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Genetic Testing for Monogenic Forms of Male Infertility Contributes to the Clinical Diagnosis of Men with Severe Idiopathic Male Infertility

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Purpose: In recent years, many genes have been associated with male infertility; however, testing of monogenic forms has not yet been clinically implemented in the diagnosis of severe forms of idiopathic male infertility, as the diagnostic utility has not been established yet. The aim of this study was therefore to answer if the implementation of genetic testing for monogenic forms of male infertility could contribute to the clinical diagnosis of men with severe forms of idiopathic male infertility.
Materials and Methods: Based on the ClinGene curation protocol, we defined a panel of genes with sufficient evidence for the involvement with severe male infertility. We tested the 21-gene panel in a representative multicentric cohort of men with significantly impaired spermatogenesis. We performed whole exome sequencing on 191 infertile men with severe forms of idiopathic male infertility; non-obstructive azoospermia, and severe oligozoospermia (<5 million spermatozoa/mL). The control group consisted of 216 men who fathered a child. DNA was prepared based on the Twist CORE exome protocol and sequenced on the Illumina NovaSeq 6000 platform. Variants were classified using the Association for Clinical Genomic Science (ACGS) Best Practice Guidelines for Variant Classification in Rare Disease 2020.
Results: We identified potential monogenic disease-causing variants in four infertile men. Pathogenic/likely pathogenic variants in *STAG3* (c.2776C>T, p.Arg926*; c.2817delG, p.Leu940fs), *MSH4* (c.1392delG, p.Ile465fs; c.2261C>T, p.Ser754Leu), *TEX15* (c.6848_6849delGA, p.Arg2283fs; c.6271dupA, p.Arg2091fs), and *TEX14* (c.1021C>T, p.Arg341*) genes were found.
Conclusions: In the present multicentric cohort study, a monogenic cause in 2.1% of infertile men was identified. These findings confirm the utility of monogenic testing and suggest the clinical use of monogenic testing for men with severe forms of idiopathic male infertility.

Keywords: Azoospermia; Genetic testing; Male; Meiosis

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INTRODUCTION

Infertility is a disease of the reproductive system, defined by the failure to conceive after at least 12 months of unprotected, regular sexual intercourse [1]. It is estimated that worldwide, around 15% of couples suffer from infertility, in which the male factor accounts for about 50% of cases [2]. The most severe forms of male infertility are defined to be severe oligoasthenoteratozoospermia (<5 million spermatozoa/mL) and azoospermia (lack of spermatozoa in the ejaculate), which can be non-obstructive (testicular failure) or obstructive (obstruction in the male reproductive tract) [3,4]. It is estimated that about 10% to 15% of severe male infertility is due to genetic disorders, including chromosomal abnormalities and Y chromosome microdeletions [5]. Still, in about 70% of patients with non-obstructive azoospermia (NOA), the cause remains unknown, also referred to as idiopathic infertility [6]. The American Urologic Association (AUA), European Association of Urology (EAU), and American Society of Reproductive Medicine (ASRM) guidelines recommend genetic testing for infertile men with NOA, severe oligozoospermia (concentration below 5 million spermatozoa/ml for American guidelines and bellow 10 million spermatozoa/mL [karyotype] or below 5 million spermatozoa/mL [Y chromosome microdeletions testing] for European guidelines) and men with no palpable vas deferens. The established genetic tests for diagnosis of those men are karyotyping, Y chromosome microdeletions testing, and testing for the *CFTR* gene variants in case of obstructive infertility [7].

In recent years, the application of next-generation sequencing technologies, like whole exome sequencing (WES), has led to the discovery of many genes involved in male infertility, and the number is still increasing [6]. However, the findings have not yet been translated into clinical practice [7], and a standardized, clinical-based gene panel has yet to be proposed. Consequently, the prevalence of monogenic forms of severe idiopathic male infertility is still unknown, and the clinical utility of testing remains to be established.

The majority of research panels nowadays are prepared based on genes previously detected in infertile men—candidate genes or using datasets like OMIM where genes associated with male infertility are included [8-10]. Estimating the causality or involvement of a gene with a disease is challenging, especially in clinically and genetically heterogeneous disorders, such as male infertility. Using guidelines in the ClinGen gene curation process can effectively evaluate the strength of a gene-disease association [11].

In the present study, we aimed to develop a diagnostic test and identify the frequency of monogenic forms in a population of men with severe idiopathic male infertility to estimate the clinical utility of genetic testing.

MATERIALS AND METHODS

1. Literature screening and gene curation process

A literature screening of the PubMed database was performed using the keywords: "nonobstructive azoospermia" OR "oligozoospermia" AND "genetic variant". All included articles were limited to the published date up to May 31, 2023. The literature screening was conducted according to the preferred reporting items for systematic review and meta-analyses [12]. Publications reporting infertile men whose infertility was due to other known genetic causes (*CFTR* mutation, chromosomal/cytological causes of infertility, and azoospermia factor microdeletions) or other forms of infertility (asthenozoospermia) were excluded. Genes observed in at least two separate studies, proposed in ClinGen's criteria, were clinically validated and scored using ClinGen's gene curation process [11]. Genes scored as strongly associated with male infertility all meet the following criteria: demonstration of the role of a gene with male infertility in at least two separate studies, multiple unrelated probands harboring variants with sufficient evidence for the involvement with severe idiopathic male infertility, and supporting experimental data. Three independent curators were involved in the curation process.

2. Participants

The analysis was performed on 191 infertile men. Of them, 154 were diagnosed with azoospermia, 35 with oligozoospermia, oligoasthenozoospermia, or oligoasthenoteratozoospermia, and two with cryptozoospermia. The study participants were recruited from regional genetic centers of five countries: Clinical Institute of Genomic Medicine, University Medical Centre Ljubljana, Slovenia; Center of Medical Genetics and Immunology, Clinical Center of Montenegro, Podgorica, Monte-

negro; Institute of Human Genetics, Medical Faculty, University of Belgrade, Belgrade, Serbia; Research Centre for Genetic Engineering and Biotechnology "Georgi D. Efremov" Macedonian Academy of Sciences and Arts, Skopje, Macedonia; Department of Medical Biology and Genetics, Faculty of Medicine, University of Rijeka, Rijeka, Croatia and from the Department of Urology, Clinical Hospital Center Rijeka, Rijeka, Croatia. The control group consisted of 216 men who fathered a child. The diagnosis of male infertility was carried out using semen analysis, the measurement of hormones, especially follicle-stimulating hormone, and in some cases also luteinizing hormone, testosterone, prolactin, and estradiol. Additional clinical parameters included the determination of the testicular volume and the evaluation of testicular biopsy results or histology if performed. The inclusion criteria for the participants were NOA or oligozoospermia/oligoasthenozoospermia/oligoasthenoteratozoospermia with a concentration <5 million spermatozoa/mL, which is based on the American and European guidelines for genetic testing (Y chromosome microdeletions and karyotyping) [7]. We excluded infertile men with obstructive azoospermia (OA), cytogenetic abnormalities, Y chromosome microdeletion, men with hypogonadotropic hypogonadism, and men with a history of testicular carcinoma.

3. Ethics statement

The study was approved by the National Medical Ethics Committee of the Republic of Slovenia (reference number: 50/03/15; 0120-213/2022/6). Written consent was given to the participants before the start of the study.

4. Whole exome sequencing and bioinformatics analysis

Peripheral whole blood samples were collected into sterile single test tubes with the anticoagulant ethylenediaminetetraacetic acid (EDTA). The genomic DNA was isolated and analyzed to determine the potential presence of a genetic cause of male infertility. To identify potential causative variants in patients, we performed WES [13] and analyzed the genes from our panel.

DNA was fragmented and enriched based on the Twist CORE exome protocol and sequenced on the Illumina NovaSeq 6000 platform in 2×100 cycles and paired-end mode. The median on-target coverage was 50× in all subjects. After demultiplexing and trimming of adaptor sequences, reads were checked for quality. The reads falling below the required quality threshold were excluded, including duplications. The reads were aligned to the reference genome (UCSC hg19), using the Burrows-Wheeler Aligner (BWA) algorithm. The calling of variants was made with the use of the Genome Analysis Toolkit (GATK) framework. For analysis, only variants exceeding the quality score of 30.0 and depth of 5 were used. The annotation of variants was performed with the use of ANNOVAR and snpEff algorithms. For the pathogenicity prediction, the dbNSEPv2 database was used, and for the structural variants, we used the CONIFER algorithm. Population-based annotations and evolutionary conversation measures were also included in the prediction of variant effects. All variants with a population frequency over 5% in 1000 genomes and ESP6500, all synonymous variants, variants outside the clinical target, and intronic variants were filtered out in the analysis. The reference gene sequences were based on the RefSeq database. Sanger sequencing was performed for cases, in which maternal and paternal samples could be obtained.

5. Identification of rare causative variations

All obtained variants were filtered. The first filter was applied to exclude variants with a population frequency above 5%. Theoretical pathogenicity predictors (SIFT, PolyPhen-2, Mutation Taster, PROVEAN, REVEL, MetaSVM, CADD, and GERP++ as the evolutionary conservation measure) were further used to filter potential interesting variants. Identification of rare hemizygous, compound heterozygous, and homozygous variants was done with the exclusion of all variants whose frequency in the Slovene Genome variation db exceeded 5%. Variants were classified using Association for Clinical Genomic Science (ACGS) Best Practice Guidelines for Variant Classification in Rare Disease 2020 [14]. The variants were annotated using the frequency data from the gnomAD exomes project v.2.1.1 https://gnomad.broadinstitute.org/.

RESULTS

1. Established gene panel and clinical findings

The literature screening resulted in 106 studies, re-

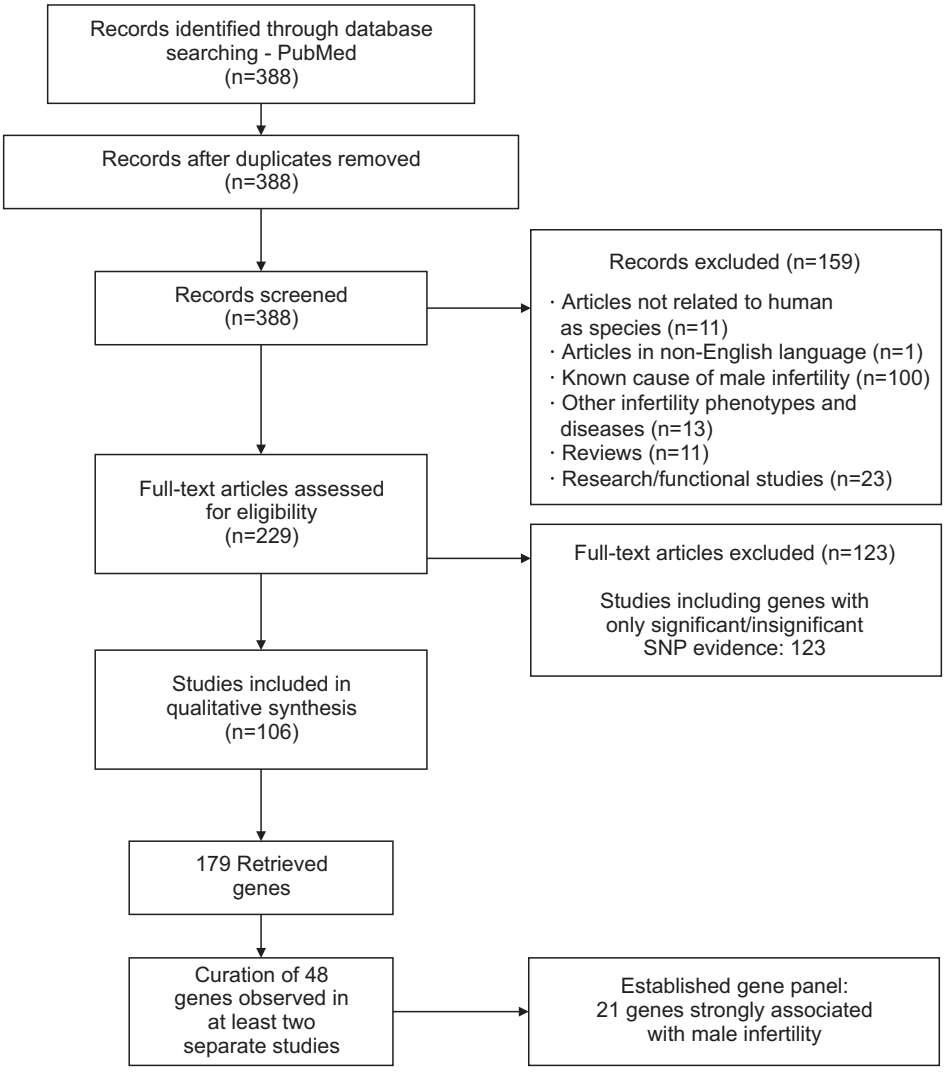

Fig. 1. PRISMA flow chart showing the selection of publications for the retrieval of genes and their further curation for the establishment of a panel of genes, strongly associated with severe idiopathic male infertility.

porting 179 genes with rare variants associated with severe idiopathic male infertility (severe oligozoospermia and NOA) (Fig. 1). Of them, 48 were reported in at least two separate studies. The retrieved 48 genes were further clinically validated and scored using ClinGen's gene curation process. The curation process resulted in 21 genes that were strongly (scored at least 12 points) associated with severe idiopathic male infertility and were therefore included in the present panel.

Genes included in the panel are reported in Table 1, and the publications gathered for the ClinGene curation in the Supplement Table 1. Although the *AR* gene is involved with androgen insensitivity syndrome [15], which can lead to genital development abnormalities, due to the idiopathic cases presenting with only azoospermia [15], the gene remained included in the panel. Similarly, we also included *NR5A1*, which is associated with disorders of sex development, but due to cases with idiopathic spermatogenic failure [16], the gene remained included.

The clinical characteristics of the patients included in this multicentric cohort study were gathered and are presented in (Table 2).

2. Potential causative variants

We identified monogenic disease-causing variants in four azoospermic men (95% confidence interval=0.45%–3.69%). Pathogenic/likely pathogenic variants were identified in *STAG3, MSH4, TEX15*, and *TEX14* genes (Table 3).

3. Candidate causative variants

A likely pathogenic variant c.832_833insA (p.Leu278fs) in *ADAD2* and a pathogenic variant

The World Journal of MEN's HEALTH

Table 1. Genes from the present panel, established as strongly associated with severe idiopathic male infertility and their corresponding inheritance

Gene	Inheritance
ADAD2	AR
AR	XLR
C14orf39	AR
DMRT1	AD
FANCM	AR
FKBP6	AR
HFM1	AR
M1AP	AR
MEI1	AR
MEIOB	AR
MSH4	AR
MSH5	AR
NR5A1	AD
PNLDC1	AR
SHOC1	AR
STAG3	AR
SYCE1	AR
TEX11	XLR
TEX14	AR
TEX15	AR
ZMYND15	AR

AR: autosomal recessive, AD: autosomal dominant, XLR: X-linked recessive.

Table 2. Clinical characteristics of included infertile men

	Value
Age (years)	35 (31–38)
Body mass index (kg/m^2)	25.0 (23.5–27.0)
Left testicular volume (mL)	13.0±4.9
Right testicular volume (mL)	12.9±4.9
FSH (IU/L)	10.4 (6.15–19.1)
Concentration:	
All infertile men (10^6/mL)	0
Oligozoospermia (10^6/mL)	0.4 (0.22–2.65)

Testicular volume is reported as mean±standard deviation (DV). Age, body mass index, FSH, and spermatozoa concentration values are reported as median with interquartile range (Q1–Q3).
FSH: follicle-stimulating hormone.

c.676dupT (p.Trp226fs) in *M1AP* were identified in two azoospermic men (Table 4). Each patient had an additional variant in the same gene, classified as a variant of uncertain significance (VUS). A homozygous in-frame deletion c.390_392delCAT (p.Ile131del), classified as a VUS, was identified in the *MSH5* gene.

Single heterozygous likely pathogenic/pathogenic variants in recessively inherited genes were identified in ten patients (Supplement Table 2). No additional variants in the particular genes or copy number variations were observed in those patients.

DISCUSSION

In this multicentric cohort study, we present a clinical-based gene panel with genes strongly associated with severe forms of idiopathic male infertility and the corresponding variants identified in those genes. With WES of 191 infertile men, potential monogenic disease-causing variants in four of them (*STAG3, MSH4, TEX14, TEX15*) were identified. The current gene panel was able to determine a monogenic cause in 2.1% of infertile men, proving the utility of monogenic testing in the clinical practice of men with severe forms of idiopathic male infertility.

Previous studies have reported that more than 600 genes were associated with male infertility [17], more than 200 with primary spermatogenetic failure [18]. In this multicentric cohort study, 179 genes with reported rare variants involved with severe idiopathic male infertility were retrieved. Currently, numerous candidate genes have been reported, many whose role in male infertility is still not sufficiently established [17]. From a clinical perspective, it is therefore a challenge to select those that are reliably associated with the given phenotype. For potential future clinical use, the genes and variants must first be defined for their clinical relevance, which was carried out in the present study, using a well-established scoring protocol. Of the 48 genes observed in more than one study, only 21 were strongly associated with severe idiopathic male infertility. Including only 21 genes in this panel indicates that even though many genes were associated with male infertility in the literature, the evidence for the majority is still insufficient. Variants identified in genes with a not yet-defined role in disease could lead to an incorrect diagnosis, leading to a premature ending of the clinical treatment of the patients [19,20]. This could prevent further evaluation and identification of the actual genetic cause. Additionally, after receiving a new genetic diagnosis, confusion among the patients and their family members could arise [19]. Therefore, caution in the diagnosis of patients with gene panels including genes without sufficient evidence is advised.

A similar scoring of genes related to male infertil-

Table 3. Causative variants identified in four azoospermic patients

Patient	Diagnosis	Gene	Transcript change	Amino acid change	Variant type	Zygosity	Variant classification	Allele frequency (Slovenian Genome Variant Browser)	Allele frequency (GnomAD exomes)	Allele frequency control population
Case 1	AZOO withMA	*STAG3*	NM_001282717.1 c.2776C>T	p.Arg926*	Nonsense	Heterozygous	Likely pathogenic (PVS1, PM2, PM3_SUP)	0.0013	0.00006	0.0031
			NM_001282717.1 c.2817delG Novel	p.Leu940fs	Frameshift	Heterozygous	Likely pathogenic (PVS1, PM2)	0.0002	0.000016	0.00156
Case 2	AZOO withMA	*TEX15*	NM_001350162.2 c.6848_6849delGA	p.Arg2283fs	Frameshift	Heterozygous (paternal origin)	Pathogenic (PVS1, PM2, PM3)	0.002	0.00081	0.0031
			NM_001350162.2 c.6271dupA Novel	p.Arg2091fs	Frameshift	Heterozygous (maternal origin)	Pathogenic (PVS1, PM2, PM3)	0.0003	0.00004	0.001557
Case 3	AZOO	*MSH4*	NM_002440.4 c.1392delG Novel	p.Ile465fs	Frameshift	Heterozygous (*de novo*)	Pathogenic (PVS1, PM6, PM2)	0.00007	0.0000042	0.00154
			NM_002440.4 c.2261C>T	p.Ser754Leu	Missense	Heterozygous (maternal origin)	Pathogenic (PM1, PM2, PM3_MOD, PP1_STR, PP3)	0.0006	0.000028	0.00617
Case 4	AZOO	*TEX14*	NM_001201457.1 c.1021C>T	p.Arg341*	Nonsense	Homozygous	Likely pathogenic (PVS1, PM2)	0.00097	0.00082	0.0046

STAG3: stromal antigen 3, *TEX15*: testis expressed 15, meiosis and synapsis associated, *MSH4*: mutS homolog 4, *TEX14*: testis expressed 14, intercellular bridge forming factor, AZOO: azoospermia, MA: meiotic arrest.

Table 4. Candidate causative variants identified in three azoospermic patients

Patient	Diagnosis	Gene	Transcript change	Amino acid change	Variant type	Zygosity	Variant classification	Allele frequency (Slovenian Genome Variant Browser)	Allele frequency (GnomAD exomes)	Allele frequency in control population
Case 5	AZOO with MA	*ADAD2*	NM_139174.4 c.1942C>T Novel	p.Gln648*	Nonsense	Heterozygous	Variant of uncertain significance (PVS1_MOD, PM2)	0.000007	-	-
			NM_139174.4 c.832_833insA Novel	p.Leu278fs	Frameshift	Heterozygous	Likely pathogenic (PVS1, PM2)	0.00003	0.000016	-
Case 6	AZOO withMA	*M1AP*	NM_001321739.2 c.676dupT	p.Trp226fs	Frameshift	Heterozygous	Pathogenic (PVS1, PS3, PM2, PM3_STR)	0.005	0.0021	0.0062
			NM_001321739.2 c.949G>A	p.Gly317Arg	Missense	Heterozygous	Variant of uncertain significance (PM2, PM3)	0.0002	0.000072	0.00157
Case 7	AZOO withMA	*MSH5*	NM_172165.3.4 c.390_392delCAT Novel	p.Ile131del	Inframe deletion	Homozygous	Variant of uncertain significance (PM2, PM4_SUP)	0.00076	0.00001	0.0015

ADAD2: adenosine deaminase domain containing 2, *M1AP*: meiosis 1 associated protein, *MSH5*: mutS homolog 5, AZOO: azoospermia, MA: meiotic arrest.

ity was performed by Houston et al [17]. They curated the genes using a simplified scoring system including genes involved with the broader term of male infertility (isolated or syndromic male infertility), therefore reporting as many as 120 genes moderately, strongly, or definitely linked with different male infertility phenotypes. As we were focused on primarily NOA and severe oligozoospermia and included only genes that were strongly or definitely involved with male infertility, the list of genes with sufficient evidence was therefore also shorter. Interestingly, some genes, such as *MSH4*, with strong evidence based on our scoring were still without evidence by the time of Houston et al [17], confirming the rapid progress in male infertility research. Especially in clinical settings, regular updating of genes is therefore needed.

Except for *DMRT1* and *NR5A1* with autosomal dominant inheritance and *TEX11* and *AR* with X-linked recessive inheritance, we observed that all additional genes in the panel indicated autosomal recessive inheritance from the present multicentric cohort study. The variants were identified in a homozygous or compound heterozygous state, which is in agreement with OMIM and literature screening, proposing an autosomal recessive inheritance. In this study, no rare deleterious variants in the two autosomal dominant genes included in the panel were observed. Previous studies reported evidence for the role of *de novo* variants in male infertility [13,21]. However, none of the genes reported in the two studies, except *MSH5* [21] met the criteria for inclusion in our panel.

Defects in meiosis-involved genes have been reported to result in meiotic arrest during spermatogenesis [22]. Similarly, genes with potential causative variants in the present study (*STAG3, MSH4, TEX14*, and *TEX15*) were also involved with the meiosis process. *MSH4* is required for reciprocal recombination and segregation of homologous chromosomes during meiosis [23]. *STAG3* is a component of the meiosis-specific cohesin complex required for maintaining chromatid cohesion, DNA repair, and synapsis between homologous chromosomes [24]. *TEX14* is required for the formation of intercellular bridges in germ cells [25], and *TEX15* for chromosome synapsis and meiotic recombination [26]. We observed that most of the genes from the panel were involved with meiosis and that various other processes essential for the establishment and preservation of fertility are still understudied. Due to the underrep-

resentation of non-meiosis-involved genes in the panel, the percentage of identified monogenic causes could be even higher, as reported in this study.

Besides the variants in the four causative genes, we identified eight likely pathogenic/pathogenic single heterozygous variants in ten patients, indicating a risk of male infertility for their potential progeny after a successful assisted reproduction outcome. Regarding other undiagnosed patients from this study, two patients harboring a likely pathogenic/pathogenic variant and a VUS in *ADAD2* and *M1AP*, and a patient carrying a VUS in *MSH5* were identified. All present good causative candidates for the patient's phenotype, as their histology results confirmed the presence of meiotic arrest. The same combination of variants in *M1AP* was previously observed in one man with spermatogenic failure, which points to a pathogenic effect of this variant [27]. However, due to the lack of functional evidence, the classification for the variants in *M1AP* and *ADAD2*, as well as the variant in *MSH5*, remains of uncertain significance. The relatively short period of studying monogenic causes of male infertility has consequences that a large number of rare variants are currently classified as VUS. Regarding this, VUSs are particularly interesting, and additional research in the form of exome sequencing of more infertile men in clinical studies will add value and help to determine their real clinical significance.

We demonstrate that the diagnostic yield of genetic testing for severe male infertility could be improved by at least 2% with the current panel. This might be estimated as a modest contribution to the current diagnostic yield of cytogenetics and Y chromosome deletion testing (10%–15%) [28]. However, timely, etiological diagnosis of patients with severe male infertility before assisted reproduction would be beneficial, as proper clinical diagnosis could enable informative genetic counseling, shorten the clinical treatment, and limit unnecessary invasive tests. Since several monogenic forms are associated with the phenotype of meiotic arrest, invasive testicular sperm extraction (TESE) could be avoided. TESE has also been associated with potential long-term androgen deficiency [29], which can lead to reduced libido, poor sexual function, gynecomastia, reduced body hair, reduced muscle mass, fatigue, impaired cognition, obesity, low mineral density, and anxiety [30]. As TESE, especially multiple ones, can have long-term adverse effects, a known prognosis due to genetic testing would not endanger the patient's health at the expense of infertility treatment. Those men would also be sooner included in the donor program, leading to a sooner obtained pregnancy and a potential child.

Based on our results, there is still limited evidence to determine factors that could predict which infertile men with severe forms of idiopathic male infertility are suitable for monogenic testing. Even though the majority of predicted causative variants and candidate causative variants were identified in men with meiotic arrest, where no spermatozoa are produced, the number of cases is still insufficient to statistically conclude the preferred use of monogenic testing for men with a negative TESE outcome. Additional confirmation would be the identification of monogenic causes in other infertility phenotypes, including oligozoospermia and even asthenozoospermia and teratozoospermia as reviewed by Cioppi et al [6] and Houston et al [17]. Until more research has been performed, we still recommend monogenic testing for all men with idiopathic severe male infertility.

This study has some limitations. In this multicentric cohort study, tested infertile men were of Slavic origin, which could be considered a strength because of the homogeneity of the study population. On the other hand, research on other populations is needed to generalize the impact of monogenic etiology. Another limitation is the strict criteria we used to develop the panel. There could be potential causative variants in genes that were not included in the present panel, considering the rarity of the variants or the lack of evidence for the involvement of a gene in the disease. Novel scientific evidence of molecular pathogenesis might result in the inclusion of additional existing candidate genes or newly identified ones.

CONCLUSIONS

In this multicentric cohort study, we identified monogenic causes in 2.1% of men with severe male infertility using a rigorously curated clinical gene panel. Our findings advocate for integrating genetic testing for monogenic etiology into the diagnostic assessment owing to its clinical utility.

Conflict of Interest

The authors have nothing to disclose.

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Author Contribution

Conceptualization: all authors. Data curation: RP, AH. Formal analysis: RP, AH, AM. Investigation: RP, AH. Methodology: RP, AH, AM. Project administration: BP. Resources: all authors. Software: AM. Supervision: BP. Visualization: all authors. Writing – original draft: RP, AH, BP. Writing – review & editing: all authors.

Supplementary Materials

Supplementary materials can be found via https://doi.org/10.5534/wjmh.240149.

Data Sharing Statement

The additional data will be shared upon request to the corresponding author.

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