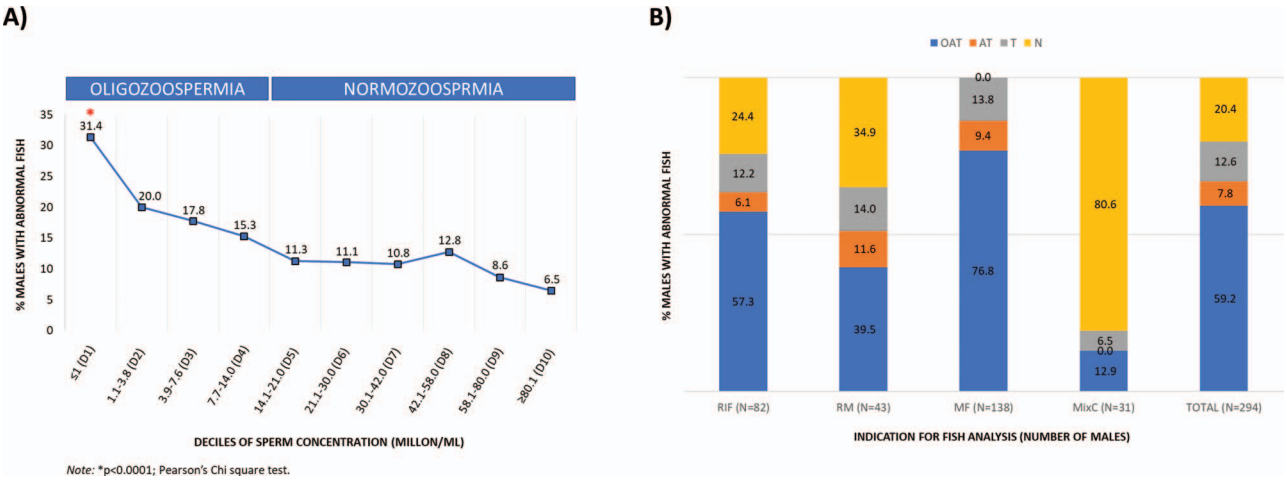

Figure 3. (A) Incidence of males with abnormal sperm FISH result in each decile of sperm concentration, from D1 with $\leq 1 \times 10^6$ sperm concentration to D10 with $\geq 80.1 \times 10^6$ of sperm concentration. (B) Percentage of males with abnormal sperm FISH result in the four groups according to the indication for FISH analysis: RIF, RM, MF, and MixC. Each bar was substratified according to the percentage of males with oligoasthenozoospermia (OAT), asthenoteratozoospermia (AT), teratozoospermia (T), and normozoospermia (N).

the mean of each category was determined for each quartile. Analysis of variance showed similar diploid and sex chromosome disomy rates in all quartiles of sperm concentration (Figure 4). However, total aneuploidy rate and total abnormality rate gradually decreased from low sperm concentration quartile (Q1, $\leq 5.9 \times 10^6$ sperm/mL) to high sperm concentration quartile (Q4, $\geq 48.7 \times 10^6$ sperm/mL). Total aneuploidy rate and total abnormality rate were significantly higher in Q1 compared to Q4 (total aneuploidy rate: 1.38 vs. 0.38%, respectively; total abnormality rate: 1.69 vs. 0.57%, respectively; $P < 0.05$).

Indication for FISH analysis. Regarding the indication for FISH analysis, incidence of an abnormal FISH result was 13.8% in males with RIF, 11% in males with RM, 18.5% in males with MF, and 12.7% in males with MixC, with significant differences in the percentage of males with abnormal FISH results only observed between MF and RM indications ($P < 0.01$). However, in the logistic regression analysis including sperm concentration as variable, the indication was not significantly correlated with the probability of having an abnormal FISH result. Sperm concentration was the only parameter associated with sperm FISH results (Supplementary Table 2).

Figure 3B shows the distribution of samples with abnormal sperm FISH results for each indication, according to sperm parameters. Despite the high incidence of oligoasthenoteratozoospermic samples (59.2%) observed in this group of 294 males with abnormal sperm FISH results, a high incidence of normozoospermic samples was observed for RIF (24.4%), RM (34.9%), and MixC (80.6%) indications.

Analysis of variance determined a similar mean number of implantation failures in RIF patients with normal and abnormal sperm FISH results (3.3 vs. 3.2, respectively), and a similar number of miscarriages in RM patients with normal and abnormal sperm FISH results (2.9 vs. 2.5, respectively). For RIF indication, the incidence of males with an abnormal sperm FISH result was similar independent of the number of previous implantation failures at the time of sperm FISH analysis (Table 1A). However, in RM indication, there was a decrease of abnormal FISH results with five or more previous miscarriages, but this trend was not statistically significant (Table 1B).

Reproductive outcome. Table 2A shows the reproductive outcomes from assisted reproduction treatments performed on the 2008 infertile males in Phase I of the study. In conventional IVF/ICSI cycles, patients with abnormal compared to normal sperm FISH results had significantly lower CPR/T (18.1 vs. 36.3%, respectively; $P < 0.0001$), IR (8.9 vs. 22.8%, respectively; $P < 0.0001$), and LBR/C (4.9 vs. 19.6%, respectively; $P < 0.0001$) and significantly higher MR (62.5 vs. 34.9%, respectively; $P < 0.0001$). In PGT-A cycles, the incidence of abnormal embryos was significantly higher in patients with normal compared to abnormal sperm FISH results (70.8 vs. 56.9%; $P < 0.0001$). However, after transference of euploid embryos, PGT-A patients with abnormal compared to normal sperm FISH results had significantly higher CPR/T (44.0 vs. 31.5%, respectively; $P < 0.01$), IR (36.2 vs. 24.9%, respectively; $P < 0.001$), and LBR/C (26.4 vs. 12.5%, respectively; $P < 0.0001$) and significantly lower MR (18.4 vs. 31.2%, respectively; $P < 0.05$).

In patients with normal sperm FISH results, IVF/ICSI compared to PGT-A was associated with signficantly higher number of embryo transfer cycles (82.6 vs. 57.5%, respectively; $P < 0.0001$) and mean number of transferred embryos (2.1 vs. 1.6, repectively; $P < 0.0001$). CPR/T, IR, and MR were similar, but LBR/C was significantly higher in conventional IVF/ICSI cycles compared to PGT-A cycles (19.6 vs. 12.5%, respectively; $P < 0.0001$). However, in patients with abnormal sperm FISH results, PGT-A compared to IVF/ICSI was associated with significantly higher CPR/T (44.0 vs. 18.1%, respectively; $P < 0.0001$), IR (36.2 vs. 8.9%, respectively; $P < 0.0001$), and LBR/C (26.4 vs. 4.9%, respectively; $P < 0.0001$) and significantly lower MR (18.4 vs. 62.5%, respectively; $P < 0.0001$).

The logistic regression analysis in conventional IVF/ICSI cycles was performed for the following outcomes: clinical pregnancy; implantation rate >50%; miscarriage; and live birth. Female age, sperm FISH result, and different types of sperm chromosomal abnormalities were included as variables. Patients with abnormal vs. normal sperm FISH results had a 2.617-fold decreased probability of achieving a clinical pregnancy ($P < 0.0001$), a 4.551-fold decreased

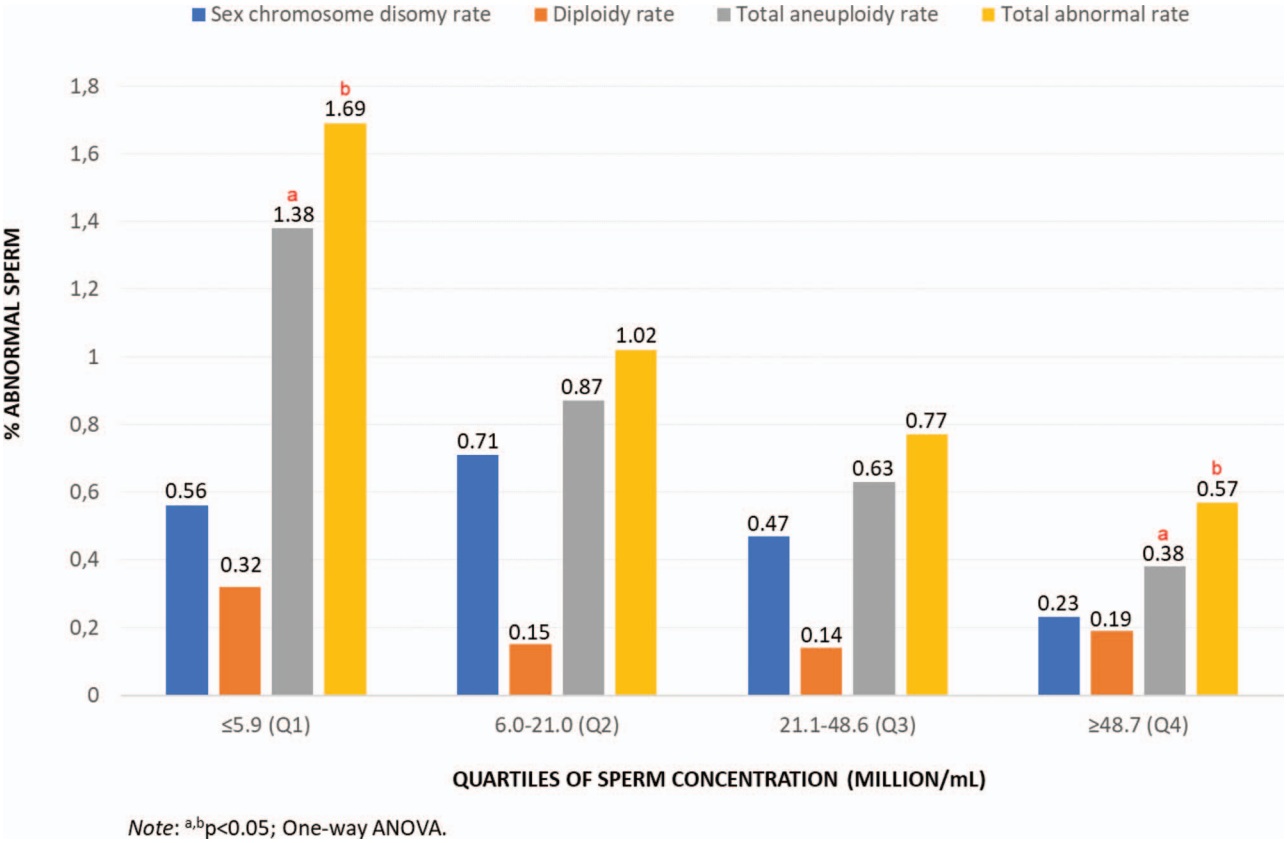

Figure 4. Incidence of four different types of sperm chromosomal abnormalities (disomy rate, diploidy rate, total aneuploidy rate, and total abnormality rate) according to the four quartiles of sperm concentration.

Table 1A. Percentage of males with abnormal sperm FISH results stratified according to the number of implantation failures

No. of implantation failures	Patients	Abnormal FISH (%)	95% Confidence interval	
			Lower limit	Upper limit
2	194	31 (15.98)	10.82	21.14
3	191	23 (12.04)	7.42	16.66
4	114	16 (14.04)	7.66	20.42
5	44	5 (11.36)	1.98	20.74
≥6	51	7 (13.73)	4.28	23.18
Total group	*594*	*82 (13.81)*	*11.04*	*16.58*

NS, no significant differences according to the number of implantation failures.

Table 1B. Percentage of males with abnormal sperm FISH results stratified according to the number of miscarriages

No. of miscarriages	Patients	Abnormal FISH (%)	95% Confidence interval	
			Lower limit	Upper limit
2	190	26 (13.68)	8.79	18.57
3	117	10 (8.55)	3.48	13.62
4	51	6 (11.76)	2.92	20.60
5	17	1 (5.88)	0	17.06
≥6	16	0	0	0
Total group	*391*	*43 (10.99)*	*7.89*	*14.09*

NS, no significant differences according to the number of miscarriages.

Table 2. A) Comparison of the reproductive outcome with conventional IVF/ICSI vs. PGT-A on day 3 biopsies analyzed by FISH, in couples with normal and abnormal sperm FISH result from the Phase I of the study. B) Comparison of the reproductive outcome in conventional IVF/ICSI, PGT-A on day 3 biopsies analyzed by FISH, PGT-A on day 3 biopsies analyzed by aCGH/NGS, and PGT-A on day 5/6 biopsies analyzed by aCGH/NGS, in the couples with abnormal sperm FISH results and female age ≤37 years from the Phase II of the study

A) PHASE I

	Normal sperm FISH result		Abnormal sperm FISH result	
	Conventional IVF/ICSI	PGT-A	Conventional IVF/ICSI	PGT-A
No. Patients	1,385	329	142	152
No. Cycles	3,841	779	302	318
Mean female age (SD)	34.1 (3.5)	36.2 (3.7)	33.8 (3.8)	33.8 (3.7)
Mean male age (SD)	37.7 (5.3)	38.9 (10.1)	37.1 (4.4)	36.8 (9.1)
No. Transfers (%)	3,174 (82.6) [a, k]	448 (57.5) [g, k]	221 (73.2) [a]	234 (73.6) [g]
Mean no. transferred embryos (SD)	2.1 (0.7) [b, l]	1.6 (0.6) [l]	2.3 (0.9) [b, m]	1.6 (0.6) [m]
Clinical Pregnancy Rate/Transfer (%)	36.3 [c]	31.5 [h]	18.1 [c, n]	44.0 [h, n]
Implantation Rate (%)	22.8 [d]	24.9 [i]	8.9 [d, o]	36.2 [i, o]
Miscarriage Rate (%)	34.9 [e]	31.2 [j]	62.5 [e, u]	18.4 [j, u]
Live Brith Rate/Cycle (%)	19.6 [q, s]	12.5 [r, s]	4.9 [q, t]	26.4 [r, t]

Note: [b-b, d-d, e-e, f-f, g-g, k-k, l-l, m-m, n-n, o-o, p-p, q-q, r-r, s-s, t-t, u-u] p<0.0001; [c-c, i-i] p<0.001; [a-a, h-h] p<0.01; [j-j] p<0.05; Chi-square test with Yate's correction, Fisher's exact test and Welch t test.

B) PHASE II

	Conventional IVF/ICSI	PGT-A FISH day-3 biopsy	PGT-A aCGH/NGS Day-3 biopsy	PGT-A aCGH/NGS day-5/6 biopsy
No. patients	127	133	155	33
Mean female age (SD)	32.8 (2.7)	32.7 (2.8)	34.1 (2.7)	34.4 (1.7)
No. cycles	276	280	175	34
No. informative embryos	--	1,349	1,190	176
% Abnormal embryos	--	61.6 [v, w]	67.0 [v, x]	39.8 [w, x]
% Cycles with transfer	75.0	77.5	74.9	82.4
Mean number of embryos transferred (SD)	2.3 (0.9)[a, b, c]	1.6 (0,6)[a]	1.7 (0,7)[b]	1.9 (1.0)[c]
Clinical Pregnancy Rate/Transfer (%)	18.4 [d, e, f]	45.6 [d, g, h]	64.9 [e, g]	89.3 [f, h]
Implantation Rate (%)	9.7 [i, j, k]	37.3 [i, l, m]	55.2 [j, l]	61.5 [k, m]
Miscarriage Rate (%)	60.5 [n, o, p]	17.2 [n]	8.2 [o]	24.0 [p]
Live Birth Rate/Cycle (%)	5.4 [q, r, s]	29.3 [q, t, u]	44.6 [r, t]	55.9 [s, u]

Note: Conventional IVF/ICSI selects embryo for transfer by morphological criteria; PGT-A by FISH selects euploid embryos for transfer, with the analysis of 9 chromosome in day-3 biopsy (chr. 13, 15, 16, 17, 18, 21, 22, X, Y); PGT-A by aCGH/NGS selects euploid embryos for transfer, with the analysis of all 24 chromosomes in day-3 biopsy; PGT-A by aCGH/NGS selects euploid embryos for transfer with the analysis of all 24 chromosomes in trophectoderm biopsy of day 5/6 embryos.
[a-a, b-b, d-d, e-e, f-f, h-h, i-i, j-j, k-k, l-l, n-n, o-o, q-q, r-r, s-s, w-w, x-x] p<0.001; [g-g, m-m, t-t] p<0.01; [c-c, p-p, u-u, v-v] p<0.05; Chi-square test, Fisher's exact test and Welch t test with Bonferroni's correction.

probability of having an implantation rate of > 50% ($P < 0.0001$), a 0.434-fold increased probability of miscarriage ($P < 0.05$), and a 3.782-fold decreased probability of having a live birth ($P < 0.0001$). Moreover, every year of increase in maternal age decreased 0.937-fold the probability of achieving a clinical pregnancy, decreased 0.929-fold the probability of having an implantation rate of > 50%, increased 1.098-fold the probability of miscarriage ($P < 0.0001$), and decreased 0.897-fold the probability of having a live birth ($P < 0.0001$). Regarding the four types of sperm chromosomal abnormalities, every unit increase in total sperm aneuploidy rate decreased 0.280-fold the probability of miscarriage, and every unit increase in total sperm abnormality rate increased 3.623-fold the probability of miscarriage and decreased 0.460-fold the probability of having a live birth ($P < 0.05$) (Supplementary Table 3).

Phase II: Reproductive outcome and FISH results

Table 2B shows reproductive outcome comparisons between the four analyzed groups in Phase II, all in women ≤37 years of age and abnormal sperm FISH results. The incidence of abnormal

embryos was significantly higher in PGT-A cycles biopsied in day 3 compared to cycles biopsied in day 5/6 (61.6% by day-3 FISH analysis and 67.0% by day-3 aCGH/NGS analysis vs. 39.8% by day-5/6 aCGH/NGS analysis; $P < 0.001$). Regarding the number of chromosomes analyzed in day 3, the incidence of abnormal embryos was significantly higher in cycles with analysis of 24 chromosomes by aCGH/NGS compared to cycles with analysis of 9 chromosomes by FISH (67.0 vs. 61.6%, respectively; $P < 0.05$). Although the mean number of embryos transferred was significantly higher in couples undergoing conventional IVF/ICSI cycles compared to the three groups of PGT-A cycles (2.3 in conventional IVF/ICSI vs. 1.6 in PGT-A day 3 by FISH, 1.7 in PGT-A day-3 by aCGH/NGS, and 1.9 in PGT-A day 5/6 by aCGH/NGS; $P < 0.05$), clinical outcomes were poorer in IVF/ICSI cycles compared to PGT-A cycles irrespective of the embryo biopsy day and number of chromosomes analyzed, with significantly lower CPR/T (18.4 vs. 45.6, 64.9 and 89.3%, respectively; $P < 0.001$), IR (9.7 vs. 37.3, 55.2 and 61.5%, respectively; $P < 0.001$), and LBR/C (5.4 vs. 29.3, 44.6 and 55.9%, respectively; $P < 0.001$) and significantly higher MR (60.5 vs. 17.2, 8.2 and 24.0%, respectively; $P < 0.05$).

Regarding PGT-A outcomes, CPR/T was significantly higher in cycles using aCGH/NGS to analyze all 24 chromosomes (64.9% in day 3 biopsy and 89.3% in day 5/6 biopsy) compared to cycles using FISH to analyze 9 chromosomes (45.6%; $P < 0.01$). This difference was also observed for IR (55.2% in aCGH/NGS day 3 biopsy and 61.5% in aCGH/NGS day 5/6 biopsy vs. 37.3% in FISH day 3 biopsy; $P < 0.01$) and LBR/C (44.6% in aCGH/NGS day 3 biopsy and 55.9% in aCGH/NGS day 5/6 biopsy vs. 29.3% in FISH day 3 biopsy; $P < 0.01$).

Discussion

Infertile males with abnormal sperm FISH results showed lower sperm concentration and progressive motility compared to males with normal FISH results. However, as described previously by Serrate and coauthors [21], only sperm concentration was associated with FISH results. We observed a gradual increase in males with abnormal sperm FISH results with decreasing sperm concentration—10% of normozoospermic males ($\geq 15 \times 10^6$ sperm/mL), ~17% of males with moderate oligozoospermia (1×10^6–14×10^6 sperm/mL), and up to 30% of males with severe oligozoospermia ($\leq 1 \times 10^6$ sperm/mL) had abnormal sperm FISH results. This incidence of males with abnormal sperm FISH result was even higher in nonobstructive azoospermic males as we previously observed [23].

Nonetheless, a high incidence of males with oligozoospermia (44.3% of the 2008 males) did not show increased aneuploid or diploid sperm, which would explain the absence of a linear correlation of incidence of aneuploid and diploid sperm with sperm concentration. However, total aneuploidy and abnormality rates were higher in severe oligozoospermic males and gradually increased with decreasing sperm concentration. Thus, classical meiotic studies of oligozoospermic males have revealed a direct correlation between abnormal chromosome pairing during meiosis and decreased sperm production [3,4]. Moreover, several studies have revealed that production of aneuploid and diploid sperm is associated with oligozoospermia [26–28], mainly at sperm concentrations of $< 5 \times 10^6$ [16,17,29].

Our study found no relationship between abnormal sperm FISH results and sperm motility or morphology, consistent with previous studies [14,21,26,30]. Regarding sperm motility, similar incidences of aneuploidy and diploidy are observed in spermatozoa with good motility compared to sperm with low motility [31] or non-motile sperm [32]. However, others have described small populations of males with severe asthenozoospermia with specific deformities involving sperm flagella, with a negative correlation between sperm motility and increased sperm aneuploidy [33,34].

Regarding sperm morphology, controversial results have been reported. Whereas some authors describe a 4-fold increase in aneuploid sperm in teratozoospermic compared to normozoospermic males [35] and a 4.4-fold increase in aneuploidies in sperm with abnormal compared to normal morphologies [36], Celik-Ozenci and coauthors [37] observed similar incidences of aneuploidies in sperm with different sizes and shapes in infertile males. However, a general consensus seems to exist regarding severe teratozoospermia with large-headed and multiple-tailed spermatozoa for a higher risk of sperm aneuploidy, diploidy, and polyploidy [18,26,38].

While our data did not show correlation between the indication for sperm FISH analysis and sperm FISH results, a tendency toward a higher incidence of males with abnormal FISH results was observed in the group of males undergoing FISH analysis for MF. Considering that only sperm concentration was directly related to sperm FISH results, the predominance of oligozoospermic males in this group (76.8%) compared to the other three indications (57.3% in RIF; 39.5% in RM; and 12.9% in MixC) could explain this tendency. However, a high incidence of normozoospermic males had abnormal sperm FISH results in each indication, suggesting that sperm parameters themselves do not identify an important proportion of infertile males at risk of sperm aneuploidies.

At the end of the 1990s, some publications suggested that the presence of chromosomal abnormalities in sperm could lead to abnormal embryos ending in implantation failures [11,39]. Since then, several studies have reported a high incidence of diploid and aneuploid sperm in couples with a clinical history of RIF [16,40,41] and RM [42–44]. Whereas our data showed similar incidence of males with abnormal sperm FISH results irrespective of the number of previous implantation failures, the number of patients with abnormal sperm FISH results decreased with increasing number of previous miscarriages. This effect was previously described by our group when analyzing the incidence of aneuploid embryos in couples with RM of unknown etiology [42], with a decrease of aneuploid embryos and implantation rate in PGT-A cycles of RM couples with > 4 previous miscarriages. These data suggest that some cases of RM may be due to increased aneuploid and diploid sperm, but there might be other causes for pregnancy loss when there is a clinical history of > 4 miscarriages.

In couples with severe MF, ICSI increases the chance of pregnancy. Prenatal testing from ICSI pregnancies has shown an increased incidence of de novo sex chromosome aneuploidies and structural rearrangements [45,46], mostly of paternal origin [47,48] and attributed to sperm quality of infertile males [49,50]. Increased incidence of sperm aneuploidies has been related to lower pregnancy and implantation rates and to higher MRs after ICSI [16,40,51–53]. Our Phase I data also showed lower reproductive success after conventional IVF/ICSI treatments in couples with abnormal compared to normal sperm FISH results. This adverse effect of numerical chromosome abnormalities in sperm on reproductive success confirms the validity of our criteria to consider a sperm sample with abnormal FISH results.

Several authors have proposed PGT-A as an alternative to improve the possibility of healthy pregnancies in MF [25,54,55]. Our Phase I data show that PGT-A cycles analyzing a panel of 9

chromosomes in couples with abnormal sperm FISH results have lower MRs and higher pregnancy, implantation, and LBRs than patients with normal sperm FISH results. Interestingly, patients with normal sperm FISH results had similar clinical outcomes regardless of conventional IVF/ICSI or PGT-A cycles. However, patients with abnormal sperm FISH results showed better clinical outcomes after PGT-A. These data suggest a potential contribution of sperm to embryo aneuploidy.

Indeed, we previously described a strong correlation between sperm chromosomal abnormalities and embryo chromosomal constitution, considering this effect as a potential cause of lower reproductive outcomes in MF infertile couples [56]. A total sperm aneuploidy rate of 6% in normozoospermic men with an average frequency of 0.12% of disomy for the autosomes and 0.31% for the sex chromosomes was previously reported [57]. Chromosome 21 and sex chromosomes have a special tendency to undergo nondisjunction [58] and along with chromosomes 13 and 18 are the most frequently studied in most sperm publications. We have considered the total sperm aneuploidy rate as the total sperm aneuploidies for chromosomes 13, 18, 21, and sex chromosomes. These aneuploidies are more related to elevated risk of generating potentially viable aneuploid embryos rather than to miscarriages and could explain the decreased probability of miscarriage that we have observed with the increase in total sperm aneuploidy rate. Therefore, it seems that the aneuploidy rate for the selected five chromosomes is a cost-effective estimation of the aneuploidy risk in a sperm sample. In our study, total sperm abnormality rate is represented by the total sperm aneuploidy rate plus the total sperm diploidy rate. Increases in diploid sperm have a higher risk of triploid embryos more related to abortions. Therefore, the presence of diploid sperm increases the total sperm abnormality rate, explaining the increased probability of miscarriage and the decreased probability of live birth that we observed with the increase in total sperm abnormality rate.

In Phase I, patients with abnormal FISH in sperm underwent a similar number of IVF/ICSI cycles than PGT-A cycles. As a consequence of the results obtained on Phase I, on the second part of the study, Phase II, more PGT-A cycles were indicated when abnormal sperm FISH results were obtained. Aneuploidy testing of 24 chromosomes in Phase II of the study performed in couples with abnormal sperm FISH results showed even better clinical pregnancy, implantation, and LBRs. Analysis of all chromosomes instead of only 9 chromosomes resulted in a higher incidence of aneuploid embryos biopsied in day 3 and, therefore, a better selection of embryos. Interphase sperm nuclei are described to be distributed in chromosomal territories, and the presence of aneuploidies for sex chromosomes partially modifies this territorial organization, leading to genetically abnormal spermatozoa that could affect other chromosomes [59]. Therefore, although we determined an abnormal sperm FISH result based on analysis of only 5 chromosomes, the presence of sperm aneuploidies in any of these chromosomes would explain detection of aneuploidies for other chromosomes in the embryos. As expected, we observed a decreased incidence of aneuploid embryos when analyzing day 5/6 blastocysts compared to day 3 cleavage-stage embryos. Similar results were previously described by other authors, suggesting a strong selection against complex aneuploidies during embryo development [60,61].

Finally, we would like to point out that sperm FISH analysis in old samples included in the historical data above discussed was performed by the same team following the same protocol and scoring criteria. However, we acknowledge that during the 18-year study period, the PGT-A methodology has evolved from day 3 biopsies to trophectoderm biopsies and from the analysis of a subset of chromosomes to a comprehensive 24 chromosome analysis. Another limitation is the retrospective nature of the study, in which follow-up of the clinical outcome after IVF/ICSI was performed in couples with a sperm FISH analysis.

Conclusions

As a clinical diagnostic tool, sperm FISH analysis offers prognostic value to evaluate reproductive possibilities in infertile couples. As described in this review of 18 years of experience, the presence of numerical chromosome abnormalities in sperm can be translated into clinical consequences, such as infertility problems or genetic risk for offspring. Therefore, when an abnormal sperm FISH result is found, couples should be offered genetic counseling and proposed with several clinical options, such as PGT-A.

Supplementary data

Supplementary data are available at *BIOLRE* online.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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