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Research Article

Vim3 and Mxi-2 as Promising New Molecular Biomarkers for the Diagnosis of Sperm Disorders

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Introduction: Male infertility remains a significant clinical challenge that requires the exploration of new diagnostics strategies. Truncated proteins, such as Vim3, a truncated variant of vimentin, show promise as a new class of biomarkers for diagnosing fertility disorders. Our objective was to assess the potential utility of Vim3 and Mxi-2, a shortened iteration of mitogen-activated protein kinase (MAPK) p38, as noninvasive biomarkers for diagnosing sperm disorders.
Methods: Semen samples of 19 fertile males (control group) and 82 infertile males with semen abnormalities and no female factor ($n = 28$ OAT [oligo–astheno–teratozoospermia] syndrome, $n = 8$ azoospermia, $n = 29$ teratozoospermia, $n = 11$ astheno–teratozoospermia, and $n = 6$ oligozoospermia) were analyzed using ELISA (enzyme-linked immunosorbent assay) and flow cytometry. Furthermore, we conducted a lateral flow assay (LFA) specific to Vim3.
Results: Our analysis revealed that Vim3 and endothelin-1 (ET-1) expression levels were significantly downregulated in non-normozoospermic patients (e.g., OAT syndrome), while Mxi-2 was overexpressed in these patients compared to the control group ($p < 0.01$). Additionally, we introduced a novel LFA that provides a user-friendly and effective method for examining Vim3 as a biomarker in clinical settings.
Conclusion: Our results suggest that Vim3 and Mxi-2 have potential as noninvasive semen biomarkers for diagnosing sperm disorders.

Keywords: male infertility; Mxi-2; truncated proteins; Vim3

1. Introduction

Male infertility affects 8% to 12% of couples worldwide, contributing to approximately 50% of all infertility cases [1, 2]. Accurate prediction of male fertility is crucial, yet molecular biomarkers with adequate sensitivity and specificity are still lacking [2]. Conventional semen analysis for predicting and diagnosing male fertility has been debated in terms of its reliability and effectiveness over the years [2–4]. This has prompted increased interest in identifying molecular biomarkers that can provide deeper insights into sperm function beyond conventional parameters like motility and morphology.

Recent advances in omics technologies, as highlighted by Llavanera et al. [2], have revealed a variety of molecular markers to improve the understanding of sperm function [2]. Non-coding RNAs, such as microRNAs (miRs), have shown excellent predictive value in various sperm quality disorders. Furthermore, miRs are involved in post-transcriptional regulation and could directly impact protein expression, including the truncation of proteins involved in biological processes [5].

Truncated proteins, such as Vim3, a truncated variant of vimentin, have emerged as potential biomarkers for diagnosing male infertility. Vim3 is primarily found in the neck and tail of human sperm, while full-length vimentin is located in the sperm head [6, 7]. Asymmetric distribution of Vim3 has been previously linked to structural abnormalities in sperm [6], suggesting that abnormal Vim3 expression could impair sperm function. Moreover, a recent study has indicated that Vim3 downregulation, potentially influenced by Epstein–Barr virus (EBV), is associated with impaired sperm parameters [8], further emphasizing its relevance in reproductive health.

Similarly, Mxi-2, a truncated variant of mitogen-activated protein kinase (MAPK) p38, represents another truncated key protein involved in biological processes [7, 9]. Our recent research demonstrates that endothelin-1 (ET-1) regulates the expression of both Vim3 and Mxi-2, via *miR-498*, and *miR-15a*, leading to transcriptional arrest [7]. Maggi et al. [10] were the first to identify ET-1 and its receptors in the human testis [10], demonstrating higher ET-1 levels in the seminal plasma of normozoospermic fertile men compared to infertile men [10].

While omics technologies offer high accuracy in identifying molecular biomarkers, their clinical application is often hindered by high costs and the need for highly specialized technicians [2]. In contrast, targeting proteins such as Vim3 and Mxi-2 offer a cost effective, noninvasive, time saving, and efficient alternative. Based on these findings, we aimed to evaluate the potential of Vim3 and Mxi-2 as molecular biomarkers for diagnosing sperm disorders and their possible clinical application.

2. Materials and Methods

2.1. Patient Cohort. Semen samples from 19 fertile males with normozoospermia (control group) and 82 infertile males with semen abnormalities and no female factor infertility were analyzed and categorized using the World Health Organization (WHO) laboratory manual for human semen analysis [11, 12]. Each patient maintained 2–7 days of sexual abstinence before providing an ejaculate sample for analysis. The study was approved by the Ethics Committee of the University of Cologne's Medical Faculty (approval number: 22-1287) and registered with the German Clinical Trials Registry (DRKS00029986). It was conducted in accordance with the Declaration of Helsinki, with all patients providing written informed consent. Table 1 provides detailed age and semen analysis data for the patient cohort, indicating no significant differences in demographic factors, including age and duration of abstinence, between groups. Furthermore, patients with male infertility factors, such as congenital bilateral absence of the vas deferens (CBAVD) or seminal duct obstructions (e.g., utricle cyst), were excluded from the study.

2.2. Enzyme-Linked Immunosorbent Assay (ELISA). The levels of ET-1, Vim3, and Mxi-2 in semen samples were determined using a semiquantitative, previously validated ELISA [6]. Samples were diluted based on the spermiogram results to reach a concentration of 6×10^5 cells/50 µl. Noncoated 96-well plates (Brands GmbH + Co. KG, Wertheim, Germany) were used. A total of 50 µl of each diluted sample was added to the wells and incubated for 1 h at room temperature. Primary antibodies against Vim3 (Davids Biotechnologie GmbH, Regensburg, Germany; dilution 1:1000, incubated for 1 h at 37°C), Mxi-2 (nanoTools Antikörpertechnik, Teinigen, Germany; dilution 1:1000, incubated for 1 h at 37°C), and ET-1 (#sc-517436, Santa Cruz Biotechnology, Dallas, Texas, USA; dilution 1:1000, incubated for 1 h at 37°C) were used according to the manufacturer's protocols [6, 8, 9, 13]. Absorbance was measured at 450 nm using the FLUOstar Omega microplate reader (BMG LABTECH, Offenburg, Germany). Each sample was measured in triplicate to ensure the reliability of the results.

2.3. Flow Cytometry. Immunostaining was performed according to standard protocols to detect Vim3 protein expression in sperm cells. For staining an antihuman Vim3 primary antibody (Davids Biotechnologie GmbH, Regensburg, Germany, dilution 1:500) and an Alexa-488-conjugated antimouse secondary antibody (#sc-516606, Santa Cruz, Dallas, Texas, USA) was used. Data were acquired with an Attune CytPix flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed using FlowJo software (BD, Version 10.8, https://www.flowjo.com). A total of 6×10^5 cells/50 µl was measured for each sample ($n = 15$).

2.4. Lateral Flow Assay (LFA). The LFA was conducted following established protocols [9, 14] to detect the presence of Vim3 protein in semen samples from patients with normozoospermia and OAT (oligo–astheno–teratozoospermia) syndrome. A total of 50 µl of semen sample was applied to each test strip. The assay was run using the ChemoStar ECL Imager (INTAS Science Imaging Instruments GmbH, Göttingen, Germany). LFA is a rapid diagnostic test that detects the presence or absence of specific target substances in liquid samples, making it ideal for point-of-care testing due to its simplicity and fast results.

2.5. Statistical Analysis. Statistical analysis was carried out using Prism statistical software (GraphPad Software, Inc., Version 9.4.0, https://www.graphpad.com/). Group comparisons were performed using the nonparametric Kruskal–Wallis test. All p-values were two-tailed, with $p < 0.05$ considered statistically significant.

3. Results and Discussion

This study analyzed semen samples from 101 males, comprising 19 fertile males with normozoospermia and 82 infertile males with semen abnormalities and no female factor infertility. Age and semen analysis data for both groups are presented in Table 1. No significant differences were observed in age or abstinence period among the patients. Male infertility factors such as CBVAD or vas deferens obstruction (e.g., utricle cyst) were excluded from the study.

We first measured ET-1, Vim3, and Mxi-2 expression in semen samples using ELISA. ET-1 and Vim3 levels were significantly lower in semen samples from infertile males, including those with OAT syndrome ($p < 0.001$, Figure 1A,B), as previously reported by Huerta et al. [8]. In contrast, Mxi-2 levels were elevated in samples with abnormal sperm parameters compared to the control group ($p < 0.001$, Figure 1C). Flow cytometry further confirmed that Vim3 expression was

Table 1: Patient age and semen analysis of fertile and infertile males.

	Fertile males ($n = 19$)	Infertile males ($n = 82$)				
	Normozoospermia ($n = 19$)	OAT syndrome ($n = 28$)	Azoospermia ($n = 8$)	Teratozoospermia ($n = 29$)	Astheno–teratozoospermia ($n = 11$)	Oligozoospermia ($n = 6$)
Age (years) (mean ± SD)	28.6 ± 3.9	30.6 ± 3.0	35.2 ± 3.2	33.2 ± 3.8	38.5 ± 8.4	32.2 ± 1.7
Semen volume (ml) (mean ± SD)	2.9 ± 0.6	3.2 ± 1.0	2.4 ± 1.2	3.2 ± 1.5	2.5 ± 0.7	1.8 ± 0.6
Sperm concentration (10^6/ml) (mean ± SD)	227 ± 116.5	10.8 ± 2.9	0	98 ± 45.0	53.5 ± 33.2	3.6 ± 2.8
Total sperm count (10^6 cells) (mean ± SD)	719.4 ± 446.5	33.1 ± 7.6	0	330.6 ± 207.4	142.8 ± 114.9	4.5 ± 3.9
Progressive sperm motility (%) (mean ± SD)	61.4 ± 7.5	23.4 ± 5.1	0	56.2 ± 6.1	19.0 ± 5.9	43.0 ± 0
Nonprogressive sperm motility (%) (mean ± SD)	6.2 ± 3.8	4.8 ± 3.6	0	2.0 ± 1.5	5.3 ± 2.8	0.5 ± 1.0
Normal sperm morphology (%) (mean ± SD)	4.8 ± 0.4	1.2 ± 1.0	0	2.0 ± 0.9	1.9 ± 1.0	5.0 ± 2.0
Serum T (ng/ml) (mean ± SD)	3.96 ± 1.45	4.37 ± 1.44	3.53 ± 1.22	4.53 ± 1.61	5.25 ± 1.68	5.76 ± 1.23
Serum LH (mIU/ml) (mean ± SD)	3.23 ± 0.94	5.23 ± 1.83	7.03 ± 1.88	3.38 ± 1.49	3.94 ± 0.85	4.35 ± 2.55
Serum FSH (mIU/ml) (mean ± SD)	3.86 ± 1.37	9.51 ± 6.22	23.59 ± 7.87	5.05 ± 2.57	5.5 ± 1.87	11.65 ± 8.35
Serum PRL (ng/ml) (mean ± SD)	7.89 ± 3.79	10.77 ± 6.49	10.34 ± 1.86	9.27 ± 2.85	12.28 ± 2.79	9.70 ± 1.00

Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone; OAT syndrome, oligo–astheno–teratozoospermia syndrome; PRL, prolactin; SD, standard deviation; T, testosterone.

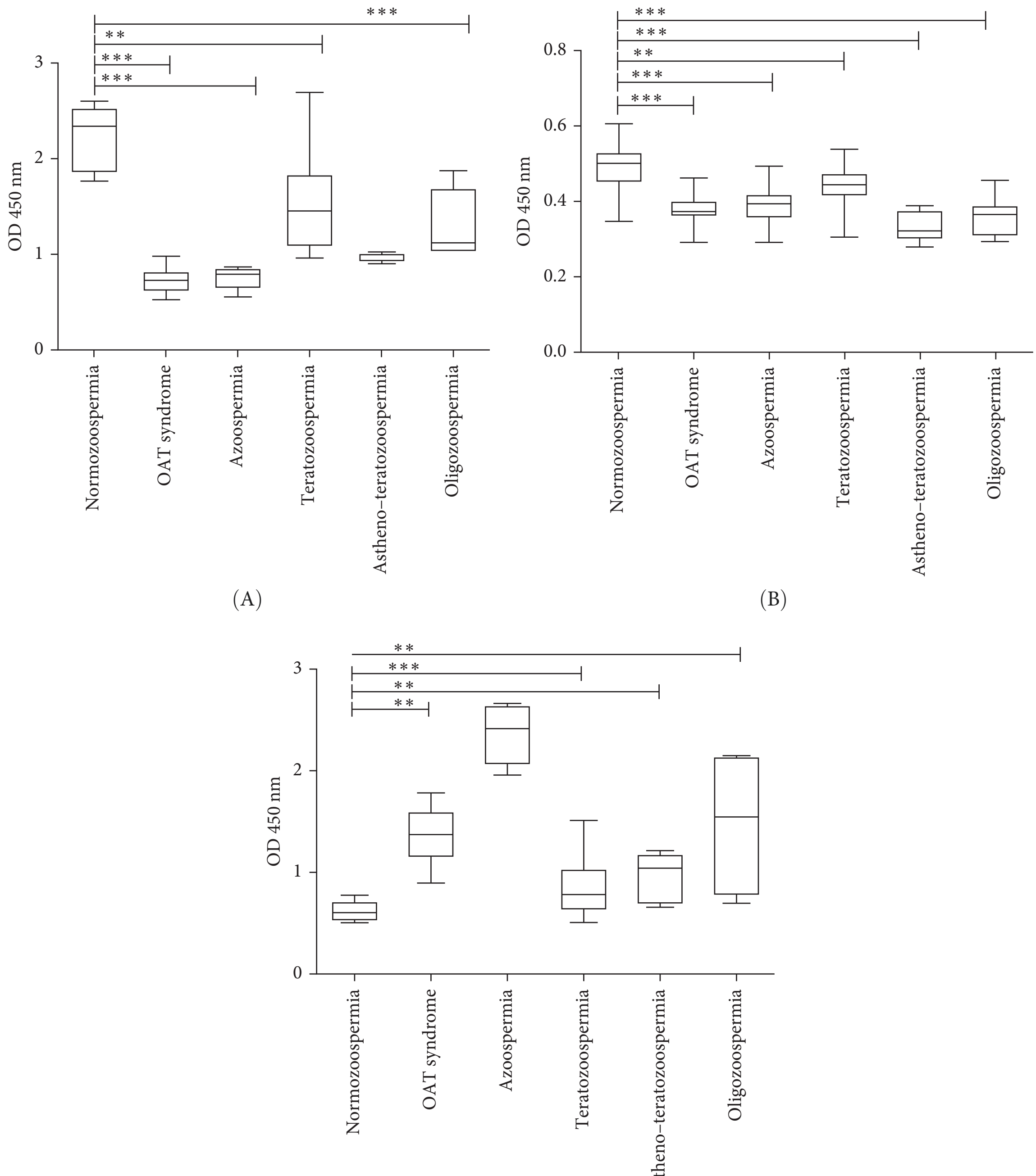

Figure 1: Differential expression of ET-1, Vim3, and Mxi-2 in semen samples from fertile and infertile males. Expression levels of ET-1 (A), Vim3 (B), and Mxi-2 (C) in semen samples ($n = 101$) were measured using ELISA (absorbance at OD 450 nm). Patients were categorized into fertile and infertile males, including those with OAT syndrome, azoospermia, teratozoospermia, astheno–teratozoospermia, and oligozoospermia. ET-1 and Vim3 levels were significantly reduced in semen samples from infertile males, particularly in those with OAT syndrome, while Mxi-2 levels were significantly elevated in samples with abnormal sperm parameters compared to the control group. Statistical analysis was performed using the nonparametric Kruskal–Wallis test ($^{**}p < 0.01$, $^{***}p < 0.001$). OAT syndrome, oligo–astheno–teratozoospermia; Vim3, vimentin 3.

significantly higher OAT syndrome patients compared to controls (Figure 2).

To complement conventional semen analysis, we developed a Vim3-specific LFA, after finding that Vim3 expression significantly decreased in OAT syndrome patients. Our results demonstrate that Vim3 is present in normozoospermic fertile patients but absent in patients with OAT syndrome (Figure 3).

Despite advancements in andrology, diagnostic challenges persist, highlighting the need for novel approaches. Truncated proteins like Vim3 and Mxi-2 are emerging as promising

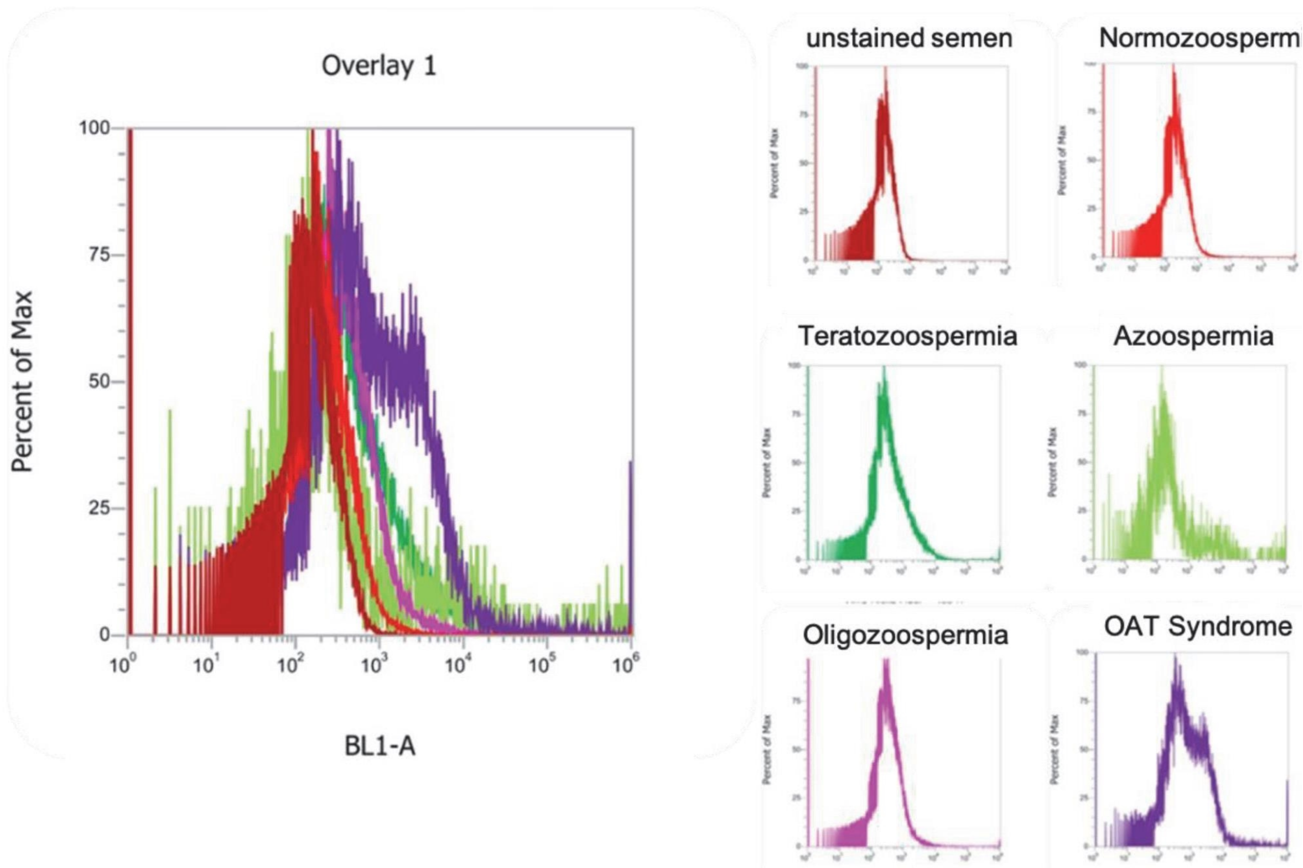

FIGURE 2: Comparative Vim3 expression in semen samples from fertile and infertile males. Normalized histogram illustrating Vim3 expression detected by flow cytometry insemen of fertile and infertile patients ($n = 15$) with OAT syndrome, azoospermia, teratozoospermia, astheno–teratozoospermia, and oligozoospermia (plus unstained control as reference). The superimposed histograms highlight differences in Vim3 expression across the various fertility conditions. OAT syndrome, oligo–astheno–teratozoospermia syndrome; Vim3, vimentin 3.

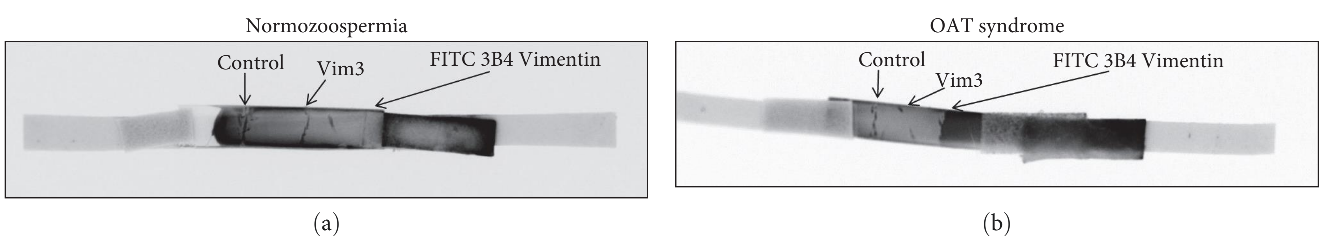

FIGURE 3: Vim3 specific lateral flow assay in semen samples from patients with normozoospermia and OAT syndrome. Vim3 was absent in the semen of patients with OAT syndrome compared to control samples (normozoospermia). In (a), both the control line and the Vim3 band were visible, indicating the presence of Vim3. In (b), only the control line was present, indicating no detectable Vim3. OAT syndrome, oligo–astheno–teratozoospermia syndrome; Vim3, vimentin 3.

biomarkers that provide cost effective, simple, and accurate diagnostic tools for identifying infertility causes and improving fertility prognosis [6]. The identification of molecular biomarkers in semen could play a crucial role in diagnosing and treating male fertility disorders [2].

Vim3 and Mxi-2 provide molecular-level insights into sperm integrity that conventional semen analysis may overlook. While traditional methods focus on sperm count, motility, and morphology, they fail to reveal the underlying molecular mechanisms contributing to infertility [2–4]. The straightforward application of ELISA and flow cytometry in detecting Vim3 and Mxi-2 makes these biomarkers highly suitable for clinical settings. Compared to more complex molecular diagnostics like omics-based technologies [2–4], which require expensive equipment and specialized expertise, Vim3 and Mxi-2 offer a more accessible and affordable alternative for routine use in fertility clinics. By complementing conventional semen analysis, these biomarkers can help identify subclinical sperm defects and enhance the precision of male infertility diagnoses.

The development of the Vim3-targeted LFA, which provides rapid and user-friendly results within minutes, represents a significant advancement in diagnostic tools for point-of-care and field applications. Conventional semen analysis often fails to identify the specific cause and etiology of male infertility, complicating prognosis and treatment [2, 4]. Incorporating molecular biomarkers like Vim3 and Mxi-2 has the potential to improve diagnostic precision and lead to better identification of male reproductive disorders. These biomarkers may be especially valuable in IVF clinics, enabling noninvasive, cost effective, and precise diagnostics. While our study presents promising results, further research is needed to fully understand the correlation between Vim3, Mxi-2, and pathological spermiograms. A deeper understanding of these biomarkers will advance male fertility diagnostics and enable targeted treatments.

Limitations of our study include the relatively small sample size, particularly in the azoospermia ($n = 8$), astheno–teratozoospermia ($n = 11$), and oligozoospermia ($n = 6$) subgroups, which may reduce the statical power of our findings. Additionally, the retrospective nature of the data collection may introduce potential bias, underscoring the need for caution when generalizing the results to broader populations.

4. Conclusion

Our results suggest that truncated proteins, particularly Vim3 and Mxi-2, hold potential as noninvasive molecular biomarkers for diagnosing sperm disorders. Additional analysis of these truncated proteins could complement conventional spermiogram findings and enhance diagnostic accuracy.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Statement

The study received approval from the Ethics Committee of the Medical Faculty at the University of Cologne (approval number: 23-1178) and was conducted in adherence to the Declaration of Helsinki.

Consent

All patients provided written informed consent.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Richard Weiten, Jan Herden, Enno Storz, and Melanie von Brandenstein contributed equally to this study.

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