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# Application of the FISH Method for Analyzing Chromosome Segregation Patterns in Preimplantation Embryos from Robertsonian Translocation Carriers

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**Abstract**

Robertsonian translocations (RTs) are very common balanced structural chromosome rearrangements in humans. Due to alterations in the chromosome segregation pattern and the formation of unbalanced gametes and embryos, the carriers of RTs have a significant risk of reproductive failure. For over 30 years, fluorescent in situ hybridization (FISH) has been used for preimplantation genetic testing for chromosomal structural rearrangement (PGT-SR) in carriers of RTs. The data obtained by performing FISH for PGT-SR can be useful for analyzing segregation patterns in preimplantation embryos. We analyzed chromosome segregation patterns in 964 embryos from 100 couples who were carriers of various types of RTs by performing FISH or using the array comparative genomic hybridization (aCGH) method. When the carriers were male, the predominant segregation mode was alternate (detected in 42.4% of embryos), whereas, when the carriers were female, alternate and adjacent types of segregation were detected in most embryos (32.3% and 26.6% of embryos, respectively). About 33% of balanced/euploid embryos in IVF cycles were formed when the carriers of RT were male, and about 24% of such embryos were formed when the carriers were female. No association was found between sperm parameters and malsegregation rates in male carriers of RT. In this study, we found that female and male RT carriers have different reproductive risks associated with chromosome segregation patterns and the rate of balanced/euploid embryos.

**Keywords**

Robertsonian translocations; chromosome segregation; aneuploidy; preimplantation genetic testing; fluorescent *in situ* hybridization; array comparative genomic hybridization

## 1. Introduction

Until recently, fluorescent in situ hybridization (FISH) was the prevalent method for detecting aneuploidy in preimplantation embryos. However, the limitation of the FISH method (analysis of a limited number of chromosomes and their loci) and the peculiarities of preimplantation embryos on the third day (D3) of development (mosaicism and a high level of aneuploidy independently corrected by the fifth or sixth day (D5/D6) of development), led to the progress of comprehensive PGT methods such as aCGH, next-generation sequencing (NGS), and nanopore sequencing. However, due to the natural selection of some forms of aneuploidy in embryos before D5 [1, 2], analysis of the D3 embryos performed by the FISH method can provide additional information on fundamental cell processes, such as chromosome segregation patterns, and the factors that affect meiotic disjunction. In this study, we performed a comparative analysis of the chromosome segregation modes of the D3 and D5/D6 human preimplantation embryos obtained in assisted reproductive technology (ART) cycles with PGT-SR by FISH and aCGH for carriers of one of the most common balanced structural chromosome rearrangements, i.e., Robertsonian translocations (RTs).

RT is a balanced rearrangement resulting from the fusion of two non-homologous or, rarely, two homologous acrocentric chromosomes, which leads to the formation of a derivative chromosome made up of the long arm chromatin of both fused chromosomes that lacks some chromatin of the

short arm. RT is named after the American zoologist William Rees Brebner Robertson [3], who first observed and described them in orthopterans. RTs are very common balanced structural rearrangements in the general population and occur at a rate of approximately 1 in 800–1,230 newborns [4, 5].

The short arms of acrocentric chromosomes 13, 14, 15, 21, and 22 contain repetitive satellite DNA I-IV and β-satellite clusters, multiple sites for DNA-binding proteins and transcription factors, rDNA cluster, and telomeric sequences [5]. The participation of acrocentric chromosomes in RT depends on the homology of the DNA sequences of their p-arm [5]. The most common RTs in humans, which include der(13;14) and der(14;21), have a frequency of 59.0–85.0% and 10.0% of all possible RTs, respectively. Other types of RTs are rare. Based on the molecular differences in the location of breakpoints, RTs might also be divided into two classes: Class 1 (with specific breakpoints and definite mechanism of formation) and Class 2 (with highly variable breakpoints and mechanism of formation). Almost all cases of der(13;14), all cases of der(14;21), and some rare RTs formed by the recombination between their p-arms with the specific breakpoints in repeat-rich regions of satellite DNA and rDNA cluster belong to Class 1 [6]. Some Class 2 RTs, which occur in less than 2% of the cases, might be attributed to non-homologous end-joining or homologous recombination in hypervariable minisatellite DNA. These processes can produce highly variable and unique breakpoints. Class 2 RTs might also occur due to telomere stress caused by the knockdown of telomere-binding protein *TRF2* that coat telomeres on the p-arm and lead to chromatin decondensation, RNA polymerase I-mediated rDNA transcription, and chromothripsis, characterized by clustered genomic rearrangements of the involved chromosomes [5, 7].

During the first meiotic division, the chromosomes of RT carriers form a special structure called a trivalent and can segregate in the alternate, adjacent, or 3:0 modes [8]. The alternate mode of segregation produces two normal or balanced chromosome sets in the embryo after fertilization. The adjacent mode of segregation produces two types of disomic and two types of nullisomic gametes. Nullisomy results in non-viable monosomy of the conceptus and leads to miscarriage. However, disomy of the gamete leads to trisomy of the conceptus that might be viable and result in birth of a child with severe congenital abnormalities. In the 3:0 mode of segregation, double nullisomy or double disomy of the gamete leads to the formation of a non-viable monosomic or trisomic embryo [8].

Due to reproductive challenges, RT carriers appeal to IVF clinics for conducting ART cycles with PGT-SR and selecting genetically balanced embryos to increase their chances of conceiving and the birth of healthy offspring. Moreover, the aneuploidy rate in embryos conceived by women above 35 years is higher [2, 9].

In this study, we analyzed the frequency of the formation of aneuploid embryos based on the chromosomes involved in RT, the sex of the RT carrier, the day of the embryo development, and the age of the female.

## 2. Materials and Methods

### *2.1 Study Design*

IVF with PGT-SR using FISH or aCGH was performed for those couples in whom an RT was detected after karyotyping of peripheral blood lymphocytes. After PGT-SR, the types of chromosome segregation were determined by a combination of fluorescence signals (FISH) or

assessing DNA dosage gain or loss (aCGH). When counting the number of signals or evaluating the DNA dosage of the autosomes involved in the RT, it was presumed that only one homolog of each chromosome was inherited from the parent with the normal karyotype, such that the number of remaining signals would help to determine the number of chromosomes inherited from the translocation carrier and specify the segregation mode. An additional analysis was performed for the chromosomes that were not involved in rearrangement, and these chromosomes were further categorized as euploid and aneuploid. The aCGH technique was used to evaluate the entire set of chromosomes, while the FISH technique was used to analyze the chromosomes 13 (or 14 or 15), 16, 18, 21, 22, X, and Y. Thus, five groups of embryos were formed: 1) RT balanced/euploid; 2) RT balanced/aneuploid; 3) RT unbalanced/euploid; 4) RT unbalanced/aneuploid; 5) "Other" group, which consisted of embryos with an uncertain segregation mode due to a mosaic karyotype or a non-diploid set of chromosomes.

### *2.2 Karyotyping and Metaphase FISH*

Karyotyping was performed using standard cytogenetic techniques on QFH/AcD-stained metaphase chromosome preparations from peripheral blood lymphocytes stimulated with phytohemagglutinin [10]. Metaphase FISH was performed to determine breakpoints, eliminate cross-hybridization of the used probes, and prepare for FISH in blastomeres. The analysis was performed according to the following protocol: after assuring that the metaphase spreads were abundant and isolated from nuclei or other metaphases, the slides were pretreated with 1 × SSC/0.02% Tween 20 for 30 sec and 1 × SSC for 2 min and 5 min, respectively. Finally, after rinsing with water, the slides were dehydrated by dipping in 70%, 96% ethanol for 30 sec each, followed by air drying. Then, 5 µL of the probe/hybridization buffer mix was applied to the target area, covered with a cover glass, and sealed with rubber cement. The metaphase spreads and probes were denatured simultaneously at 75 °C for 5 min on a heat block and hybridized at 37 °C overnight in a humidity chamber. After removing the cover glass, the slides were equilibrated in 1 × SSC three times for 30 sec each time at 42 °C, rinsed in distilled water, and dehydrated by rinsing them with 70% and 96% ethanol for 1 min each. Then, the slides were air-dried and covered with coverslips. Centromeric and locus-specific probes flanking the breakpoints on the chromosomes involved in translocation and seven other chromosomes (13 (or 14 or 15), 16, 18, 21, 22, X, and Y) were used for performing metaphase FISH (Abbott Molecular, United States; Kreatech, Netherlands).

### *2.3 In Vitro Fertilization Laboratory Methods*

Embryological procedures, including egg retrieval, in vitro fertilization, embryo culture, and blastomere and trophectoderm biopsy, were performed based on the standard protocols routinely followed by the International Centre for Reproductive Medicine [11]. To maximize the efficacy of the treatment cycle, female patients underwent controlled ovarian stimulation followed by a recombinant human chorionic gonadotropin (hCG) injection (Ovitrelle, MERCK SERONO, Italy). Transvaginal egg collection was performed 36 h after hCG was triggered. The eggs were fertilized either by the conventional IVF method or by the intracytoplasmic sperm injection (ICSI) procedure (day 0). A fertilization check was performed on day 1 (16–18 h after fertilization). The normally fertilized zygotes with two pronuclei were cultured in sequential media (Cleavage and Blastocyst Medium, Cook Medical, USA) at 37 °C in a humidified atmosphere with 6% $CO_2$, 5% $O_2$, and 89% $N_2$.

#### 2.3.1 Biopsy and Fixation of Blastomeres Before FISH Analysis (Day 3)

For conducting the ART cycles with PGT-SR by FISH, the embryos were biopsied 66–68 h post-fertilization (D3). The embryos were considered to be suitable for biopsy if they consisted of six or more blastomeres. The selected embryos were placed in a calcium-free and magnesium-free biopsy medium (Origio, Denmark) to perform a biopsy. After mechanical hatching, one blastomere from each embryo was removed and fixed following the protocol of Tarkowski [12], with some modifications.

#### 2.3.2 Trophectoderm Biopsy (Days 5 and 6)

For conducting the ART cycles with PGT-SR by aCGH, the embryos were cultured until the blastocyst stage (D5/D6). Only good-quality embryos, determined by Gardner's blastocyst morphology assessment system [13], were selected for biopsy. After laser-assisted hatching, 5–7 trophectoderm cells were removed from each embryo. The trophectoderm sample was then washed in PBS, transferred to an Eppendorf tube with 2 µL of PBS, and frozen at –20 °C. Successfully biopsied blastocysts were cryopreserved by vitrification.

### *2.4 Blastomere FISH Analysis*

The blastomere FISH analysis was performed following the protocol described in the guidelines for clinical embryology [11]. Initially, the slides were pretreated with 1 × SSC/0.02% Tween 20 for 1 min, then immersed in 1 × SSC for 2 min, washed with purified water, and dehydrated in an ethanol series (70%, 96%). Next, 0.5 µL of the probe/hybridization buffer mix tested on metaphase spreads were placed on the slide area with fixed blastomeres, covered with a glass coverslip, and sealed with rubber glue. The samples were co-denatured at 78 °C for 9 min on a heat block during the first hybridization round, at 75 °C for 5 min during the second hybridization round, and at 70 °C–72 °C for 2–3 min during the third round. The duration of hybridization at 38 °C in a humidity chamber depended on the composition of the probe/hybridization buffer mix. As a part of the post-hybridization treatment, after removing the cover glass, the slides were placed in a Coplin jar containing 1 × SSC and washed thrice for 1 min at 42°C. Next, after rinsing with water, dehydrating in an ethanol series, and air-drying, the Vectashield antifade medium (Vector Laboratories, USA) was applied without counterstaining. Finally, each slide was covered with a cover glass.

#### 2.4.1 Image Acquisition and Analysis

Post-hybridization fluorescence images of metaphase spreads and blastomeres were obtained using an Axio Imager.A1 microscope (Carl Zeiss) equipped with fluorescence filters and an AxioCam MRc camera with a CCD matrix for signal accumulation (Carl Zeiss). The digital images were evaluated using AxioVision Rel 4.8 (Carl Zeiss) and Leica Application SuiteV.4.1.0 (Leica Microsystems, Germany).

### *2.5 Whole-genome Amplification and Array CGH*

Whole-genome amplification (WGA) of DNA from the trophectoderm cell lysate was performed using the PicoPlex SingleCell WGA Kit (Rubicon Genomics) and the SmarTer PicoPLEX WGA kit

(Takara Bio, United States) following the manufacturer's protocol. The concentration of the WGA product was measured using the Qubit dsDNA Broad-Range Assay kit (Life Technologies, United States) and the Qubit 4.0 fluorimeter (Life Technologies, United States). The WGA products were labeled using the SureTag Complete DNA Labeling kit (Agilent, United States). The labeled DNA was hybridized to the G5963 GenetiSure Pre-Screen Microarray 8 × 60 K following the manufacturer's protocol (Agilent Technologies, Santa Clara, CA, USA). The arrays were scanned using ScanRI (PerkinElmer, Waltham, MA, USA) and then processed and analyzed using the Agilent Cytogenomics software v5.2 (Agilent Technologies, Santa Clara, CA, USA).

### *2.6 Semen Samples*

Semen samples were collected from male RT carriers after sexual abstinence for 2–7 days. The parameters regarding sperm quality (volume, motility, and concentration) were assessed following the criteria of the World Health Organization [14]. The morphological characteristics of spermatozoa were evaluated according to Kruger's strict criteria [15].

### *2.7 Statistical Analysis*

Fisher's exact test was conducted to compare the differences between segregation patterns in male and female carriers, based on the following parameters: the type of RT, the day of embryo development, the aneuploidy rate for the chromosomes not involved in RT combined with balance/unbalance for RT-autosomes, and the age of females. A chi-squared test with Yates's correction was performed to compare the segregation patterns and sperm parameters in male RT carriers. All differences were considered to be statistically significant at $p < 0.05$.

All procedures were performed according to The Declaration of Helsinki. The study was approved by the institutional ethics committee of the Almazov National Medical Research Center (no. 02–21 from February 15, 2021). All couples signed the informed consent form.

## 3. Results

### *3.1 General Information*

A total of 100 couples, who were carriers of different types of RTs (47 females and 53 males) and underwent ART treatment between 2014 and 2021 in two reproductive centers (International Centre for Reproductive Medicine and Scandinavia Ava-Peter), were included in this study. The study was performed using the large-scale research facility # 3076082, "Human Reproductive Health". A total of 964 embryos were analyzed; 805 embryos were evaluated on D3 of development by performing FISH, and 159 embryos were evaluated on D5/D6 of development by performing aCGH. The mean age of female and male RT carriers was 33.3 ±5.2 and 32.7 ±3.9 years, respectively.

### *3.2 Chromosome Segregation Analysis*

An example of the PGT-SR data analysis for segregation pattern and aneuploidy for chromosomes not involved in RT is shown in Figure 1.

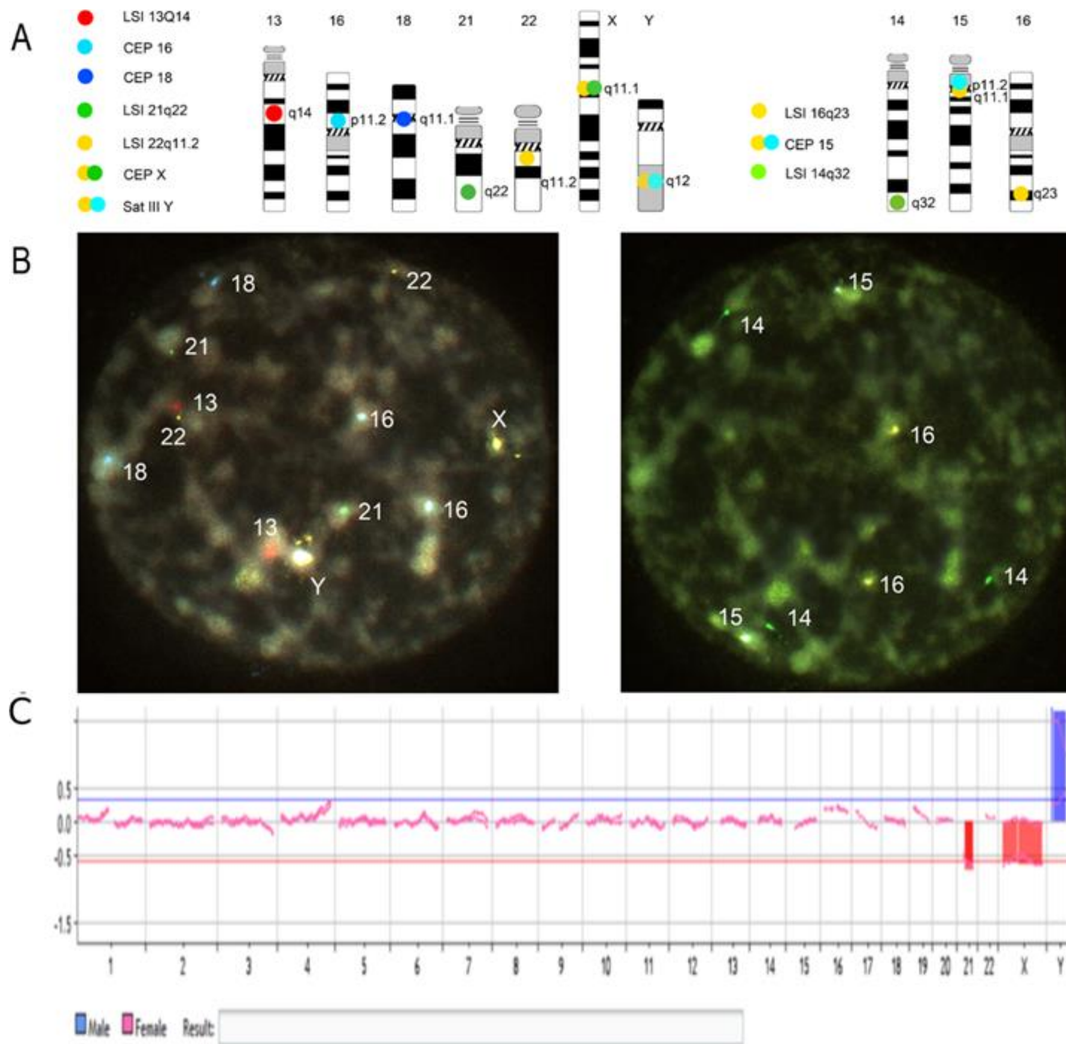


**Figure 1** The scheme of hybridization mixtures (A), the results of FISH on a blastomere for der(13;14) carrier (B), and the results of aCGH on trophectoderm cells for der(14;21) carrier (C). B) The number of fluorescent signals corresponds to the adjacent mode of segregation. C) The aCGH profile shows chromosome 21 monosomy resulting from an adjacent segregation mode.

The results of the PGT-SR analysis and the distribution of embryos depending on the chromosomes involved in the formation of RT and segregation mode are shown in Tables S1a-b and S2a-b and Figures 2A-C.

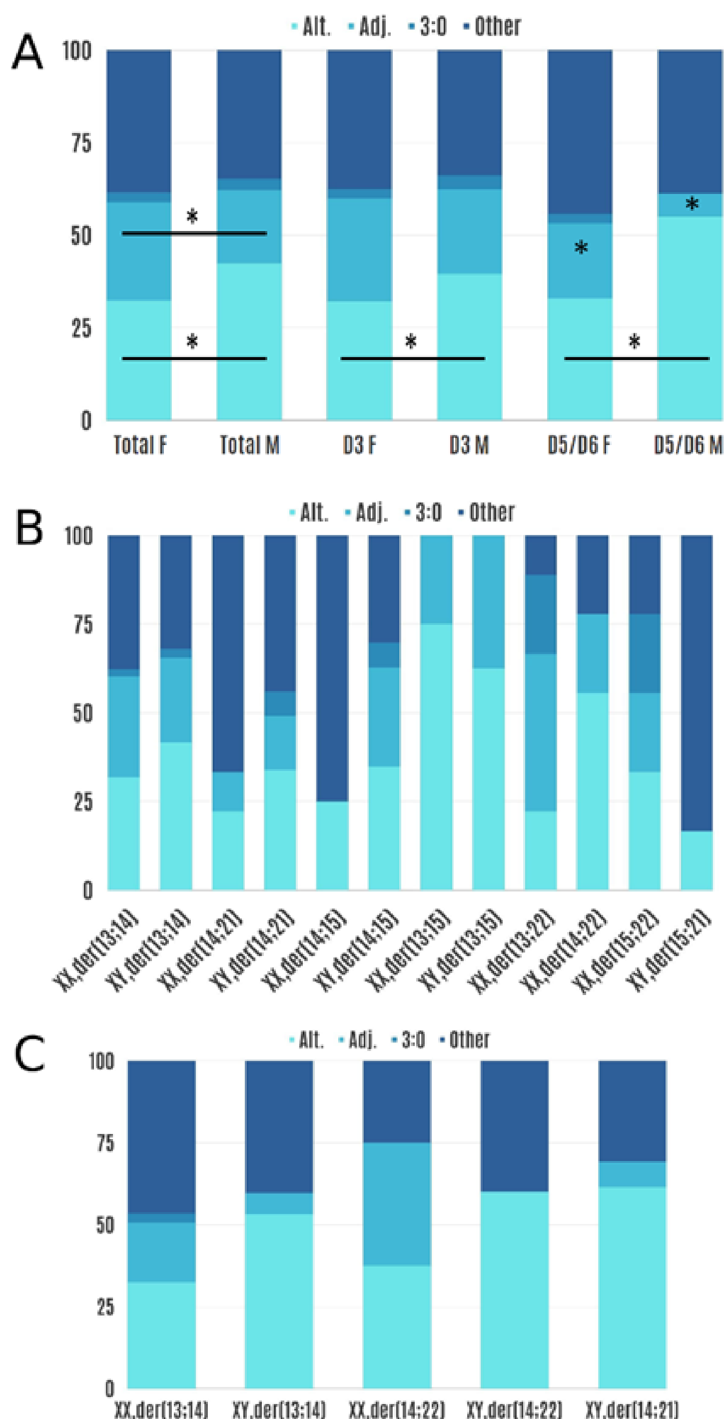


**Figure 2** The chromosome segregation modes in embryos were based on the sex of the carriers and the day of embryo development (A), the type of RT, and the day of embryo development (B and C). B) The embryos were analyzed on D3 of development. C) The embryos were analyzed on D5/D6 of development. Alt: alternate mode; Adj: adjacent mode. Asterisks indicate statistically significant differences. Total F and Total M denote total embryos for female and male RT carriers, respectively.

Overall, the alternate mode of segregation was predominant in male RT carriers and occurred in 42.4% of the analyzed embryos, irrespective of the involved chromosomes and the day of embryo development. The pathological modes of segregation (adjacent and 3:0) were detected in 19.8%

and 3% of embryos, respectively. In female RT carriers, the alternate and adjacent segregation modes were observed in 32.3% and 26.6% of the embryos, respectively, and were predominant. The 3:0 mode of segregation was detected only in 2.6% of the embryos. In more than one-third of all analyzed embryos, determining the type of chromosome segregation was not possible (group “other”) due to mosaicism and/or ploidy aberration. Female and male carriers showed significant differences in the distribution of segregation modes of embryos for alternate (p = 0.0013) and adjacent (p = 0.0146) segregants, but not for the 3:0 (p = 0.845) and “other” (p = 0.2541) segregants (Figure 2A).

Analysis of the distribution of the embryos depending on the sex of the carriers and the day of development also showed differences between male and female carriers. The alternate mode of segregation was detected in 32.2% and 39.5% (p = 0.0314) of D3 embryos from female and male carriers, respectively. The adjacent segregation mode was found in 27.7% and 22.9% (p = 0.1229) of D3 embryos from female and male carriers, respectively. The 3:0 mode of segregation was observed in 2.6% and 3.7% (p = 0.4212) of D3 embryos from female and male RT carriers, respectively (Figure 2A).

In D5/D6 embryos from female and male carriers, the distribution was as follows: alternate mode in 32.9% vs. 55% (p = 0.0066); adjacent mode in 20.3% vs. 6.3% (p = 0.0103); 3:0 mode in 2.5% vs. 0% (p = 0.2453) (Figure 2A).

Moreover, male carriers had a higher frequency of the alternate mode of segregation than female carriers with the same type of RT, irrespective of the day of embryo development (Figures 2B and C).

Additionally, a detailed comparison of the segregation patterns was performed based on the day of embryo development, type of RT, and the sex of the carriers for der(13;14), der(14;21), and der(14;22), because for these translocations, the PGT-SR data were available for both D3 and D5/D6 embryos. In the female carriers of der(13;14), the frequency of the alternate mode of segregation was not significantly different between D3 and D5/D6 embryos (31.8% vs. 32.4%, p = 0.8912). For the adjacent mode of segregation, the frequency was 28.5% vs. 18.3% (p = 0.0824), and for the 3:0 mode, the frequency was 2% vs. 2.8% (p = 0.6503) for D3 and D5/D6 embryos, respectively. The opposite pattern was detected for the female carriers of der(14;22). The D5/D6 embryos had a lower frequency of the alternate mode of segregation than the D3 embryos (55.6% vs. 37.5%, p = 0.6372), while the frequency of the adjacent mode was higher (22.2% vs. 37.5%; p = 0.6199) (Figures 2B and C). On the contrary, in the male carriers of der(13;14), the comparison between D3 and D5/D6 embryos showed an increase in the frequency of the alternate mode of segregation (41.6% vs. 53.2%, p = 0.1148) and a decrease in the frequency of pathological segregation modes, i.e., the adjacent mode: 23.9% vs. 6.5% (p = 0.0014) and the 3:0 mode: 2.5% vs. 0% (p = 0.6049). A similar pattern was observed for the male carriers of der(14;21), where alternate segregation was found in 33.9% and 61.5% (p = 0.1135), adjacent segregation was found in 15.3% and 7.7% (p = 0.6774), and 3:0 segregation was found in 6.8% and 0% (p = 1.000) of the D3 and D5/D6 embryos, respectively.

A detailed analysis of the distribution of embryos with pathological modes of segregation showed that in the case of male RT carriers, the derivative chromosomes (trisomy A, trisomy B, and trisomy A+B variants) were inherited by 42.8% of the embryos, and in the case of female carriers, the derivative chromosomes were inherited by 39.4% (p = 0.6012) of the embryos, irrespective of the day of embryo development and acrocentric autosomes involved in the formation of RT (Table S3).

### *3.3 Analysis of the Aneuploidy Rate for Non-RT Chromosomes Depending on the Balance/Unbalance of RT-autosomes*

The analysis of the distribution of the embryos between groups based on the combination of autosomes involved in the rearrangement by their balance/unbalance and euploidy/aneuploidy of the chromosomes not involved in the rearrangement showed that the number of balanced/euploid embryos was 23.6% and 32.9% for female and male RT carriers, respectively ($p = 0.0185$), irrespective of the type of RT, female age, and the day of embryo development (Tables S4a-b). In the group with maternal age ≤35 years, the number of balanced/euploid embryos was 23.7% for female RT carriers and 33.9% for male RT carriers ($p = 0.0002$). In the group with maternal age ≥36 years, the number of balanced/euploid embryos was 23.3% and 29.4% ($p = 0.7977$) in the case of female and male RT carriers, respectively (Tables S4a-b).

The total aneuploidy rate for chromosomes not involved in the rearrangement (irrespective of the balance or unbalance for autosomes involved in rearrangement) was 42.7% and 39.9% ($p = 0.3935$) for embryos from female and male carriers, respectively. The distribution of aneuploid embryos with maternal age was 41.8% for female RT carriers and 38.7% for male RT carriers in the group with maternal age ≤35 years ($p = 0.777$). In the group with maternal age ≥36 years, the frequency of aneuploid embryos was 44.7% and 44.3% for female and male carriers of RT, respectively ($p = 0.0869$) (Tables S4a-b).

In general, the frequency of balanced/euploid embryos was higher for the male RT carriers than for the female RT carriers, irrespective of maternal age.

### *3.4 Analysis of Sperm Parameters Based on Chromosome Segregation Patterns in Embryos Derived from Male RT Carriers*

We compared the frequency of the segregation patterns in embryos from male carriers depending on the parameters of the spermogram. The segregation modes detected in embryos obtained in the ART cycles from 15 male carriers of RT were analyzed regarding sperm count and motility. Ten of the carriers had oligozoospermia, and nine of them had reduced sperm motility (asthenozoospermia). The segregation products observed in the embryos obtained in the IVF cycles from male carriers with oligozoospermia and/or asthenozoospermia are shown in Table S5 and Figure 3.

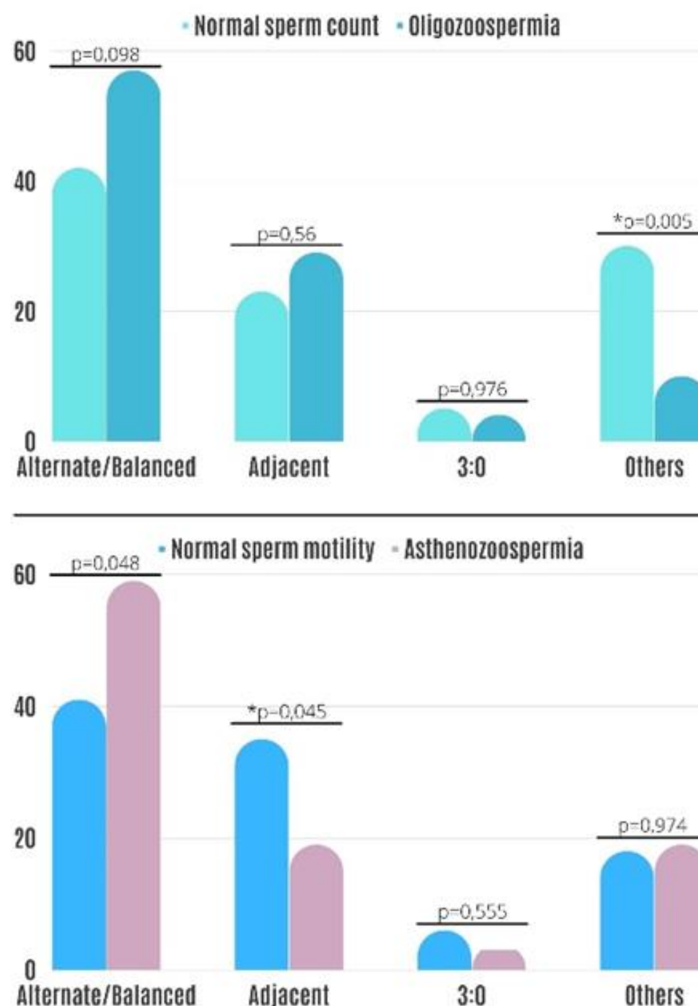


**Figure 3** The relationship between the segregation modes and spermogram parameters in male RT carriers. The top graph shows the association between the frequency of segregation types and sperm count. The bottom graph shows the association between the frequency of segregation types and sperm motility.

No significant difference in the mean frequencies of normal/balanced embryos (due to the alternate mode of segregation) depending on the sperm concentration was found (42% in carriers with normal sperm count vs. 57% in carriers with oligozoospermia, p = 0.098). The alternate segregation frequency also did not depend on sperm motility, with a frequency of 41% in carriers with normal sperm motility and 59% in those with asthenozoospermia (p = 0.048).

The frequency of the observed adjacent and 3:0 segregation modes was also similar in the groups of male carriers with oligozoospermia and normal sperm count (3.5% vs. 5%, p = 0.976). The frequency of the adjacent segregation pattern was higher in patients with asthenozoospermia than in patients with normal motility (35% vs. 19%; p = 0.045%). The frequency of uncertain segregation patterns in aneuploid cells was significantly higher in patients with normal sperm count than in patients with oligozoospermia (30% vs. 10%, “other” group, p = 0.005) (Figure 3).

Overall, the alternate mode was predominantly observed in embryos of male RT carriers with abnormal semen concentration and sperm motility.

## 4. Discussion

### *4.1 Female and Male RT Carriers Have Different Reproductive Risks Related to Chromosome Segregation Pattern and Rate of Balanced/Euploid Embryos*

Traditionally, FISH of the oocyte polar bodies or the blastomere of a D3 embryo was used for PGT. PGT-A is now performed using comprehensive methods such as aCGH and NGS, but the data obtained by FISH in PGT-SR can still be used to analyze the segregation pattern in preimplantation embryos. In this study, we found that female and male carriers of RT have different reproductive risks associated with chromosome segregation patterns. The alternate mode of segregation was more common in the embryos from male RT carriers than in those from female RT carriers. On the other hand, the adjacent mode of segregation was detected significantly more often in embryos from female RT carriers than in those from male RT carriers. Moreover, the predominance of alternate segregation in males was detected irrespective of the autosomes involved in the formation of RT. Similar results were found for male [7] and female [16, 17] gametogenesis and D5/D6 preimplantation embryos [18-20]. The predominance of the alternate mode of segregation in male RT carriers might be due to the association with the specific orientation of the trivalent in cis-configuration and strict meiotic checkpoints and/or post-meiotic differentiation [7]. Additionally, in male carriers, the number of balanced/normal sperm due to the alternate mode of segregation is formed in equal ratios for most of the studied RTs [21] (der(13;14); der(13;15); der(21;22)), except for der(14;21), in which more normal sperm than balanced sperm is present [21]. However, during *in vivo* fertilization, spermatozoa carrying a balanced chromosome set are preferred, as it was shown for der(13;14), based on prenatal data and the frequency of the offspring with a balanced karyotype from XY,der(13;14) carriers [22].

Natural selection might lead to an increase in the number of balanced embryos from D3 to D5/D6 [23]. The results of our study supported this hypothesis, as we found that the unbalanced segregation rates decreased from D3 to D5/D6. Additionally, our results suggested that this process might depend on the sex of the RT carriers and the chromosomes involved in the formation of RT. For male carriers, the rate of balanced embryos increased by 1.4 times from D3 to D5/D6 (39.5% vs. 55%), while for female carriers, it increased by 1.02 times (32.2% vs. 32.9%), irrespective of the chromosomes involved in the formation of RT. In contrast, the frequency of unbalanced embryos decreased by 1.27 times from D3 to D5/D6 for female carriers (30.3% vs. 23.8%) and by 4.22 times for male carriers (26.6% vs. 6.3%), irrespective of the chromosomes involved in the formation of RT. Similar results were found in the embryos depending on the sex of the RT carrier and the chromosomes involved in the formation of RT, as was shown for der(13;14) carriers. Moreover, for XX, der(14;22), the frequency of balanced embryos decreased by D5/D6 of development, while the frequency of unbalanced ones increased. This suggested a high genetic risk for female carriers of this translocation. Also, RT carriers with a history of infertility might have a family member with the same translocation but without reproductive problems [24]. That can partly be explained by the segregation pattern of the autosomes involved in the formation of RT and the natural selection of balanced/unbalanced embryos based on the sex (if the relative is of different sex) and type of RT, as shown in our study. However, variations in fertility levels in same-sex carriers of the same RT remain unclear. Studies on the nuclear organization of the carrier gametes might provide some information regarding this issue [4].

Inheritance of the derivative autosomes forming Robertsonian structures could be sex-dependent. For example, der(13;14) in the balanced form has a predominantly paternal inheritance, while in the unbalanced form, it is passed to the offspring without the influence of the sex of the RT carriers [22, 25]. If der(14;21) is inherited from females, it is often accompanied by trisomy 21. In contrast, the risks of an unbalanced karyotype in an offspring are lower if der(14;21) is inherited from males [22, 25, 26]. Our detailed analysis of the frequency of pathological segregants containing the derivative RT autosomes (trisomy A; trisomy B and trisomy A+B variants, Table S3) confirmed that segregation occurred irrespective of the sex of the carriers. This was shown for both female and male carriers and for der(13;14) in particular. Thus, in general, for both male and female carriers, an unbalanced inheritance of the RT was predominantly caused by the formation of gametes without derivative autosomes. Such variants led to monosomy for the chromosomes involved in the formation of RT that would have terminated embryo development before implantation or *in utero*. Analysis of a larger number of embryos and RT types could prove the presence of and the biological reasons for the selection against the unbalanced inheritance of the derivative autosomes.

The female reproductive age determines the frequency of the formation of euploid eggs and embryos [27]. Thus, we determined the rate of occurrence of aneuploid embryos from female and male carriers in general (without selection by female age, termed “Total F” and “Total M”) and also according to the female age in two groups: 1) up to 35 years (≤35) and 2) 36 years and older (≥36). We found that the greatest number of balanced/euploid embryos (about 33%) were obtained from male carriers of RT, while the least number of such embryos (about 24%) were obtained from female carriers. Similar results with a larger number of balanced/euploid embryos from male RT carriers were demonstrated depending on the female age and the day of embryo development. Additionally, a dramatic increase in the aneuploidy rate in older females was not detected. The major differences in D5 embryos in the group with maternal age up to 35 years (45.2% vs. 9.8% for female and male RT carriers, respectively) probably resulted from the number of analyzed embryos. Data on the rate of occurrence of aneuploid gametes or embryos in RT carriers with abnormalities for chromosomes not involved in translocation (interchromosomal effects) are contradictory. Some studies found no influence of the age of the carrier or female age on the segregation patterns of the trivalent [18], while other studies demonstrated an increase in interchromosomal effects in young male carriers [20]. This indicated a possible role of additional factors on the interchromosomal effects (for example, chromosomes involved in rearrangement), as well as the need for further studies on larger samples and types of RTs.

Thus, female carriers of RTs might have higher reproductive risk related to chromosome segregation patterns and the rate of occurrence of balanced/euploid embryos than male carriers of the same type of RTs.

### *4.2 High Frequency of Balanced/Euploid Embryos in Male RT Carriers Regardless of the Spermogram Abnormality can Indicate a Positive Outcome of the IVF Program*

The morphological characteristics and maturation of spermatozoa do not affect the aneuploidy rate and the observation frequency of the adjacent and 3:0 segregation patterns [28, 29]. The lack of a clear association between a decrease in sperm count and sperm aneuploidy rate in our study was consistent with the results of a previous study, which demonstrated the same frequency of formation of balanced gametes in male RT carriers with oligozoospermia and even

oligoasthenoteratozoospermia, i.e., carriers with a sperm count above $20 \times 10^6$/mL [30]. At the same time, a study conducted on a relatively large sample of 29 male RT carriers found that the aneuploidy rate was two times higher in oligozoospermic patients than in normozoospermic carriers (18.0% vs. 9.7%) [31].

However, despite the high frequency of alternate segregation, there were differences in the observed pathological types of chromosome segregation. For example, the adjacent segregation pattern in asthenozoospermic carriers was a very common pathological type, while uncertain segregation was more typical for patients with normal spermogram parameters. This tendency might partly be explained by chromosome malsegregation during gametogenesis in the second partner; also, the small sample size cannot be ignored.

The frequency of the 3:0 mode of segregation was low, and it occurred with the same frequency regardless of sperm count or motility. Since no spermogram parameters were predisposing to a 3:0 segregation pattern, it might be considered to be sporadic in male RT carriers.

## 5. Conclusions

This study has some limitations. Most of the embryos were obtained in ART cycles with PGT-SR for carriers of the most common variant of RT, i.e., der(13;14), on D3 of embryo development. It was presumed that only one homologue of each RT-autosomes was inherited from the parent with the normal karyotype. In D3 embryos, only seven additional non-RT chromosomes were analyzed, which could misrepresent the total number of embryos with balanced or unbalanced RT-autosome sets along with aneuploidy for other chromosomes. The spermogram data were available only for 15 out of 53 male carriers. The parental origin of the chromosomes involved in aneuploidy was not analyzed. Some normal/balanced embryos could mask uniparental disomy (UPD) that might have appeared after correction of trisomy or monosomy of autosomes with imprinted genes, for example, chromosomes 14 and/or 15, which could be a (rare) important reproductive risk in couples carrying RTs [32].

Although a successful natural pregnancy is possible in patients with an RT, the ART cycles with PGT-SR of embryos might be a better option for carriers with a history of infertility. Our results suggested that female and male RT carriers have different reproductive risks related to chromosome segregation patterns and the rate of occurrence of balanced/euploid embryos. The rate of occurrence of embryos with the alternate mode of segregation and euploid for other chromosomes was higher in male carriers than in female carriers, regardless of the type of RT. Moreover, our results suggested that there was no association between sperm parameters and malsegregation rates in male RT carriers. Thus, the high rate of formation of balanced/euploid embryos in male carriers of RTs gives hope for a positive outcome of the IVF program without the use of donor sperm.

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## Author Contributions

I.L.P., Z.N.T., A.N.P. designed the study, analyzed the data, and prepared the manuscript. A.A.K. and S.V.K. analyzed the data and prepared the manuscript. A.F.S., Y.A.L., J.R.P., O.A.L., O.G.C., O.V.M., E.M.F., N.K.B. performed laboratory studies, reviewed, and modified the manuscript. T.V.V. critically reviewed and modified the manuscript. All authors have approved the final manuscript.

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## Competing Interests

The authors have declared that no competing interests exist.

## Additional Materials

The following additional materials are uploaded at the page of this paper.

1. Table S1a: Analysis of the segregation modes in D3 cleavage stage embryos derived from female carriers of various types of RTs based on the FISH method.
2. Table S1b: Analysis of the segregation modes in D5/D6 embryos derived from female carriers of various types of RTs based on the aCGH method.
3. Table S2a: Analysis of the segregation modes in D3 cleavage stage embryos derived from male carriers of various types of RTs based on the FISH method.
4. Table S2b: Analysis of the segregation modes in D5/D6 embryos derived from male carriers of various types of RTs based on the aCGH method.
5. Table S3: The incidence of aneuploidies for the chromosomes involved in RTs* in D3 and D5/D6 embryos.
6. Table S4a: The genetic status of the embryos derived from female carriers of RTs based on female age (≤35 years/≥36 years).
7. Table S4b: The genetic status of the embryos derived from male carriers of RTs based on female age (≤35 years/≥36 years).
8. Table S5: The segregation products in male carriers with oligozoospermia/asthenozoospermia.

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