

## Chromosomal abnormalities in men with azoospermia

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### Abstract

**Background:** Infertility affects about 15 percent of all couples attempting pregnancy, with the man responsible in approximately half the cases. Azoospermia is detected in up to 8% of male infertility situations. The prevalence of chromosomal abnormalities is increased in azoospermic men.

**Material and methods:** We performed cytogenetic analysis in a group of 128 infertile men with azoospermia from the Republic of Moldova during 2013-2018 period. Karyotyping was performed on peripheral blood lymphocytes according to standard methods of G-banding of metaphase chromosomes. For reporting the results, the 2016 *International System of Cytogenetic Nomenclature* was used.

**Results:** Chromosomal variations were identified in 48 infertile men with azoospermia. In 38 cases were found abnormalities of gonosomes and in 10 cases abnormalities of autosomes. The most common sex chromosomal abnormality was Klinefelter syndrome: in 21 (55.3%, 95CI 47.23-63.37) cases homogeneous form 47,XXY and in 4 (10.5%, 95CI 5.52-15.48) cases mosaic form. Y-chromosome aberrations were also identified: in 7 (18.4%, 95CI 12.11-24.69) cases was noticed duplication of distal arm 46,XYqh+ and in 3 (7.9%, 95CI 3.53-12.27) cases deletion of the same arm 46,X,del(Y). Additionally, 45,X/46,XY and 46,XX karyotypes were found.

**Conclusions:** 38% of the studied group have chromosomal variations that may explain the origin of infertility. All men with azoospermia should be offered cytogenetic screening followed by appropriate genetic counseling before infertility treatment.

**Key words:** infertility, azoospermia, chromosomal abnormalities.

### Cite this article

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### Introduction

Globally, it is estimated that about 15% of couples of reproductive age face fertility problems [1]. Infertility affects both men and women, and in about half of the cases a male factor can be identified [2]. The term "male infertility" is not a clinically defined syndrome, but rather a collection of heterogeneous conditions, most commonly caused by disorders of spermatogenesis, clinically manifested by asthenozoospermia, teratozoospermia, oligozoospermia and azoospermia [3].

Azoospermia is found in about 8% of infertile men and 1% in the male population [4]. The role of genetic factors in the pathogenesis of male infertility has become increasingly recognized by reproductive specialists. The individual's genome contributes to infertility by influencing the anatomy of the urogenital tract and physiological processes, including hormonal homeostasis, spermatogenesis and sperm quality. The most common genetic causes of azoospermia are chromosomal abnormalities and their frequency is negatively correlated with the concentration of sperm. In azoospermic patients the prevalence of reported chromosomal variations was between 15% and 25%, depending on the subgroup of

azoospermic men studied [5]. Chromosomal abnormalities in infertile men can be numerical or structural, with the involvement of sex chromosomes or autosomes [6].

The introduction of assisted reproduction techniques such as intracytoplasmic sperm injection (ICSI) and microsurgical sperm extraction (micro-TESE) presents an option for infertile couples to overcome the factor of male infertility [7]. The use of these ICSI and micro-TESE techniques can overcome the barrier in the process of natural fertilization, but there are many concerns about the safety of ICSI and the likely transmission of genetic abnormalities to offspring [8].

Before resorting to assisted reproduction techniques, cytogenetic examination is mandatory to detect the cause of male infertility with severely affected spermiogram. The identification of an abnormal karyotype as well as chromosomal polymorphisms should lead to a comprehensive genetic counseling, which should include all information about the individual type of abnormality, its clinical relevance, possible inheritance / transmission, genetic risk for offspring [9]. This allows infertile couples to make an informed decision when opting for medically assisted reproduction. Therefore, cytogenetic screening continues to

Table 1

## Distribution of chromosomal abnormalities in men with azoospermia, years 2013–2018

Years	Abs. No men with azoospermia	The average age/ years	46,XY		Karyotype with chromosomal variations	
			Abs. No	%, 95 <sub>CI</sub>	Abs. No	%, 95 <sub>CI</sub>
2013	22	35	15	11.7%, 95CI 8.86-14.54	6	4.7%, 95CI 2.83-6.65
2014	23	35	13	10.2%, 95CI 7.53-12.87	8	6.3%, 95CI 4.16-8.44
2015	22	33	12	9.4%, 95CI 6.82-11.98	10	7.8%, 95CI 5.43-10.17
2016	21	33	12	9.4%, 95CI 6.82-11.98	9	7.0%, 95CI 4.74-9.26
2017	22	26	13	10.2%, 95CI 7.53-12.87	8	6.3%, 95CI 4.16-8.46
2018	18	32	13	10.2%, 95CI 7.53-12.87	5	3.9%, 95CI 2.19-5.61
Total	128	32	80	62.0%, 95CI 58.22-66.78	48	38.0% 95CI 5.33.22-41.78

remain a good practice for proper diagnosis, treatment, evaluation and prognosis [9, 10].

**The aim of the study:** to evaluate the frequency of chromosomal variations in azoospermic men and to confirm the cytogenetic exploration of infertile men for diagnosis, treatment and prognosis.

### Material and methods

The research presents a retrospective descriptive study of a selected group of 128 infertile men with azoospermia, from the population of the Republic of Moldova during the years 2013-2018. Patients come from infertile couples who are referred to the National Center for Reproductive Health and Medical Genetics. The spermogram was performed after a period of 2–7 days of sexual abstinence, according to the reference criteria of the 2010 sperm analysis of the World Health Organization (WHO). All patients were cytogenetically investigated by the classic G-banding technique, on 15 peripheral blood lymphocytes being analyzed 15 metaphases of which 5 karyotyped. Nomenclature according to 2016 ISCN (International System of Cytogenetic Nomenclature) was used for reporting the results.

### Results

128 men with azoospermia were cytogenetically investigated in 2013–2018 at the department of the National Center for Reproductive Health and Medical Genetics (tab. 1). The number of azoospermic men investigated cytogenetically was distributed by years as follows: 22 patients in 2013, 23 patients in 2014, 22 patients in 2015, 21 patients in 2016, 22 patients in 2017 and 18 patients in 2018, which shows that the number of cytogenetic investigations is relatively constant. The same homogeneity is observed at the age at which patients were referred for consultation: in 2013–2014 the average age was 35 years, in 2015–2016 – 33 years, in 2017 – 26 years, in 2018 – 32 years.

Of the total number of 128 infertile men with azoospermia, 80 (62%) showed normal karyotype 46,XY (tab. 1, fig. 1) and 48 (38%) showed variations in the number or structure of chromosomes. 38 patients (30%) showed variations in the X or Y sex chromosomes, and 10 patients (8%) had variations in the autosomal chromosomes (fig. 1, tab. 2, tab. 3).

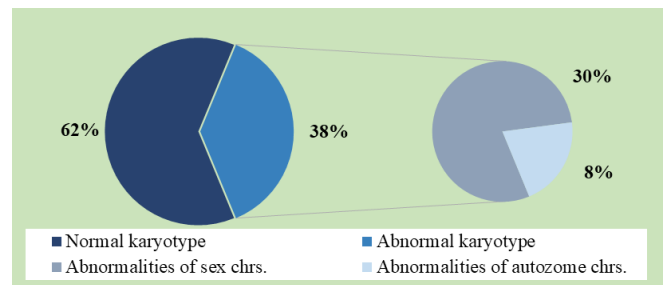


Fig. 1. Frequency of azoospermic men with chromosomal abnormalities in 2013-2018.

In the 38 patients identified with sex chromosomes abnormalities (tab. 2), in 28 cases numerical abnormalities were detected and in 10 cases – structural variations. Among the numerical chromosomal abnormalities, in 25 cases was identified aneuploidy X (Klinefelter Syndrome), in 2 cases – mosaic 45,X/46,XY and in one case – male 46,XX. The structural variations of sex chromosomes detected were in 7 cases duplications of the distal arm of the Y chromosome and in 3 cases deletions of the distal arm of the Y chromosome.

Table 2

### Distribution of infertile men with azoospermia by sex chromosomal abnormalities

Karyotype	Abs. No. (n=38)	%, 95 <sub>CI</sub>
47,XXY	21	55.3%, 95CI 47.23-63.37
47,XXY/46,XY	3	7.9%, 95CI 3.53-12.27
47,XXY/46,XX(80%/20%)	1	2.6%, 95CI 0-5.2
45,X/46,XY	2	5.3%, 95CI 1.68-8.92
46,XX	1	2.6%, 95CI 0-5.2
46,XYqh+	2	5.3%, 95CI 1.68-8.92
46,XYqh+ (Yqh≤18q)	3	7.9%, 95CI 3.53-12.27
46,XYqh+ (Yqh<18q)	1	2.6%, 95CI 0-5.2
46,XYqh+, 22 ps+ (Yqh=18q)	1	2.6%, 95CI 0-5.2
46,Xdel(Y)(q11.23→qter)	2	5.3%, 95CI 1.68-8.92
46,Xdel(Y)(q11.22→qter)	1	2.6%, 95CI 0-5.2

The most common cytogenetic variant of Klinefelter Syndrome identified was the classical form 47,XXY in 21 cases (84%) (Figure 3), followed by the forms: mosaic

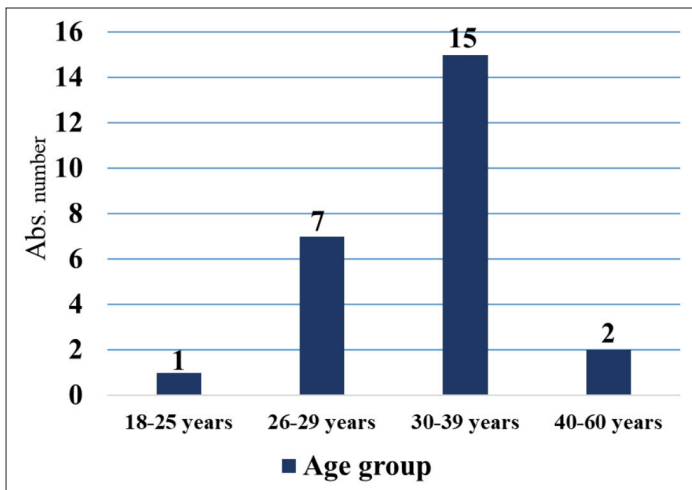


Fig. 2. Distribution of diagnosed cases with Klinefelter Syndrome by age groups

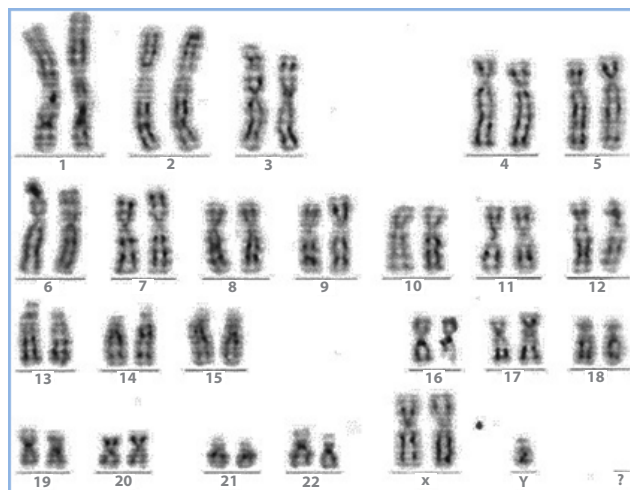


Fig. 3. Karyotype with 47,XXY Klinefelter Syndrome, in a 31-year-old patient

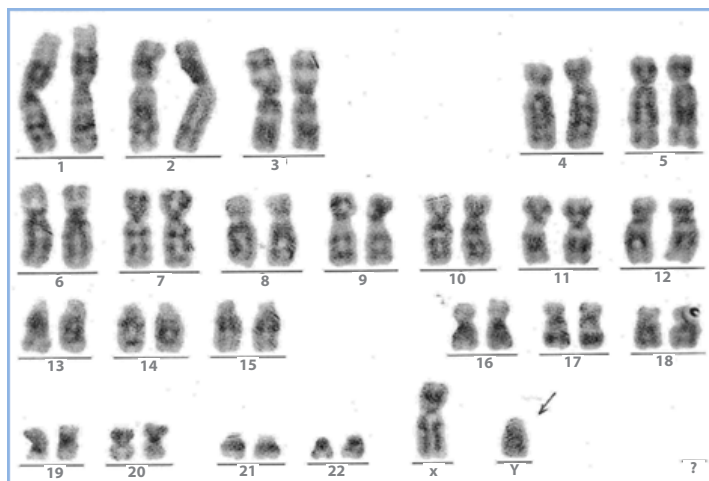


Fig. 4. Karyotype 46,XYqh+ in azoospermic male, 36 years.



Fig. 5. Karyotype 46,Xdel(Y)(q11.23->qter) in male with azoospermia, 45 years.

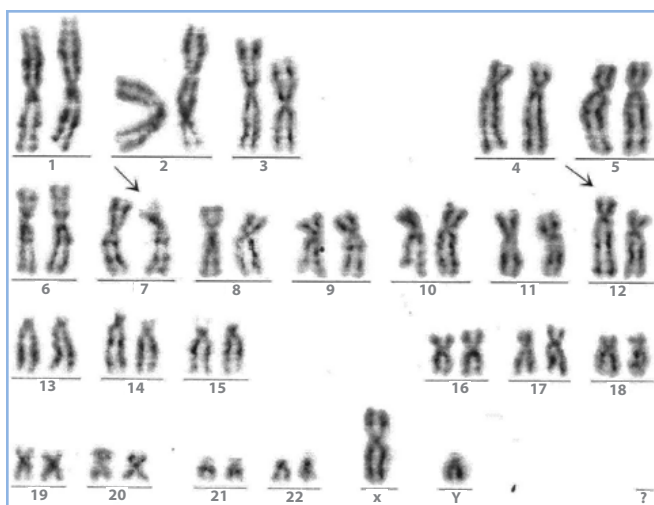


Fig. 6. 46,XY,der(7),t(12;7)(12qter::7p21->pter) karyotype in male, 30 years.



Fig. 7. 46,XY,t(8;7)(8qter::7q336->qter), karyotype in male, 31 years.



47,XXY/46,XY in 3 cases (12%) and in 1 case 47,XXY/46,XX (4%). Most cases – 15 patients – were diagnosed at the post-pubertal age of 30–39 years, 7 cases at the age of 26–29 years, 2 cases 40 and more and one case at 24 years.

Table 2

**Distribution of autosomal chromosome variations in men with azoospermia**

Karyotype	Abs. No (n=10)
46,XY,der(7),t(12;7)(12qter::7p21→pter)	1
46,XY,der(15), t(13;15) (13qter::15q23→qter)	1
46,XY,t(8;7)(8qter::7q336→qter)	1
46,XY-15-12,+der(15),+rec(12;15),t(13;12)7p+	1
46,XY,der(5),t(9;5)(9pter::5q23.3→qter)	1
46,XY,15ps+	1
46,XY,14 ps+	1
46,XY,13 ps+	1
46,XYinv(9)(p;q)	1
46,XY,1q+	1

In 10 cases, variations of autosomal chromosomes were detected, including duplicate satellites of acrocentric chromosomes 13, 14, 15, and 22 (tab. 3), and in one patient it was accompanied by changes in the sex chromosome.

### Discussion

The frequency of chromosomal abnormalities identified in the selected group with azoospermic infertile men in 2013–2018 was (38%), higher than that cited in other bibliographic sources [6, 11]. The results of our study are probably due to thorough clinical selection prior to cytogenetic investigation and, of course, due to the selective group of men with azoospermia. The most common chromosomal abnormalities were identified by sex chromosome abnormalities in 30%. Autosomal chromosome abnormalities were detected in 8% (fig. 1, tab. 2, tab. 3).

Of the 128 cytogenetically investigated azoospermic men, 25 had X disomy with a frequency of 32%. This high frequency of Klinefelter Syndrome among infertile men is also reported in bibliographic sources [11]. The results obtained in our study are similar to the data in the literature which reports the same high incidence of 80–90% for the classic form 47,XXY of Klinefelter Syndrome and in about 20% the mosaic forms are described [12].

Klinefelter syndrome is characterized by both cytogenetic and phenotypic diversity, with age the clinical picture worsens, so the diagnosis of patients at an early age can be failed. The same phenomenon is observed in our study (fig. 2, fig. 3), most cases were diagnosed post-pubertal, which is an unfavorable factor for the success rate of sperm recovery.

The genetic cause of Klinefelter Syndrome is the presence of one or more additional X chromosomes obtained by non-disjunction during maternal or paternal gametogenesis. The severity of the clinical picture is directly proportional to the number of additional X chromosomes. The

genes on the additional X chromosomes are inactivated, but in more than 15% they escape the inactivation process, including genes from the pseudoautosomal regions PAR1 and PAR2. A gene imbalance is determined by a higher level of gene expression that can compromise testicular function or influence the meiotic process playing an important role in the pathogenesis of this syndrome [13].

At the same time, 7–8% of individuals with 47, XXY can produce spermatozoa, in 30–50% micro-TESE allows the recovery of testicular sperm in young people, which helps patients with Klinefelter to conceive their own genetic children; these can be explained by: 1) testicular mosaicism – some spermatogonia lose the supernumerary X chromosome becoming 46,XY ensuring a normal spermatogenesis; 2) selective and variable inactivation of linked X genes that are expressed in the testicles; 3) polymorphisms in the AR gene – a number of trinucleotide repeats CAG from 9 to 37 times – determine normal testosterone levels and, implicitly, normal gonadotropin concentrations that will support the normal functioning of germ cells 47,XXY including spermatogenesis. With age, the chance of sperm recovery in people with Klinefelter syndrome decreases, but studies show that the average age of detection of people with this syndrome is around 25 years, indicating the importance of an early diagnosis that would allow preventive cryopreservation of sperm ejaculated or obtained by micro-TESE to maintain fertility [14].

In 2 male patients, the rare type of the 45,X/46,XY mosaic was identified. The significance of mosaic 45, X/46,XY in bibliographic sources is controversial and presents a great clinical challenge, because it can affect growth, hormonal balance, gonadal development, but also in some cases may have a normal phenotype [15]. Therefore, the detection of these cases without clinical changes can be quite late, as in our study, the first case was detected at the age of 30 and the second at the age of 35. Both patients were investigated cytogenetically due to azoospermia.

A karyotype 46,XX was identified in a 23-year-old azoospermic man. The male phenotype can be explained by the translocation of the masculinization SRY region (Yp11.32) to the X chromosome or one of the other chromosomes, but due to the lack of Yq and AZF genes involved in spermatogenesis – men 46,XX are infertile. The frequency of men with XX in the general population being very rare (1 in 10000, from Chapelle et al. 1990), it is identified only in the case of azoospermic men.

The polymorphic variant of the Y chromosome (Yqh+) was diagnosed in 7 patients. Y chromosomal polymorphisms are mentioned in several studies on male infertility mainly in azoospermia and severe oligozoospermia. This topic is becoming increasingly controversial due to the role of heterochromatin, without having a fully elucidated clinical relevance. The Yqh+ chromosome was associated with an increased risk of pregnancy loss, while in another study this relationship was not found [16, 17]. These patients probably need additional molecular investigations to investigate the involvement of the AZF region.

The prevalence of Y chromosome deletions is estimated at approximately 1: 2000 to 1: 3000 in men [18]. It is the second most common genetic cause of spermatogenesis failure in infertile men after Klinefelter syndrome. In this study, 3 out of 128 azoospermic men are found with a frequency of 2.3% (tab. 2, fig. 5). The association between long arm deletions of the Y chromosome and azoospermia was initially suggested by Tiepolo and Zuffardi in 1976 [18]. In two cases (fig. 5) deletions were detected in the Yq11.23 region. In this locus are located the genes of spermatogenesis of the Y chromosome and designated as Azoospermia Factor (AZF).

Numerous variations of autosomal chromosomes are identified in patients with azoospermia, which are often not expressed by detectable phenotypic changes. Azoospermia in these men can be explained by the involvement of thousands of autosomal genes in the direct or indirect control of testicular formation, their functioning and spermatogenesis. The most common autosomal chromosomal abnormalities detected were balanced chromosomal rearrangements in 9 cases and 1 case being unbalanced. Reciprocal translocations are the most common balanced chromosomal abnormalities, being reported in 0.9 out of 1000 newborns and in about 1% of infertile men [19]. In our study of 128 men with azoospermia balanced simple translocations were detected in 5 cases (3.9%) – t (13; 15), t (12; 7), t (8; 7), t (9; 5), t (13; 12). Balanced chromosomal translocations involve breaking points in two chromosomes and abnormal rearrangement of chromosomal fragments that lead to the transposition of genetic material from one chromosome to another without loss of genetic material, which explains that in most cases carriers with translocations had a normal phenotype [20]. Azoospermia in these cases can be explained by: 1) one of the breaking points interrupts a gene that controls spermatogenesis and leads to blockade of spermatogenesis or incomplete spermatogenesis; 2) chromosomes with translocations conjugate abnormally in prophase I of meiosis which makes chromosome disjunction difficult and gametogenesis is blocked [19, 20]. As with chromosomal translocations, inversions can cause infertility in men. The consequences of this are not to be neglected because there are associated risks: pregnancy loss, children with genetic abnormalities, offspring with fertility problems.

In 3 cases, duplications were detected in the satellites of the acrocentric chromosomes 13, 14, 15, and 22, and in one patient it was accompanied by changes in the sex chromosome. The involvement of these polymorphisms of the listed chromosomes in male infertility is also reported in other specialized studies, such as in the study of S. Penna Videau et al. 2001 [17]. Although satellites are component parts of heterochromatin, in some studies a positive correlation has been shown between the frequency of acrocentric chromosome variants with satellites and sterility, due to their association with the risk of nondisjunction leading to gametes with aneuploidy [21].

## Conclusions

The incidence of chromosomal abnormalities as a cause of male infertility was 38%. Chromosomal rearrangements affect both autosomal chromosomes and X and Y chromosomes. Therefore, the negative prognostic effects of chromosomal abnormalities/variations on spermatogenesis should be clearly explained to individuals with azoospermia during counseling for assisted reproduction. Future studies are certainly needed to identify any new genetic abnormalities and to help a deeper understanding of the causes of male infertility. Cases of infertility with normal karyotype (62% – 46,XY) can be explained by other genetic causes, such as point gene mutations, deletions and nucleotide duplications, which are below the threshold of detection by karyotyping, but are currently identified by various molecular genetic tests.

Given the high frequency of chromosomal abnormalities in infertile men as well as the genetic risks for future generations, the importance of a thorough cytogenetic assessment of them before resorting to assisted reproduction techniques, such as ICSI is mandatory.

## References

1. Azimi C, Khaleghian M, Farzanfar F. A retrospective chromosome studies among Iranian infertile women: Report of 21 years. *Iran J Reprod Med.* 2013;11(4):315-324.
2. Dohle GR, Colpi GM, Hargreave TB, Papp GK, Jungwirth A, Weidner W. EAU guidelines on male infertility. *Eur Urol.* 2005;48(5):703-711. doi: 10.1016/j.eururo.2005.06.002.
3. Poongothai J, Gopenath T S, Manonayaki S. Genetics of human male infertility. *Singapore Med J.* 2009;50(4):336-47.
4. Lee JY, Dada R, Sabanegh E, Carpi A, Agarwal A. Role of genetics in azoospermia. *Urology.* 2011;77(3):598-601.
5. Donker RB, Vloeberghs V, Groen H, Tournaye H, van Ravenswaaij-Arts CMA, Land JA. Chromosomal abnormalities in 1663 infertile men with azoospermia: the clinical consequences. *Hum Reprod.* 2017;32(12):2574-2580. doi: 10.1093/humrep/dex307.
6. Balkan M, Tekes S, Gedik A. Cytogenetic and Y chromosome microdeletion screening studies in infertile males with oligozoospermia and azoospermia in Southeast Turkey. *J Assist Reprod Genet.* 2008;25(11-12):559-565. doi: 10.1007/s10815-008-9272-8.
7. Pylyp LY, Spinenko LO, Verhoglyad NV, Zukin VD. Chromosomal abnormalities in patients with oligozoospermia and non-obstructive azoospermia. *J Assist Reprod Genet.* 2013;30(5):729-732. doi: 10.1007/s10815-013-9990-4.
8. Georgiou I, Syrrou M, Pardalidis N, Karakitsios K, Mantzavinos T, Giotsitsas N, et al. Genetic and epigenetic risks of intracytoplasmic sperm injection method. *Asian J Androl.* 2006;8(6):643-73. doi: 10.1111/j.1745-7262.2006.00231.x.
9. Mau-Holzmann UA. Somatic chromosomal abnormalities in infertile men and women. *Cytogenet Genome Res.* 2005;111(3-4):317-36. doi: 10.1159/000086906.
10. Forti G, Krausz C. Clinical Review 100: Evaluation and treatment of the infertile couples. *J Clin Endocrinol Metab.* 1998;83(12):4177-88. doi: 10.1210/jcem.83.12.5296.
11. Ferlin A, Raicu F, Gatta V, Zuccarello D, Palka G, Foresta C. Male infertility: role of genetic background. *Reprod BioMed Online.* 2007;14(6):734-745. doi: 10.1016/s1472-6483(10)60677-3.
12. Vogt PH, Fernandes S. Polymorphic DAZ gene family in polymorphic structure of AZFc locus: artwork or functional for human spermatogenesis? *Acta Pathol Microbiol Immunol Scand.* 2003;111:115-127.
13. Belling K, Russo F, Jensen AB, Dalgaard MD, Westergaard D, Rajpert-De Meyts E, Skakkebaek NE, Juul A, Brunak S. Klinefelter syndrome

- comorbidities linked to increased X chromosome gene dosage and altered protein interactome activity. *Hum Mol Genet.* 2017;26(7):1219-1229. doi: 10.1093/hmg/ddx014.
14. Krausz C. Male infertility: pathogenesis and clinical diagnosis. *Best Pract Res Clin Endocrinol Metab.* 2011;25(2):271-285. doi: 10.1016/j.beem.2010.08.006.
15. Gassó-Matoses A, Picó-Alfonso A, Fernández-García J, Lobato-Encinas J, Mira-Llinares A. 45,X/46,XY gonadal dysgenesis in an infertile adult male. *Urol Int.* 1992;48(2):239-241. doi:10.1159/000282343.
16. DeBraekeleer M, Dao T. Cytogenetics studies in male infertility: a review. *Hum Reprod.* 1991;6(2):245-250.
17. Penna Videau S, Araujo H, Ballesta F, Ballescà JL, Vanrell JA. Chromosomal abnormalities and polymorphisms in infertile men. *Arch Androl.* 2001;46(3):205-210.
18. Micic M, Micic S, Diklic V. Chromosomal constitution of infertile male. *Clin Genet.* 1984;25(1):33-36. doi: 10.1111/j.1399-0004.1984.tb00459.x.
19. Tahmasbpour E, Balasubramanian D, Agarwal A. A multi-faceted approach to understanding male infertility: gene mutations, molecular defects and assisted reproductive techniques (ART). *J Assist Reprod Genet.* 2014;31(9):1115-1137. doi: 10.1007/s10815-014-0280-6.
20. Harton GL, Tempest HG. Chromosomal disorders and male infertility. *Asian J Androl.* 2012;14(1):32-39. doi: 10.1038/aja.2011.66.
21. Kamischke A, Baumgardt A, Horst J, Nieschlag E. Clinical and diagnostic features of patients with suspected Klinefelter syndrome. *J Androl.* 2003;24(1):41-48.

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#### Authors' contribution

SR – designed the research, did statistics and interpreted the data, drafted the manuscript; VM – conceptualized the project and designed the research; SC – collected and interpreted the data; AM – conducted/performed the laboratory work; MS – conducted the laboratory work, revised the manuscript critically. All the authors revised and approved the final version of the manuscript.

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#### Ethics approval and consent to participate

The research was approved by the Research Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy, protocol No 48 of April 12, 2018.

#### Conflict of Interests

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