Serum miRNAs as Potential Diagnostic Biomarkers for Non-Obstructive Azoospermia

Fatima Mahdi Kadhum 1*, Ali Ibrahim Rahim 2, Ula Al-Kawaz 1

Abstract
Background: Infertility in couples shows a significant challenge worldwide, with non-obstructive azoospermia (NOA) being a prevalent condition characterized by defective spermatogenesis. Research into genetic factors contributing to NOA and the exploration of non-invasive diagnostic biomarkers are crucial for effective management. Method: Thirty-five NOA men underwent testicular biopsy and physical assessments. Serum miRNA (miR-211, miR-429, miR-34c-5p) levels were measured using RT-PCR to evaluate their potential as diagnostic biomarkers. Results: Among the patients, 77.14% showed positive biopsy results. Serum miR-211 levels were significantly lower in NOA patients compared to controls (p < 0.001), while miR-429 and miR-34c-5p showed slight decreases without significance. ROC analysis revealed diagnostic potential for miR-211. Discussion: Dysregulation of miRNAs, particularly miR-211, has been associated with spermatogenesis disorders. However, limited research exists on miRNA profiles in NOA. Our findings align with previous studies, suggesting miRNA-211’s potential as a biomarker. Further studies with larger cohorts are warranted. Conclusion: Serum miR-211 demonstrates promise as a non-invasive diagnostic biomarker for NOA. This study underscores the importance of miRNAs in male infertility diagnostics and lays the groundwork for future research in this area.

Keywords: Non-obstructive azoospermia, miRNA biomarkers, male infertility, diagnostic technique, spermatogenesis, miR-211, miR-34c-5p, and miRNA-429

Introduction
Infertility in couples is defined as the inability to achieve a successful pregnancy after 12 months of unprotected sexual intercourse (Vander Borght & Wyns, 2018). Non-obstructive azoospermia (NOA), characterized by defective spermatogenesis, affects approximately 60% of individuals with azoospermia. It results from a complete or partial loss of sperm production, often referred to as testicular failure (TF) (Cao et al., 2023). Since many cases of azoospermia have unknown causes, there is a pressing need for research into the genetic factors contributing to this condition and the exploration of viable treatment options (Shapiro & Ohlander, 2018). Additionally, it is crucial to identify a non-invasive diagnostic biomarker for azoospermia to prevent the need for further diagnostic biopsies.

miRNAs have the ability to control gene expression and play significant roles in various biological processes, including cellular differentiation, proliferation, and apoptosis (Pop & Almquist, 2021, Monika et al. 2023). These molecules are crucial in sperm production and male reproduction, and they are present in significant amounts in seminal fluid and blood serum. Several studies have examined various microRNAs in serum and plasma samples of infertile males. miR-211, miR-34c-5p, and miR-429 have shown potential as new and effective non-invasive diagnostic biomarkers for male infertility.

Significance
Identifying serum miRNAs aids in diagnosing non-obstructive azoospermia non-invasively, crucial for effective male infertility management.

*Correspondence. Fatima Mahdi Kadhum, High Institute for Infertility Diagnosis and Assisted Reproductive Technology, Al-Nahrain University, Baghdad, Iraq. E-mail: Fatima.altamim88@gmail.com

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infertility. Recent research has explored the possible link between these microRNAs and male infertility, noting their downregulation in the testes of patients with non-obstructive azoospermia (NOA) and oligozoospermia in testis, seminal plasma, and blood serum (Saebnia, Neshati & Bahrami, 2021).

Materials and Methods
Thirty-five men with non-obstructive azoospermia were included in a clinical trial study conducted at the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies at Al-Nahrain University from June 1, 2022, to December 2023. Each participant underwent a comprehensive physical assessment and scrotal ultrasonography to determine testicular dimensions and rule out any obstructive or varicose conditions within the seminal tract. This study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. All participants provided informed consent prior to their inclusion in the study. The research protocol was reviewed and approved by the Institutional Review Board of the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies at Al-Nahrain University. Participants’ confidentiality and anonymity were maintained throughout the study, and all data were securely stored and accessed only by authorized personnel. The study aimed to minimize any potential risks to participants, ensuring that the benefits of the research outweighed any potential harm.

miRNA was extracted using the miRNeasy Serum/Plasma Advanced Kit (Qiagen, Germany) according to the manufacturer’s instructions. Complementary DNA (cDNA) synthesis was performed using the GoScript™ Reverse Transcription System (Promega, USA), and the expression levels of miR-211, miR-429, and miR-34c-5p were analyzed using two-step real-time PCR (RT-PCR). Specific lyophilized primers were prepared for each marker (Table 1).

Statistical Analysis
Statistical analyses were performed using SPSS software version 25.0 (SPSS, Chicago). The receiver operating characteristic curve (ROC) was used to evaluate the diagnostic value of (miRNA211, miRNA429, and miRNA-34c-5p) in the context of discrimination between patients and controls. A p-value less than 0.05 indicated a statistically significant difference.

Results
Thirty-five infertile males were enrolled in this interventional comparative study. The results were expressed as mean ± standard deviation. The mean age of the patients was 34.23 ± 8.02 years, which did not differ significantly from that of the controls (35.0 ± 5.53 years). Out of the thirty-five patients, 27 (77.14%) had positive biopsy results, while 8 (22.86%) had negative biopsy results (Figure 1). The median miR-211 expression in patients was significantly lower than in controls (P-value < 0.001). The median expression of miR-429 in patients was slightly lower than that of controls, with no significant difference (P-value 0.356). Similarly, the expression of miR-34c in patients was slightly lower than in controls, with no significant difference (P-value 0.884), as shown in Table 2.

Discussion
Non-obstructive azoospermia (NOA) is a condition characterized by testicular failure that cannot be treated. It can be caused by various factors and is categorized into primary or secondary forms. Research indicates a strong association between the dysregulation of miRNAs and human diseases (Krausz & Ciopp, 2021). Studies have shown that miRNAs play significant roles in the process of spermatogenesis (Chen & Han, 2023). However, there are limited reports on this topic, and more investigation is needed to understand the specific changes in miRNA profiles in individuals with NOA.

Alterations in the expression of miRNA-429 have been linked to various histopathologic forms of azoospermia in male patients, suggesting that miRNAs could serve as targeted biomarkers for assessing male fertility (Khawar, Mehmood & Roohi, 2019). We observed a non-significant downregulation in the expression of miRNA-429 in the blood serum of individuals with non-obstructive azoospermia (NOA) compared to the control group (P-value 0.356). Our findings align with those of Zhang et al., who conducted a comparative analysis of miRNA-429 expression levels in spermatozoa from 10 NOA males and compared them to data from 10 fertile males with normal sperm quality (Zhang et al., 2022). However, this observation was made in testicular tissue samples rather than blood serum samples.

According to different investigations examining the expression of miRNA-429 in seminal plasma from individuals with azoospermia, there was no notable change in expression between patients and the control group (P-value 0.459) (Abu-Halima et al., 2020). The likely cause of this discrepancy is the limited range of participants in the research and the ethnic diversity, which may lead to contrasting miRNA expression in distinct directions.

According to multiple studies, the expression of miR-34c-5p is cell-specific in mammals, particularly humans. miR-34c is highly expressed in spermatocytes and round spermatids, indicating its significance in the later stages of spermatogenesis. The expression of miR-34c-5p is strongly associated with the total number of germ cells (Fu et al., 2023). In this study, the presence of miR-34c-5p in blood serum was further examined using RT-PCR. The findings indicated a statistically nonsignificant downregulation in miR-34c-
Figure 1. Biopsy result in patients with azoospermia.

Table 1. Primers sequences and annealing temperature

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR429 R</td>
<td>GTTGGCTCTGGTGCAAGGCGAGGTATTC</td>
<td>+55</td>
</tr>
<tr>
<td></td>
<td>GCACCAGAGCCAACACGGTT</td>
<td></td>
</tr>
<tr>
<td>miR429 F</td>
<td>GTTTTTGGGTAATACGTCTGTTAA</td>
<td></td>
</tr>
<tr>
<td>miR-34c-5p R</td>
<td>GTTGGCTCTGGTGCAAGGCGAGGTATTC</td>
<td>+60</td>
</tr>
<tr>
<td></td>
<td>GCACCAGAGCCAACGGAATC</td>
<td></td>
</tr>
<tr>
<td>miR-34c-5p F</td>
<td>GGAGGCAGTGTAGTTAGCT</td>
<td></td>
</tr>
<tr>
<td>miRNA-211 R</td>
<td>TCGGCAGGTCCCTTTTGTATCC</td>
<td></td>
</tr>
<tr>
<td>miRNA-211 F</td>
<td>TGCAGGTCAGCTTGCTGTCGT</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Fold expression of miR-211, miR-429 and miR-34c in patients and controls.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Patients (n=35)</th>
<th>Controls (n=20)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-211 Mean±SD</td>
<td>1.3±1.4</td>
<td>5.7±3.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Median</td>
<td>1.25</td>
<td>4.47</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0.01-4.55</td>
<td>1.04-12.6</td>
<td></td>
</tr>
<tr>
<td>miR-34c Mean±SD</td>
<td>2.6±6.9</td>
<td>3.6±3.8</td>
<td>0.884</td>
</tr>
<tr>
<td>Median</td>
<td>2.58</td>
<td>2.72</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0.01-33.38</td>
<td>0.1-13.28</td>
<td></td>
</tr>
<tr>
<td>miR-429 Mean±SD</td>
<td>5.3±11.3</td>
<td>8.8±17.9</td>
<td>0.356</td>
</tr>
<tr>
<td>Median</td>
<td>2.35</td>
<td>3.78</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0.0-48</td>
<td>0.02-57.61</td>
<td></td>
</tr>
</tbody>
</table>
5p levels in individuals with non-obstructive azoospermia (NOA) compared to normal fertile controls.

In a recent study by Pantos et al., it was found that individuals with NOA who were unable to retrieve sperm had decreased expression of miR-34c-5p in their testicular tissue compared to those who were successful in retrieving sperm (Pantos et al., 2021). Furthermore, the expression pattern of miR-34c-5p was comparable between testicular tissue obtained from individual patients and blood serum samples. Another study by Li et al. identified 173 miRNAs with distinct expression patterns across spermatids and spermatocytes in human azoospermic patients (Li et al., 2020). The disparity in statistical outcomes between our research and others may be attributed to the restricted number of cases. Conducting further studies with larger and more diverse sample sizes will be beneficial in validating our findings.

Research has shown that miR-211 inhibits the spread and invasiveness of various malignancies, including cervical, breast, melanoma, and ovarian cancer. In these cancers, decreased levels of miR-211 expression (downregulation) have been observed in the tissues (Ye et al., 2022). Our study indicates that infertile men exhibit significantly lower levels of miR-211 expression compared to controls. The reduced expression levels in infertile males with non-obstructive azoospermia (NOA) suggest impairment in the process of sperm production, with the downregulation of miRNA-211 playing a crucial role in male infertility. Zhang and colleagues demonstrated that miRNA-211, found in human blood serum, has the potential to serve as a novel biomarker for human spermatogenetic disease (Zhang et al., 2023).

### Conclusion

Developing a non-invasive diagnostic technique for male infertility is imperative. miRNA is increasingly recognized as a crucial tool in diagnosing male infertility. The bioinformatics and correlation analyses conducted in this study have laid a crucial foundation for future investigations into the direct or indirect roles of miRNAs in spermatogenesis. Our findings indicated a notable decrease in serum miR-211 levels among infertile males compared to the control group. This work highlights the potential of serum miR-211 as a non-intrusive and cost-effective biomarker for male infertility.

### Author contributions

F.M.K., A.I.R., U.A.K. developed the Study design, and wrote, reviewed, and edited the paper.

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### Competing financial interests

The authors have no conflict of interest.

### References


