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ORIGINAL PAPER

Multifactorial analysis of male infertility: Sperm DNA damage, semen parameters, and genetic testing in iranian infertile men

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Summary ***Introduction: Male infertility is a multifactorial condition influenced by genetic, physiological, and environmental factors. While semen analysis is the cornerstone of diagnosis, additional tests like sperm DNA fragmentation (DFI) assessment and genetic testing are increasingly recognized for their diagnostic and prognostic value. This study investigates the relationships between DFI, semen parameters, and genetic abnormalities in Iranian men.***

Methods: A retrospective cross-sectional study analyzed 6,397 men (5,196 infertile and 1,201 fertile controls) at the MOM Infertility Center between December 2022 and August 2023. Semen analysis evaluated sperm concentration, motility, morphology, Y chromosome AZF microdeletion testing, and whole-exome sequencing (WES) on subsets of infertile men. Correlations between age, sperm parameters, and DFI were assessed, and logistic regression evaluated the likelihood of abnormal sperm conditions.

Results: Infertile men showed significantly lower sperm concentration, motility, and morphology, along with higher DFI, compared to controls. DFI was negatively correlated with sperm concentration, motility, and morphology. Age was positively correlated with DFI and negatively correlated with motility and morphology. Genetic abnormalities, including chromosomal anomalies, AZF deletions, and WES-detected mutations, were identified in subsets of infertile men, underscoring the value of genetic testing.

Conclusions: This study highlights significant associations between DFI, age, semen parameters, and genetic abnormalities in male infertility. Incorporating advanced diagnostic tools, such as DFI assessment and genetic testing, into clinical practice can improve diagnostic accuracy and guide personalized treatment strategies for infertile men.

KEY WORDS: Male infertility; DFI; Y chromosome microdeletion; AZF; Karyotype; WES.

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INTRODUCTION

Infertility is a common and complex reproductive health issue, affecting approximately 15% of couples worldwide (1). Both male and female factors contribute to infertility, with male factors contributing to nearly 40% to 50% of cases and male factors alone accounting for approximately 20-30% of cases (2). Male infertility is generally linked to abnormalities in sperm quality and/or quantity, and understanding the underlying causes is critical for both diagnosing and treating infertility. Unexplained male infertility occurs despite normal findings, while idiopathic infertility involves reduced sperm quality with no identifiable cause (3). Among the factors contributing to male infertility, genetic abnormalities play a particularly crucial role, especially in cases of unexplained infertility or recurrent early pregnancy losses (4). Sperm DNA is protected by protamines, repair enzymes, antioxidants, and structural defenses, but impaired apoptosis, chromatin defects, and oxidative stress can weaken these mechanisms and lead to DNA damage (5-8). Sperm integrity is strongly affected by environmental and lifestyle factors, with oxidative stress as the main damaging pathway. Smoking, toxins (pesticides, heavy metals, heat), and obesity or inactivity impair sperm quality and increase DNA fragmentation. These changes reduce fertility potential, leading to lower fertilization rates and higher miscarriage risk (9-12). Genetic causes of male infertility include chromosomal abnormalities (2-8% of cases) such as Klinefelter syndrome, 46 XX male syndrome, and translocations that impair spermatogenesis (13).

Spermatogenesis is regulated by genes on the Y chromosome's *azoospermia factor* (AZF) region, which includes three subregions: AZFa, AZFb, and AZFc and defects in any of them can disrupt sperm production and cause infertility (14). Y chromosome microdeletions in the AZF region affect 5-15% of men with azoospermia or severe oligozoospermia, but since 40% of cases remain unexplained, further research into novel genetic variants is needed (15-20). Advanced genetic testing like NGS helps identify mutations causing male infertility, with CFTR mutations as a key example, and over 120 genes have now been linked to this condition (20, 21). Traditional

semen analysis is essential but limited, since ~15% of infertile men have normal results; assessing sperm DNA integrity offers deeper diagnostic insight. An emerging diagnostic focus is the assessment of sperm DNA integrity, which could provide additional insights into fertility (22-23). *Sperm DNA fragmentation* (SDF) can occur during spermatogenesis or post-testicular transit through three main mechanisms: abortive apoptosis, where germ cells meant to die survive with fragmented DNA; defective chromatin remodeling, where incomplete replacement of histones with protamines leaves DNA vulnerable; and oxidative stress, caused by excessive *reactive oxygen species* (ROS) overwhelming antioxidant defenses. High SDF levels are associated with lower fertilization rates, poor embryo quality, increased miscarriage risk, and potential adverse effects on offspring health.

The assessment of SDF has become an essential diagnostic tool in evaluating male infertility. Previous research indicates that elevated SDF are associated with poorer outcomes in *assisted reproductive technologies* (ART), including *in vitro fertilization* (IVF) and *intracytoplasmic sperm injection* (ICSI) (22, 24). By delivering additional information regarding sperm quality, SDF testing can guide treatment decisions and enhance the likelihood of successful ART outcomes.

This study aimed to fill gaps in understanding and diagnosing male infertility in Iranian men by examining sperm parameters, *DNA fragmentation index* (DFI), karyotype abnormalities, Y chromosome AZF microdeletions, and monogenic mutations. DFI, an important predictor of infertility, is not routinely assessed in many Iranian labs. The study highlights DFI's potential as a key diagnostic tool and emphasizes the underuse of genetic testing in reproductive medicine. We evaluated the feasibility of incorporating karyotyping, AZF testing, and whole-exome sequencing into clinical practice. Ultimately, the findings aim to guide personalized treatment and improve understanding of genetic contributions to male infertility in Iran.

Materials and methods

Study design

This retrospective single-center cross-sectional cohort study included 6,397 male participants, aged 25 to 55 years, who underwent semen analysis at the MOM Infertility Center in Iran between December 2022 and August 2023.These men were tested due to various reasons. Some were tested due to infertility assessment, some were tested as pre-IVF evaluations which were due to female-related infertility, consanguineous marriage or sex-selection. Fertility confirmation was done using positive History of pregnancy under one year or under 6 months if the female partner is over 36 years old and also normal sperm parameters. In infertile men, all assessments were done after confirming normal hormonal levels in each individual, including LH, FSH, Testosterone, etc to exclude endocrine factors. Exclusion criteria included any known correctable causes (e.g., varicocele, obstruction), Active urogenital infection, history involving exposure to gonadotoxins, smoking, Use of anabolic steroids or testosterone therapy, a history of cryptorchidism, testicular torsion or hypogonadotropic hypogonadism. This cross-sectional study was approved by the institutional review board protocol of MOM Fertility& Infertility Treatment Center (the ethics code: IR.IAU.TMU.REC.1402.159) and adhered to the tenets of the declaration of Helsinki. All experiments were performed in accordance with relevant guidelines and regulations. informed consent was obtained at the time of original sample collection from each individual. The study population was divided into two groups: the case group, which included 5,196 infertile patients with abnormal semen parameters (including morphology, motility rate, sperm count or DFI), and the control group, consisting of 1,201 individuals with normal sperm parameters.

Further detailed investigations were carried out on specific patient subsets. Subsets included 500 infertile patients from case group who were studied through karyotype analysis, 100 patients with normal karyotype results who were tested for AZF microdeletions, and finally 50 patients with normal karyotype and AZF test results who then proceeded to undergo *Whole Exome Sequencing* (WES) for more comprehensive genetic evaluation.

Sperm analysis

Semen specimens were collected via masturbation after an ejaculatory abstinence period of 2 to 5 days. Sperm concentration, motility, and morphology were evaluated according to World Health Organization (WHO, 2010) criteria.

DFI was assessed using flow cytometry in combination with the *Sperm Chromatin Structure Assay* (SCSA). Data were analyzed using FlowJo software (*Version 7.6, FlowJo LLC, United States*).

Cytogenetic and molecular analysis

Chromosomal analysis was performed using High resolution G-banding techniques, with chromosomal abnormalities documented according to the internationally recognized ISCN 2020 guidelines to ensure consistent reporting. Then we investigated Y chromosome microdeletions associated with infertility in 100 men who had normal karyotype results and sperm concentration below 5 million/mL, which is considered the criteria for AZF microdeletion assessment (25). these men were randomly selected from a larger cohort based on predefined criteria, including confirmed infertility and low sperm concentration and availability of blood samples. Randomization was performed to reduce selection bias.

Genomic DNA was extracted from *peripheral blood lymphocytes* (PBLs) and analyzed using multiplex PCR to detect specific DNA markers on the Y chromosome linked to sperm production, specifically in the AZFa, AZFb, and AZFc regions. Additionally, WES was performed on a cohort of 50 patients with normal karyotypes and no detectable AZF microdeletions. These 50 men went under WES due to either consanguineous marriage, history of a suspected genetic disorder which their treating physician wanted to investigate the genetic basis for, history of having a child or late-pregnancy abortions or

IUFDs with a suspected genetic problem or due to use of donated egg in which case the couple wanted to investigate common variants between the man and the egg donator.

Genomic DNA was extracted from peripheral blood leukocytes following standard protocols. The DNA underwent WES using the Illumina NovaSeq platform (*Illumina, Inc, United States*), enabling high-throughput sequencing and comprehensive coverage of the exonic regions. Variant callings were performed by using HaplotypeCaller and DeepVariant tools (*https://github.com/google/deepvariant*). The vcf files were annotated using ANNOVAR (26) and our custom annotation tool. Subsequently, variant filtering was conducted through filtering out the variants with *minor allele frequency* (MAF) of greater than 0.01 in the selected databases (1,000 Genomes project (*https://www.internationalgenome.org/*), Exome Aggregation Consortium database (*https://exac.broadinstitute.org/*), the Genome Aggregation Database (gnomAD) (*https://gnomad.broadinstitute.org/*), NHLBI GO *Exome Sequencing Project* (ESP) (*https://esp.gs.washington.edu/drupal/*), Iranome (*https://www.iranome.com/*), and our in-house database including 2500 individuals. Subsequently, we excluded those variants located in intronic regions and reported as benign in Clinvar database (*https://www.ncbi.nlm.nih.gov/clinvar*) (access date: November 2023). The prioritization of variants was based on criteria such as allele frequency, predicted effects on protein structure and function, and prior associations with male infertility in literature review and disease-based online resources like CFTR mutation database (*http://www.genet.sickkids.on.ca/StatisticsPage.html*) and CFTR2 database (*https://cftr2.org/*). We recruited ACMG guideline for variant classification and evaluation of their pathogenicity and ACMG SFv3.0 for considering secondary findings (27).

Statistical analyses

Data are reported as frequencies (n, %) or means with *standard deviations* (SD) and medians with ranges. Group comparisons for categorical variables were conducted using the chi-square (χ^2) test, while the Mann-Whitney U test and Kruskal-Wallis (followed by post-hoc pairwise comparisons using the Mann-Whitney U test when appropriate) were applied for continuous variables to identify significant differences. Spearman's correlation analysis was used to explore relationships between parameters. Additionally, logistic regression assessed the influence of multiple factors on outcomes, and the Wald-Wolfowitz test evaluated the randomness of data distribution. A two-tailed p-value of less than 0.05 was considered statistically significant for all analyses. All Statistical analyses were performed using SPSS version 27.0.1 (*SPSS Inc., Chicago, IL, USA*).

Results

A total of 6,397 participants were enrolled in this study including 1,201 individuals whom exhibited normal sperm parameters, while the remaining 5,196 individuals were reported with at least one abnormality in sperm parameters (including elevated DFI, reduced concentration, impaired motility, or atypical morphology).

Based on the DFI results, specimens were classified into four categories reflecting varying levels of DNA integrity:

a. < 15% DFI: Excellent to Good DNA integrity
b. ≥ 15% to < 25% DFI: Good to Fair Sperm DNA integrity
c. ≥ 25% to < 30% DFI: Fair Sperm DNA integrity
d. ≥ 30% DFI: Poor Sperm DNA integrity.

Population characteristics and sperm analysis

The mean age of participants was 38.25 years, with a standard deviation of 5.87. The average sperm concentration was 35.16 million/mL (SD = 16.74), and the mean total progressive motility was 40.64% (SD = 17.21). The average percentage of normal sperm morphology was 2.41% (SD = 1.37), and the DFI had a mean of 23.79% (SD = 11.85), displaying a heavy-tailed distribution (Table 1).

The mean and median age of the infertile patients were 38.61 years (SD = 5.94) and 38 respectively. Their average and median sperm concentration were 32.66 (SD = 16.97) million/mL and 35, and the mean and median total progressive motility, normal sperm morphology and DFI were 37.43% (SD = 17.17) and 40%, 1.88% (SD = 0.84) and 2, 26.20% (SD = 11.76) and 23.8%, respectively.

Approximately 14.5% of the patients were diagnosed with only oligozoospermia or azoospermia, while 34.1% had asthenozoospermia.

Nearly all patients (99.3%) exhibited teratozoospermia. In terms of DNA integrity, 12.4% of patients showed excellent to good integrity, whereas 29.2% displayed poor sperm DNA integrity (Tables 2, 3).

The control group had a mean age of 36.69 (SD = 5.29) years, with an average sperm concentration of 45.99

	Age	Sperm concentration /mL (×10^6)	Total motility (%)	Normal morphology (%)	DFI (%)
Mean	38.25	35.16	40.64	2.41	23.7925
Median	38.00	35.00	45.00	2.00	21.2000
Std. Deviation	5.876	16.741	17.213	1.374	11.85857
Skewness	.564	2.727	-.713	.715	1.309
Kurtosis	1.011	80.416	-.271	-.341	2.265
Range	54	485	80	6	89.46
Minimum	18	0	0	0	2.74
Maximum	72	485	80	6	92.20

Table 1.
Total study population characteristics.

	Age	Sperm concentration /mL (×10^6)	Total motility (%)	Normal morphology (%)	DFI (%)
Mean	38.61	32.66	37.43	1.88	26.2045
Median	38.00	35.00	40.00	2.00	23.8000
Std. Deviation	5.945	16.972	17.178	.843	11.76802
Skewness	.558	3.463	-.557	.163	1.251
Kurtosis	.945	95.882	-.559	-1.083	2.149
Range	54	485	80	4	89.46
Minimum	18	0	0	0	2.74
Maximum	72	485	80	4	92.20

Table 2.
Case group characteristics.

Table 3.
Sperm analysis parameters in infertile men.

Category	Subcategory	Frequency	Percentage
Sperm Count	Azoospermia	2	0
	Severe/Moderate Oligo	229	4.4
	Mild Oligo	522	10
	Normal	4443	85.5
Sperm Motility	Severe Astheno	492	9.5
	Moderate Astheno	357	6.9
	Mild Astheno	924	17.8
	Normal	3423	65.9
DFI Categories	Poor	1517	29.2
	Fair	871	16.8
	Fair to good	2163	41.6
	Excellent	645	12.4
Sperm Morphology	Teratozoospermia	5158	99.3
	Normal Morphology	38	0.7

(SD = 10.11) million cells/mL. Total progressive motility averaged 54.53% (SD = 7.98), while the mean normal morphology was 4.73% (SD = 0.61). DFI averaged 13.357% (SD = 3.97). These parameters fell within moderate ranges, reflecting overall normal sperm characteristics in the control group population.

Age and sperm parameters (concentration, motility, morphology) and DFI were significantly different between case and control group (Table 4).

Table 5 shows significant differences between different DFI groups. Age and sperm parameters differed across DFI groups. Post-hoc pairwise comparisons were conducted following a significant Kruskal-Wallis test. Significant differences in age, sperm concentration, motility, and normal morphology were observed between most DFI group pairs. The only non-significant comparison was sperm concentration between the DFI group with 25-30% and the group with > 30% DFI (U = 633,886, Z = -1.77, p = 0.076).

The correlation analysis among sperm parameters, age, and DFI revealed significant relationships. Age exhibits slightly negative correlations with normal sperm morphology and motility, which were not significant ($r < 0.2$, $p < 0.001$). In contrast, age positively correlates with DFI ($r = 0.248$, $p < 0.001$), indicating that increased age is associated with declining sperm DNA integrity. Sperm concentration positively correlates with total motility ($r = 0.774$, $p < 0.001$) and normal morphology ($r = 0.637$,

	Age	Sperm concentration /mL (×10^6)	Total motility (%)	Normal morphology (%)	DFI (%)
Study group Mean (median)	38.61 (38.00)	32.66 (35.00)	37.43 (40.00)	1.88 (2.00)	26.2045 (23.8000)
Control group Mean (median)	36.69 (36.00)	45.99 (45.00)	54.53 (55.00)	4.73 (5.00)	13.3574 (13.0000)
P-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Table 4.
Comparison of different parameters between case and control group. Variables represented as means and medians.

	DFI Category (DNA integrity condition)				P-value
	Poor	Fair	Good	Excellent	
Age - Mean (median)	40.48 (40.00)	38.94 (39.00)	37.75 (37.00)	36.46 (36.00)	< 0.001
Sperm Concentration /mL (×10x^6) - Mean (median)	30.38 (30.00)	31.59 (30.00)	35.75 (35.00)	41.03 (45.00)	< 0.001
Total Motility - Mean (median)	31.31 (35.00)	36.61 (40.00)	42.40 (45.00)	49.38 (50.00)	< 0.001
Normal Forms - Mean (median)	1.60 (1.00)	1.80 (2.00)	2.38 (2.00)	3.64 (4.00)	< 0.001

Table 5.
Comparison of age and sperm parameters in different DNA integrity conditions (DFI categories). Variables represented as means and medians.

		Age	Sperm concentration /mL (×10^6)	Total motility (%)	Normal forms (%)	DFI (%)
Age	Coefficient	1.000	-0.010	-0.059	-0.091	0.248
	p-value	-	0.435	.000	.000	0.000
Sperm Concentration/mL (×10x^6)	Coefficient	-0.010	1.000	0.774	0.637	-0.251
	p-value	0.435	-	0.000	0.000	0.000
Total Motility (%)	Coefficient	-0.059	0.774	1.000	0.756	-0.405
	p-value	0.000	0.000	-	0.000	0.000
Normal Forms (%)	Coefficient	-.091	0.637	0.756	1.000	-0.514
	p-value	0.000	0.000	0.000	-	0.000
DFI (%)	Coefficient	0.248	-0.251	-0.405	-0.514	1.000
	p-value	0.000	0.000	0.000	0.000	-

Table 6.
Comparison of different parameters between case and control group. Variables represented as means and medians.

$p < 0.001$), suggesting that higher sperm concentration is associated with improved motility and morphology. Additionally, sperm concentration shows a negative correlation with DFI ($r = -0.251$, $p < 0.001$), indicating that higher concentrations are associated with lower DNA fragmentation. Total progressive motility was correlated with normal sperm morphology ($r = 0.756$, $p < 0.001$) and negatively correlated with DFI ($r = -0.405$, $p < 0.001$), suggesting that greater motility is associated with better morphology and lower DNA fragmentation. Finally, normal sperm morphology is negatively correlated with DFI ($r = -0.514$, $p < 0.001$), highlighting that better sperm morphology is associated with lower DNA fragmentation levels and better sperm DNA integrity. All correlations are statistically significant at the 0.01 level (Table 6).

The 3D scatter plot of age, DFI, and normal sperm morphology reveals that younger individuals tend to cluster with lower DFI and normal morphology. A slight negative correlation between normal morphology and DFI is stronger in older participants, who also exhibited a wider range of DFI values (Figure 1).

We evaluated the impact of age on the likelihood of abnormal sperm conditions, including DFI and teratozoospermia using logistic regression analysis. The DFI model was statistically significant ($\chi^2 = 110.081$, $p < .001$) and well-fitted (Hosmer and Lemeshow test: $\chi^2 = 12.538$, $p = .129$), with age as a significant predictor ($B = -0.060$, $p < 0.001$), indicating that each additional year reduces the odds of normal DFI by approximately 5.8%. The model accurately classified 81.2% of cases. Similarly, the teratozoospermia model was significant ($\chi^2(1) = 105.535$, $p < .001$) with a relatively good fit ($\chi^2(8) = 12.309$, $p = .138$), explaining 2.6% of the variance and classifying 80.6% of cases. Despite age's significance, low R^2 values suggest that other factors are also important, highlighting the need for further research to identify additional predictive factors (Figure 2).

An ordinal logistic regression analysis was conducted to examine the effect of age on categorical DFI. The model

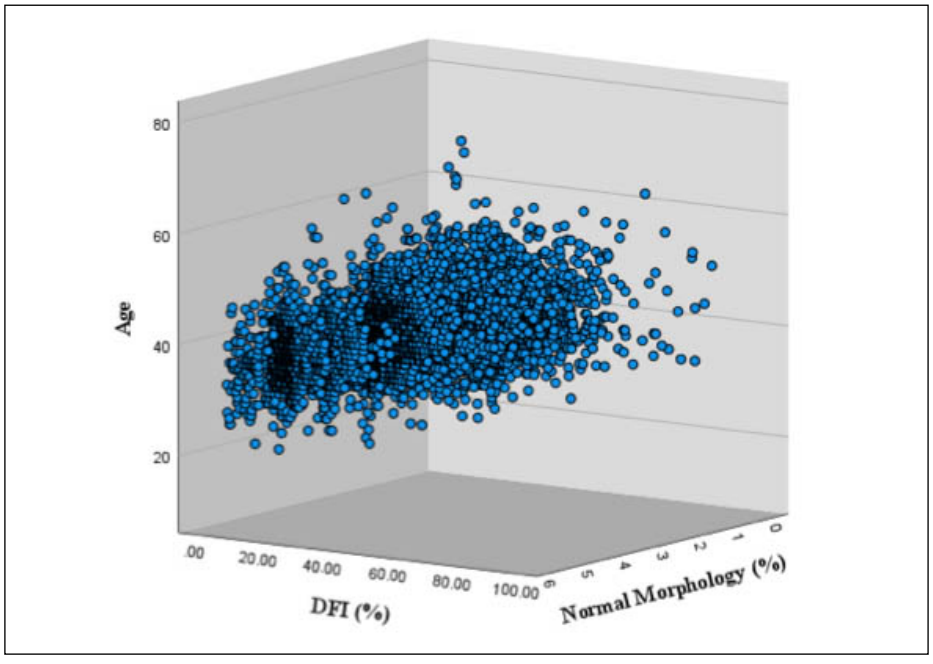
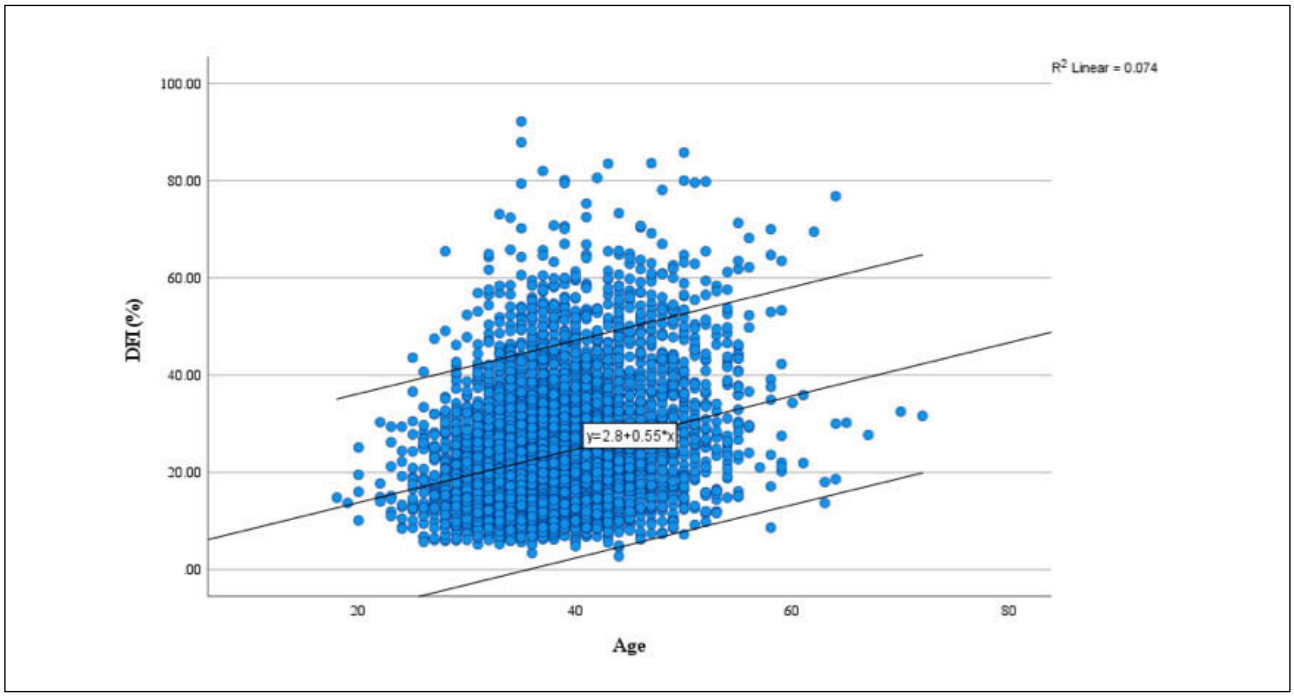


Figure 1.
Relationship between age, DFI and normal morphology.

Figure 2.
Age and DFI linear regression plot.



was statistically significant compared to the null model (χ^2 = 395.039, p < 0.001). Goodness-of-fit tests showed satisfactory results (Pearson χ^2 = 130.151, p = 0.822; Deviance χ^2 = 120.173, p = 0.942), suggesting a good fit for the data. The pseudo-R-squared values indicated a modest explanation of variance (Cox and Snell R^2 = 0.060, Nagelkerke R^2 = 0.064, McFadden R^2 = 0.023) (Figure 3).

The parameter estimate for age was significant (Estimate = 0.079, SE = 0.004, Wald χ^2 = 380.429, p < 0.001), indicating that each additional year of age significantly increases the likelihood of being in a higher DFI category (worse DNA integrity). The threshold estimates for the DFI categories were as follows:

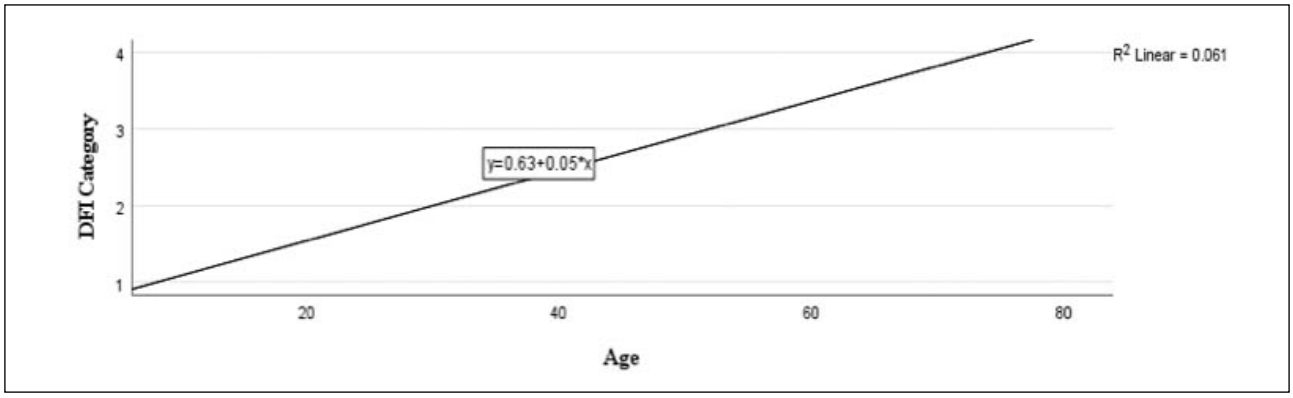
Excellent condition:
Estimate = 1.788 (SE = 0.153, p < 0.001)

Good condition:
Estimate = 3.544 (SE = 0.158, p < 0.001)

Fair condition:
Estimate = 4.227 (SE = 0.161, p < 0.001).

The ordinal logistic regression analysis found that age does not significantly predict the likelihood of being in different categories of oligozoospermia/azoospermia, as the model fit was not statistically significant, and the parameter estimate for age was non-significant (p > 0.05). The analysis of age's impact on motility condition categories revealed a significant model improvement (χ^2 = 9.935, p = 0.002). Goodness-of-fit statistics showed adequate model fit (Pearson χ^2 = 138.010, p = 0.669; Deviance χ^2 = 131.512, p = 0.799). However, the pseudo-R-squared values were low (Cox and Snell R^2 = 0.002, Nagelkerke R^2 = 0.002, McFadden R^2 = 0.001), suggest-

Figure 3.
Age and DFI ordinal regression plot.



ing that age accounts for only a small proportion of the variance in motility conditions.
The threshold estimates for motility conditions were:
Severe asthenozoospermia:
-3.049 (SE = 0.185, $p < 0.001$)
Moderate asthenozoospermia:
-2.441 (SE = 0.183, $p < 0.001$)
Mild asthenozoospermia:
-1.518 (SE = 0.181, $p < 0.001$)..

The parameter estimate for age was -0.015 (SE = 0.005, $p = 0.002$), indicating that as age increases, the likelihood of being in a higher motility condition category (better progressive motility) will be decreased. While age is a significant predictor of motility conditions, the low pseudo-R-squared values suggest the need for further research to identify additional predictors or interactions that may better explain variations in motility conditions.

Cytogenetic and molecular analysis

Among the 500 patients, predominantly diagnosed with azoospermia or oligozoospermia, 14 patients (2.8%) were found to have abnormal karyotypes, while the remaining 486 patients (97.2%) exhibited normal karyotypes. Among those with abnormal karyotypes, six patients had numerical chromosomal abnormalities, and eight had structural abnormalities of autosomal chromosomes, including t (4; 12), inv (11), inv (2), t (1; 3), t (2; 10), t (2; 11), t (3; 14) and t (4; 7). Specifically, three patients were diagnosed with Jacobs syndrome (XYY), one patient had Klinefelter syndrome (XXY), and another demonstrated mosaicism including XYY and XO chromosome complement. Additionally, one patient exhibited mosaicism for XYY alone. Of the patients with normal karyotypes, 48 patients carried marker chromosomes with no documented pathogenic significance, and 19 (14.2%) had chromosome 9 inversions, which are not associated with any reported pathogenic clinical condition (Table 7).
Multiplex PCR analysis conducted on 100 infertile males revealed deletions in AZF regions, including AZFa, AZFb, AZFc, AZFb+c, and AZFa+b+c, in 17 patients. The prevalence of *Y chromosome microdeletions* (YCM) was 17% in azoospermia/oligospermia cases. Among the detected deletions, AZFc region microdeletions were the most common, occurring in 16% of the patients. Specifically, 16 patients carry AZFc microdeletions; of these individuals, one also had an AZFb microdeletion, one patient had an AZFb microdeletion, and another had additional microdeletions in both AZFb and AZFa regions (Table 8).
WES was performed on 50 patients presenting with infertility, abnormal sperm parameters, and normal chromosome complement with no abnormalities detected in the AZF regions on Y chromosome. The mean age of such participants was 39.67 years, ranging from 29 to 60 years, with a standard deviation of 5.645. Among these patients, 26.1% had a history of varicocele, and all exhibited abnormalities in their sperm analysis reports. Additionally, 45.7% of these patients were in consanguineous marriages.
We identified mutations in 23 individuals across 19 genes linked, some of them loosely, to male infertility in OMIM database, including CFTR, TDRD9, DNHD1, DNAH5, DNAH9, NME8, CFAP43, CATIP, IFT74, MSH5, DRC1,

Normal	Frequency	Percentage	Abnormal	Frequency	Percentage
46 XY	419	83.8	46, XY (91)/ 47, XYY (8), 45, X (1)	1	0.2
46 XY 1(qh+)	13	2.6	46, XY (93)/ 47, XYY (7)	1	0.2
46 XY 13 (Ps+)	1	0.2	47, XYY	3	0.6
46 XY 22 (Ps+)	1	0.2	46, XY, t (4;12) (q31.1; q24.1)	1	0.6
46 XY 9 (qh+)	6	1.2	46, XY, inv (11) (p13q14)	1	0.6
46 XY Y(qh+)	3	0.6	46, XY, inv (2) (p16; q21)	1	0.6
46, XY, inv (9) (p11.2q13)	9	1.8	46, XY, t (1;3) (p31.2; qTer)	1	0.6
46, XY, inv (9) (p11.2q13) x2	2	0.4	46, XY, t (2;10) (q11; q24)	1	0.6
46, XY, inv (9) (p11.q12)	8	1.6	46, XY, t (2;11) (q32.3; q14)	1	0.6
46XY 14 (Ps+)	11	2.2	46, XY, t (3;14) (p25; q22)	1	0.6
46XY 15(Ps+)	1	0.2	46, XY, t(4;7) (q13;p21)	1	0.6
46XY 16 (qh+)	12	2.4	47, XXY	1	0.6

Table 7.
Karyotype results in infertile men.

Microdeletion	Frequency	Percentage
AZFa (sY84 & sY86) and AZFb (sY127&sY134) and AZFc (SY254 & SY255) microdeletion	1	1.0
AZFb (sY127 & sY134) and AZFc (sY254&sY255) microdeletion	1	1.0
AZFb (sY127 & SY134) microdeletion	1	1.0
AZFc (sY254 & SY255) microdeletion	14	14.0
No microdeletions on AZF region	83	83.0
Total	100	100.0

Table 8.
AZF region microdeletions found in 100 patients.

Genes	Frequency	Mutation	Pathogenicity
CFTR	2	Chr7:117611595T>G; NM_000492: exon20: c.3154T>G: p. Phe1052Val	LP
TDRD9	1	Chr14:104022263GT>G; NM_153046.3: exon24: c.2541del: p. His848IlefsTer13	LP
DNHD1	1	Chr11:6557183C>T; NM_144666.3: exon25: c.7888C>T: p. Arg2630Ter	LP
DNAH5	1	Chr5:13814615G>A; NM_001369: exon43: c.7220C>T: p. Pro2407Leu	VUS
DNAH9	1	Chr17:11961948C>T; NM_001372.4: exon68: c.12925C>T: p. Arg4309Ter	LP
NME8	1	Chr7:37850259G>A; NM_016616.5: intron2: c.-7-1G>A	VUS
CFAP43	1	Chr10:104143452G>A; NM_025145: exon32: c.4132C>T: p. Arg1378Ter	P
CATIP	1	Chr2:218357172T>A; NM_198559: exon2: c.103T>A: p. Phe35Ile	VUS
IFT74	1	Chr9:27056338T>G; NM_025103.4: exon18: c.1502T>G: p. Leu501Ter	LP
MSH5	2	Chr6:31758201C>G; NM_172166.4: exon13: c.1051C>G: p. Arg351Gly	VUS
DRC1	1	Chr2:26450086G>A; NM_145038: intron12: c.1599+1G>A	LP
DNAH8	2	Chr6:38926054G>A; NM_001206927.2: intron73: c.10963-1G>A	LP
DNAH11	1	Chr7:21677278G>T; NM_001277115.2: exon27: c.4775G>T: p. Cys1592Phe	VUS
LHCGR	1	Chr2:48687820G>C; NM_000233: exon11: c.1977C>G: p. Tyr659Ter	VUS
CYP21A2	5	Chr6:32040723G>A; NM_000500: exon9: c.1174G>A: p. Ala392Thr Chr6:32006281C>T; NM_000500: exon1: c.82C>T: p. Leu28Phe Chr6:32040723G>A; NM_000500: exon9: c.1174G>A: p. Ala392Thr Chr6:32040110G>T; NM_000500: exon7: c.844G>T: p. Val282Leu Chr6:32041006C>T; NM_000500.9: exon10: c.1360C>T: p. Pro454Ser	LP/P
HSF2	1	NM004506.3: c.456_459delTGAG	VUS
DNAAF5	1	Chr7:726923ct>c; NM_017802: exon1: c.206del: p. phe69serfsTer17	VUS

Table 9.
Heterozygous mutations in genes associated with male infertility.

		History of infertility or recurrent 1st trimester pregnancy losses				Total	P-value
			N	Not married	Y		
Consanguineous marriage		1	0	0	0	1	< 0.001
	No	0	11	1	11	23	
	N/A	0	2	0	0	2	
	Yes	0	10	0	11	21	
Total	1	23	1	22	47		

Table 10.
Frequency of infertility or recurrent 1st trimester pregnancy losses in consanguineous and non- consanguineous married.

DNAH8, DNAH11, LHCGR, CYP21A2, HSF2, DNAAF5, BBS4 and CFAP300 genes (Table 9).

The analysis of sperm parameters across individuals with and without mutations using Wald-Wolfowitz Runs Test revealed significant deviations from randomness in several parameters. Specifically, the following sperm characteristics exhibited non-random distributions: Progressive Motility, Non-Progressive Motility, Normal Morphology and Round Cells Count (P-value < 0.001).

Conversely, sperm concentration, total sperm number, total motility, non-motile sperm, and round cells (leukocytes) count did not show significant deviations from randomness, with p-values exceeding 0.05. Total motility approached significance with a p-value of 0.071, while the DNA fragmentation index (DFI) showed no significant deviations (p = 0.984).

The Pearson chi-square test indicated a significant association between consanguineous marriages and recurrent miscarriage or infertility ($\chi^2 = 50.160$, $p < 0.001$).

However, the likelihood ratio test did not yield significant results ($\chi^2 = 13.885$, $p = 0.126$), and the Fisher-Freeman-Halton Exact Test also did not reach significance (p = 0.063) (Table 10).

Discussion

Infertility is a growing global public health issue, with rising rates over recent decades due to factors like delayed pregnancy, environmental pollution, and lifestyle and nutritional changes (28). Male infertility significantly contributes to overall infertility, traditionally assessed using WHO-recommended semen analysis, with sperm DFI serving as a key marker, where higher DFI indicates lower chances of conception, embryo development, and implantation (29).

In our study, DFI was analyzed alongside age and sperm parameters (concentration, motility, morphology). Normozoospermic men had significantly lower DFI than infertile patients. Age showed a slight negative correlation with progressive motility and normal morphology, and a positive correlation with DFI, indicating declining sperm quality and DNA integrity with age. Logistic regression

revealed that each additional year increased the odds of abnormal DFI by ~5.8%. Scatter plots highlighted a stronger negative correlation between normal morphology and DFI in older men, with notable outliers showing high DFI and low morphology, emphasizing age-related impacts on sperm (30-32).

We also reported a negative correlation between DFI and sperm concentration, motility, and morphology; normal sperm morphology and motility were negatively correlated with DFI, indicating that better sperm morphology and motility are associated with lower DNA fragmentation levels and better sperm DNA integrity. Previous research supports this result, showing that DFI and other sperm analysis parameters (morphology, motility, concentration) are often negatively correlated (33) and high DFI levels often lead to poor sperm parameters and pregnancy outcomes (29, 32).

Our study found a significant link between consanguineous marriages and recurrent miscarriage or infertility, though the impact of consanguinity remains debated. Some research suggests inbreeding increases risks of stillbirth, preterm labor, and genetic disorders, while older studies report no clear effect on fertility outcomes (34, 36). This controversy highlights the need for further investigation. Using conventional cytogenetics on 500 men, we detected chromosomal abnormalities in 2.8% of patients. Six men (1.2%) had sex chromosome numerical abnormalities, including Jacob's, Klinefelter, and Turner syndromes with a male phenotype. Eight men (1.6%) carried structural autosomal abnormalities, such as translocations and inversions.

Recent studies reported genetic abnormalities in 15-30% of male infertility cases (37, 38). These genetic factors are including chromosomal abnormalities (numerical and/or structural) and single-gene defects (39). According to previous research, chromosomal abnormalities are responsible for 2-14% of male infertility cases (40) and up to 10% of female infertility cases (41). A recent study conducted on 1750 infertile men reported 4% chromosomal abnormality among Iranian infertile men, including 2% numerical abnormalities and 2% structural abnormalities (42).

We investigated Y chromosome microdeletions in 100 oligo/azoospermic patients with normal karyotypes, as these are the second most common genetic cause of male infertility after Klinefelter syndrome. Yq microdeletions are more prevalent in azoospermic men and range from 12-32% in Iranian infertile males (43-47). In line with previous studies, we detected AZF microdeletions in 17% of patients, reflecting the vulnerability of the haploid Y chromosome during spermatogenesis. Previously, WES was routinely recruited for discovering genetic defects in infertile men. Accordingly, mutations in HSF2 and TDRD9 were reported in males with idiopathic and non-obstructive azoospemia (48, 49). In addition, mutations in CYP21A2 and LHCGR can cause variable clinical spectrum and affect multiple hormonal pathways as well as tissues beyond the testis in infertile males (21). Another study reported CFAP43 to be confidently linked to multiple morphological abnormalities of the sperm flagellum (MMAF) in male infertility (50, 51). In our study, 23 out of 50 patients in the WES group were found to carry mutations in 19 genes linked to male infertility. Eleven genes, including DNHD1, DNAH5, DNAH8, DNAH9, DNAH11, CFAP43, IFT74, DRC1, CFAP300, DNAAF5, and CATIP, are critical for the structure, motility, and function of cilia and sperm flagella (52-58). Mutations in these genes can cause primary ciliary dyskinesia (PCD) and related male infertility with variable severity. DNHD1, DNAH5, DNAH8, DNAH9, and DNAH11 encode dynein heavy chains, while DRC1 regulates dynein synthesis. DNAH5 and DNAH9 work together, and mutations can disrupt outer dynein arms, impairing cilia and sperm flagella movement. One patient with DNAH5/DNAH9 mutations showed severe asthenozoospermia, motility defects, and high DFI. A heterozygous DNAH11 mutation caused severe azoospermia, although PCD symptoms usually require homozygous or compound heterozygous mutations. Recent studies show heterozygous DNAH11 mutations can impair cilia function even without ultrastructural defects. Overall, homozygous or compound heterozygous variants disrupt dynein arm function and cause infertility, while the impact of heterozygous mutations on azoospermia is not well documented (58-63).

Mutations in CFAP43 and CFAP300 typically cause PCD in an autosomal recessive manner, with symptoms usually seen in biallelic carriers. Our patient with a heterozygous CFAP300 mutation exhibited low sperm motility, supporting studies suggesting heterozygous mutations may contribute to disease. Heterozygous CFAP43 mutations have also been linked to conditions beyond PCD, such as normal pressure hydrocephalus (NPH), indicating broader ciliary dysfunction. The p.Arg1378Ter CFAP43 mutation in our patient may underlie infertility, teratozoospermia, and reduced sperm motility. These findings suggest a potential role of heterozygous CFAP43 mutations in male infertility, warranting further study (65-66). One patient carried a mutation in IFT74, part of the intraflagellar transport complex, which can cause ciliopathies affecting multiple systems, including fertility and hearing. This patient showed progressive hearing loss, moderate teratozoospermia, and reduced sperm motility, consistent with previous reports of heterozygous IFT74 mutations in ciliopathy syndromes (67). We also identified heterozygous mutations in TDRD9, NME8, and HSF2, genes important for spermatogenesis. TDRD9 mutations are linked to spermatogenic failure or azoospermia, and NME8 is involved in sperm development. However, the role of heterozygous mutations in TDRD9 and NME8 in male infertility remains unclear and requires further investigation (68, 70).

The HSF2 gene regulates spermatogenesis, and our patients with heterozygous HSF2 mutations had non-obstructive azoospermia. Previous studies identified heterozygous HSF2 mutations causing spermatogenic blockage via dominant-negative effects. Another patient carried a heterozygous deletion-insertion variation linked to azoospermia. Functional validation is still needed to confirm the pathogenicity of these mutations (71-72). The CFTR gene encodes a chloride channel essential for epithelial salt and water balance, and mutations can cause CFTR-Related Disorders like CBAVD and cystic fibrosis (73, 74). CFTR abnormalities in men can lead to infertil-

ity through obstructive sperm transport issues or oligospermia. Even mild heterozygous CFTR mutations may cause isolated infertility without tract obstruction (75). We identified two infertile patients with heterozygous CFTR mutations presenting with azoospermia, highlighting the need for further study on their impact on male fertility.

The MSH5 gene encodes a protein that is a part of the MutS homolog family, which is involved in DNA mismatch repair and homologous recombination during meiosis. Bi-allelic mutations in MSH5 are typically associated with defects in meiosis, potentially leading to issues such as impaired fertility due to problems with chromosomal segregation (76, 77). The LHCGR gene regulates reproductive hormone signaling, and mutations can cause familial male-limited precocious puberty (testotoxicosis) with variable effects on fertility. Prolonged high testosterone may lead to testicular exhaustion and reproductive dysfunction. Our patient showed oligozoospermia, teratozoospermia, asthenozoospermia, and high DFI. Studies in Iranian men suggest certain LHCGR variants are more frequent in infertile individuals, indicating a possible link to male infertility (78, 79).

One patient with mild teratozoospermia, asthenozoospermia, and very poor DFI carried a mutation in CYP21A2, which encodes 21-hydroxylase, essential for corticosteroid and sex hormone biosynthesis. Mutations in CYP21A2 cause *congenital adrenal hyperplasia* (CAH), typically recessive (80, 81), though heterozygous mutations can also contribute, as shown in studies by *Espinosa et al.* (2020), *Gangodkar et al.* (2021), and a Chinese population study linking heterozygous variants to hyperandrogenism symptoms like acne and hirsutism. Infertility related to 21-hydroxylase deficiency is documented in both sexes, indicating heterozygous CYP21A2 mutations may impair fertility (81-85). Additionally, mutations in CYP21A2 and LHCGR can cause variable phenotypes, including precocious puberty, Leydig cell hypoplasia, and CAH, affecting multiple hormonal pathways and tissues beyond the testes (21, 86).

Recent studies have indicated that mutations in the BBS4 gene can exhibit a complex inheritance pattern. Katsanis et al. conducted a research involving 175 families with Bardet-Biedl syndrome that identified cases where individuals had a single heterozygous BBS4 mutation but presented with symptoms typical of the disease. This observation suggests that BBS4 mutations may sometimes contribute to the disease in a non-classical manner, beyond the traditional autosomal recessive model (87). Bardet-Biedl Syndrome is known to affect both males and females, often leading to infertility due to its systemic manifestations. Given the established link between BBS and infertility, the presence of BBS4 mutations in a heterozygous state, raises the possibility that these mutations could contribute to male infertility (88).

In this study, mutations in 19 genes linked to male infertility were reported and explored. While the exact relationship between some of these mutations and infertility remains to be fully understood and some of these reported mutations can be incidental findings with no significant clinical implication, our findings underscore the complexity of genetic factors in reproductive health. The presence of these mutations in nearly half of our cohort highlights the potential utility of WES as a diagnostic tool, particularly for patients with no known acquired etiology, with normal karyotype and no AZF region microdeletions.

Conclusions

Our comprehensive study involving over 5,000 infertile and 1200 fertile men provides insights into the relationships between sperm parameters, DFI, and age. By analyzing karyotypes, AZF microdeletions, and WES in a subset of participants, we identified significant genetic abnormalities, including chromosomal anomalies, AZF region microdeletions, and monogenic mutations. These findings underscore the importance of integrating molecular assessments, such as DFI assessment and genetic analysis, into the diagnostic protocols for male infertility. Future research should continue to explore these molecular factors to enhance the accuracy of infertility diagnosis and improve treatment outcomes.

Declarations

Ethical approval and consent for participate: *This study was approved by the Institutional Review Board protocol of MOM Fertility & Infertility Treatment Center and adhered to the tenets of the Declaration of Helsinki (the ethics code: IR.IAU.TMU.REC.1402.159). All experiments were performed in accordance with relevant guidelines and regulations.*

Consent for publication: *As this is a retrospective study, informed consent was not required from individual participants. However, all data were anonymized to ensure participant confidentiality and privacy. The study adhered to the guidelines set forth by the ethical committee, and all necessary precautions were taken to protect the rights and well-being of the participants.*

Availability of data and material: *All relevant data are included within the article. The data supporting the findings of this study are available from the corresponding author upon reasonable request.*

Competing interests: *We declare that we have no conflicts of interest or competing interests in relation to this research.*

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Authors' contributions: *MM and AF: Conceptualized and designed the study, Assisted with data collection, Performed statistical analysis and wrote the manuscript. SP and AB: Assisted with data collection. ASh and AS: Contributed to the writing of the manuscript and performed statistical analysis. MH and SM and SB and MK: Assisted with data interpretation and reviewed the manuscript. NY and NY: Provided critical feedback on the study design. HM and FF: Supervised the project, reviewed the manuscript and approved the final version for submission. All authors read and approved the manuscript*

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