

## Original Research

**Differential Expression of MiR-21 and MiR-19 in Biliary Atresia: Diagnostic Potential and Therapeutic Implications**

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**Abstract**

Biliary atresia (BA), a severe pediatric liver disease of unknown etiology, causes neonatal jaundice, progressive cholestasis, and life-threatening liver fibrosis. Emerging evidence suggests microRNAs (miRNAs) – small non-coding RNAs that regulate gene expression by binding target mRNAs may play a role in BA pathogenesis. Circulating miRNAs have shown diagnostic potential for various diseases, prompting our investigation of miR-21 and miR-19 as potential biomarkers in BA. We conducted a case-control study comparing 18 BA patients from the Shiraz Pediatric Liver Cirrhosis Cohort Study (SPLCCS) with 18 age-matched healthy controls. Serum samples were subjected to RNA extraction, followed by cDNA synthesis with stem-loop primers. Quantitative real-time PCR was performed to measure miR-21 and miR-19 expression levels, with U6 snRNA as an endogenous control. Quantitative PCR analysis revealed distinct miRNA expression patterns in BA patients compared to healthy controls. miR-21 expression was significantly elevated in the BA group, demonstrating a 5-fold increase relative to controls ( $p = 0.0002$ ). Conversely, miR-19 showed a 2.1-fold downregulation (0.47-



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fold change) in BA patients, though this decrease did not reach statistical significance ( $p = 0.2118$ ). Quantitative PCR analysis revealed distinct miRNA expression patterns in BA patients compared to healthy controls. miR-21 expression was significantly elevated in the BA group, demonstrating a 5-fold increase relative to controls ( $p = 0.0002$ ). Conversely, miR-19 showed a 2.1-fold downregulation (0.47-fold change) in BA patients, though this decrease did not reach statistical significance ( $p = 0.2118$ ).

## Keywords

MicroRNAs; biliary atresia; liver fibrosis

## 1. Background

Biliary atresia (BA) is one of the most common pediatric liver diseases with unknown etiology [1]. While the exact etiology of BA remains elusive in many cases, well-documented triggers of the inflammatory process include perinatal cytomegalovirus (CMV) infection and aflatoxin-induced cholangiopathy in neonates with a GST M1 detoxification defect, also known as Kotb disease [2].

In BA, the bile ducts outside and inside the liver are scarred and blocked, and bile cannot flow into the intestine; consequently, the liver is damaged, causing hyperbilirubinemia, alcoholic stools, neonatal jaundice, life-threatening cholestasis, progressive and rapid liver fibrosis, and death of children under 2 years of age [3]. The only effective treatments are operations such as the Kasai procedure and liver transplantation [4].

Various hypotheses have been proposed regarding the role of epigenetic factors such as microRNAs (miRNAs) in BA pathogenesis [5]. MicroRNAs are small, endogenous non-coding RNAs consisting of approximately 19 to 23 nucleotides [6]. They bind to target mRNA and regulate gene expression at the post-transcriptional and translational levels [7]. These molecules play a crucial role in various biological processes, including cellular differentiation and proliferation [8]. Recently, microRNA was described as a potential marker of hepatic diseases, such as BA, and is currently being evaluated as a non-invasive diagnostic marker in children [9]. Some research demonstrated differential regulation of microRNA during the development of biliary atresia in an animal model [10].

Both miR-21 and miR-19 are involved in the signaling pathways involved in BA pathogenesis [11]. One of the leading hypotheses is that miR-21 expression is increased in the livers of patients with BA [12]. This elevated miR-21 expression inhibits the phosphatase and tensin homolog deleted on chromosome ten (PTEN) through its 3'-untranslated region (UTR), thereby enhancing the expression of alpha-smooth muscle actin ( $\alpha$ -SMA); both are directly associated with experimental liver fibrogenesis and human fibrosis in chronic liver disease [12].

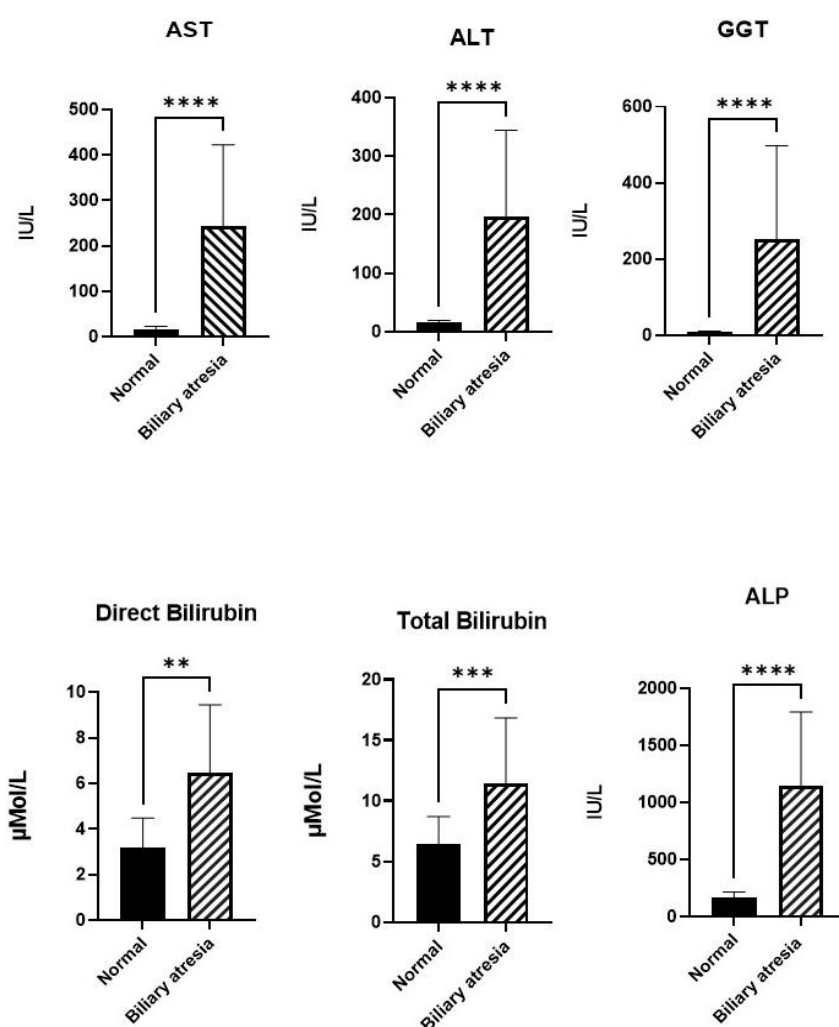
Conversely, miR-19 is identified as a negative regulator of profibrotic TGF- $\beta$  signaling, playing a crucial role in the inflammatory and fibrotic pathways affecting the extrahepatic bile ducts [13].

Additionally, studies indicate that the expressions of miR-21 and miR-19 may vary among different ethnic groups and populations worldwide [14-16]. Consequently, we sought to examine the expression levels of miR-21 and miR-19 in the livers of children with Biliary Atresia (BA).

## 2. Materials and Methods

### 2.1 Patients

The study analyzed blood samples from a rigorously selected cohort of 18 children with biliary atresia (BA) enrolled in the Shiraz Pediatric Liver Cirrhosis Cohort Study (SPLCCS). The diagnosis of BA was definitively confirmed in all included patients by intraoperative cholangiography, with inclusion contingent upon the availability of a detailed operative report explicitly documenting the cholangiographic findings. Standard pre-operative evaluations (clinical presentation, laboratory tests, imaging, and liver biopsy) were performed as part of the diagnostic workup, with detailed ultrasonography findings and PELD scores provided in Figure 1 and Table 1 [17] to characterize the cohort's clinical presentation.



**Figure 1** Serum level of AST, ALT, GGT, total and direct bilirubin, and ALP in BA patients in comparison with the normal group (p value < 0.05). AST (Aspartate Aminotransferase), ALT (Alanine Aminotransferase), GGT (Gamma-Glutamyl Transferase), ALP (Alkaline Phosphatase), BA (Biliary Atresia).

**Table 1** Abdominal ultrasonography findings in the biliary atresia group.

| Abdominal Ultrasonography Findings |     |      |
|------------------------------------|-----|------|
| Liver size                         | No. | %    |
| Abnormal size                      | 9   | 69.2 |
| Normal size                        | 4   | 30.8 |
| Irregular Liver border             | 4   | 30.8 |
| Coarse Liver parenchyma            | 11  | 84.6 |
| Ascites                            | 4   | 30.8 |
| Splenomegaly                       | 12  | 92.3 |
| PELD Score in BA patients          |     |      |
| Mean                               |     | 14.7 |
| Std. Deviation                     |     | 9.3  |

For the disease control group, 18 children with progressive familial intrahepatic cholestasis (PFIC) were selected from a previously published cohort [18]. A definitive clinical genetics report from a certified laboratory genetically confirmed the diagnosis of PFIC in all these selected cases. This selection was performed to create a control group matched in size to the BA patient group.

Written informed consent was obtained from all participants' parents or guardians, and the study protocol was approved by the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1398.142). Blood samples were stored at -80°C, and clinical data were extracted from the pediatric liver cirrhosis registry (IR.SUMS.REC.1399.530).

## 2.2 RNA Isolation and cDNA Synthesis

MicroRNA extraction and cDNA synthesis were performed on serum samples from all study participants, including biliary atresia patients, PFIC patients, and healthy controls. Total RNA was isolated using RNX-Plus reagent (Cinnagen, Iran), followed by cDNA synthesis with the RB-Micro RNA Synthesis kit (RNA Biotechnology Company, Iran). The 25-μL reverse transcription reaction contained 500 ng of total RNA, stem-loop primers, RT primers, dNTPs, Reverse Transcriptase Buffer, and M-MLV reverse transcriptase enzyme. The reaction proceeded in a thermal cycler (ABI) under optimized conditions: 25°C for 10 minutes, 50°C for 60 minutes, 80°C for 5 minutes, and a final 12°C hold.

## 2.3 Quantitative Real-Time PCR (qRT-PCR)

For quantitative analysis, real-time PCR was conducted using Step-One ABI Applied Biosystems (Life Technologies) with U6 snRNA as the endogenous control. The thermal profile included an initial 95°C step for 15 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 60 seconds. Each sample was run in triplicate, and the average Ct value was determined. Relative expression levels of miR-21 and miR-19 were calculated using the  $2^{-\Delta\Delta Ct}$  (Livak) method, with primer details provided in Table 2.

**Table 2** primer sequence.

| Primer name | Sequence                               |
|-------------|----------------------------------------|
| *miR-21     | Forward: 5'-CACGCATAGCTTATCAGACTG-3'   |
| *miR-19     | Forward: 5'-CAGACAGCAGGTTTGCATCCAGC-3' |
| RNU6 (U6)   | Forward: 5'-GCTTCGGCAGCACATATACTA-3'   |
|             | Reverse: 5'-CGAATTTGCGTGTCTCCTTG-3'    |

\*We use the universal primer 5'-CCAGTGCAGGGTCCGAGGTA-3' as the reverse primer for mir-21 and mir-19 [19].

## 2.4 Statistics

The expression levels of miR-21 and miR-19 were analyzed and presented as mean  $\pm$  SD. Statistical comparisons between groups were performed using either Student's t-test or Mann-Whitney U test, depending on data distribution. All analyses were conducted using GraphPad Prism software (version 9.0), with statistical significance set at  $p < 0.05$ .

To evaluate the diagnostic potential of these microRNAs in biliary atresia (BA) patients, receiver operating characteristic (ROC) curve analysis was performed. This analysis assessed both the specificity and sensitivity of miR-21 and miR-19 expression levels as potential biomarkers for BA diagnosis.

## 2.5 Ethics Approval and Consent to Participate

Written informed consent was obtained from the parents or guardians of the participant for providing a blood sample. This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1398.142).

## 3. Results

### 3.1 Demographic and Clinical Characteristics

The biliary atresia (BA) cohort consisted of 18 pediatric patients with a mean age of  $2.6 \pm 1.1$  years, showing an equal gender distribution (9 males, 9 females). This carefully matched control group of 18 healthy children had a comparable mean age of  $2.3 \pm 1.3$  years and an identical sex distribution, ensuring minimal demographic confounding. Comprehensive biochemical profiling revealed pronounced hepatic dysfunction in BA patients, with serum levels of alkaline phosphatase (ALP) increasing to  $1126.1667 \pm 653.62$  U/L, alanine aminotransferase (ALT) to  $157.48 \pm 143.73$  U/L, and aspartate aminotransferase (AST) to  $209.54 \pm 187.63$  U/L - all markedly elevated compared to control values ( $p < 0.0001$ ). The cholestatic profile was particularly striking, with total bilirubin reaching  $9.3 \pm 7.15$  mg/dL and direct bilirubin  $6.7 \pm 8.75$  mg/dL, along with  $\gamma$ -GGT levels of  $277.6 \pm 355.7$  U/L, collectively demonstrating the severe hepatobiliary impairment characteristic of BA (Figure 1).

### **3.2 MicroRNA Expression Profiling**

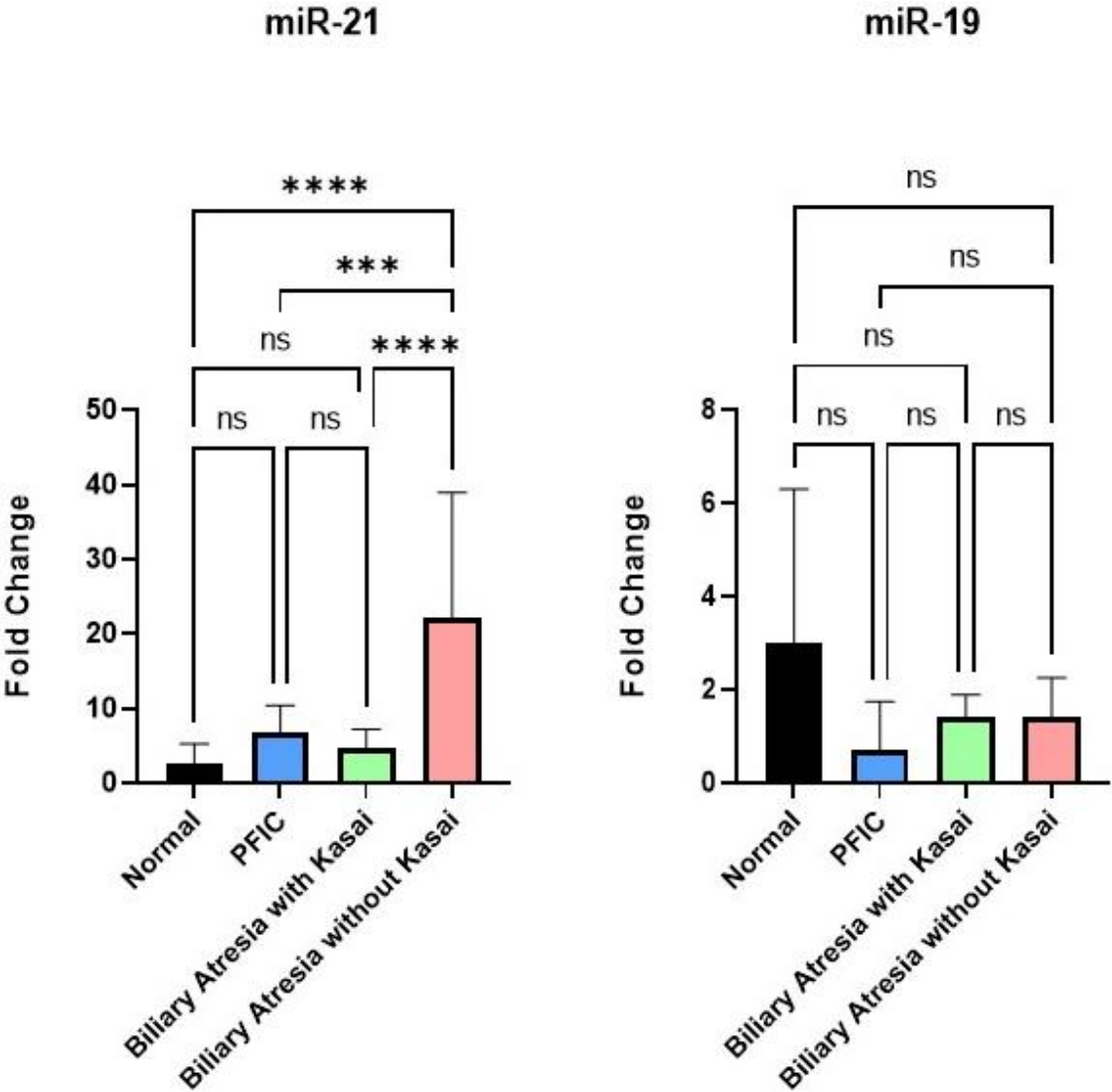
Quantitative PCR analysis uncovered significant dysregulation of candidate miRNAs in the BA cohort:

#### **3.2.1 miR-21 Dynamics**

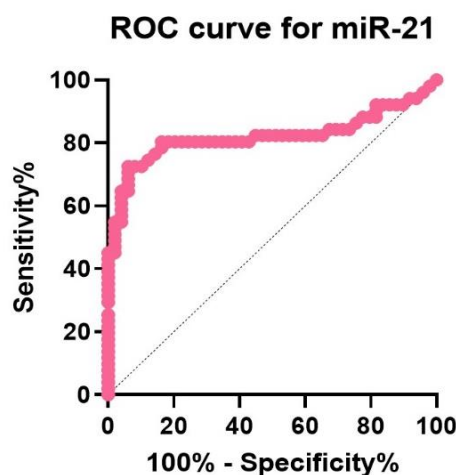
The most notable finding was a dramatic 5-fold upregulation of miR-21 expression in BA patients compared to controls ( $p = 0.0002$ ) (Table 3), suggesting its potential role in BA pathogenesis (Figure 2). This elevation showed particular clinical relevance when analyzing post-Kasai portoenterostomy outcomes: patients who underwent the procedure demonstrated miR-21 levels that approached normal ranges, representing a significant decrease from pre-operative levels ( $p < 0.0001$ ). The diagnostic potential of miR-21 was further underscored by ROC curve analysis, which identified an optimal cutoff of 3.069-fold change with 72.55% sensitivity and 93.88% specificity (Figure 3). Notably, miR-21 expression patterns effectively discriminated BA from progressive familial intrahepatic cholestasis (PFIC) cases ( $p = 0.0003$ ), though this distinction disappeared in post-Kasai patients ( $p = 0.9314$  vs. PFIC).

**Table 3** The miR-21 expression in the BA patients and the control groups.

| Comparison (Case vs. Reference)        | Mean $\Delta Ct$<br>(Case Group) | Mean $\Delta Ct$<br>(Reference Group) | Fold Change<br>( $2^{-\Delta\Delta Ct}$ ) | 95% CI<br>( $2^{-\Delta\Delta Ct}$ ) | p-value | Sig. |
|----------------------------------------|----------------------------------|---------------------------------------|-------------------------------------------|--------------------------------------|---------|------|
| PFIC vs. Normal                        | 10.24 $\pm$ 1.8                  | 6.12 $\pm$ 0.9                        | -4.116                                    | -12.63 to 4.400                      | 0.57    | ns   |
| BA (with Kasai) vs. Normal             | 8.20 $\pm$ 1.2                   | 6.12 $\pm$ 0.9                        | -2.082                                    | -9.153 to 4.989                      | 0.86    | ns   |
| BA (without Kasai) vs. Normal          | 25.80 $\pm$ 3.1                  | 6.12 $\pm$ 0.9                        | -19.68                                    | -27.21 to -12.15                     | 0.0002  | **** |
| BA (with Kasai) vs. PFIC               | 8.20 $\pm$ 1.2                   | 10.24 $\pm$ 1.8                       | 2.034                                     | -6.933 to 11.00                      | 0.93    | ns   |
| BA (without Kasai) vs. PFIC            | 25.80 $\pm$ 3.1                  | 10.24 $\pm$ 1.8                       | -15.57                                    | -24.90 to -6.234                     | 0.0003  | ***  |
| BA (without Kasai) vs. BA (with Kasai) | 25.80 $\pm$ 3.1                  | 8.20 $\pm$ 1.2                        | -17.60                                    | -25.64 to -9.564                     | <0.0001 | **** |



**Figure 2** Expression of miR-19 and miR-21 in biliary atresia patients compared with normal and PFIC groups. P-values indicate statistical significance:  $p < 0.05$  (\*),  $p < 0.01$  (\*\*),  $p < 0.001$  (\*\*\*),  $p < 0.0001$  (\*\*\*\*). PIFC (Progressive Familial Intrahepatic Cholestasis).



| Cutoff  | Sensitivity% | 95% CI           | Specificity% | 95% CI           | Likelihood ratio | Sensitivity + Specificity |
|---------|--------------|------------------|--------------|------------------|------------------|---------------------------|
| > 3.069 | 72.55        | 59.05% to 82.89% | 93.88        | 83.48% to 97.90% | 11.85            | 1.6643                    |

**Figure 3** ROC curve for miR-21 showed a fold change of 3.069 at a cutoff, with a sensitivity of 72.55% and a specificity of 93.88%. ROC (Receiver Operating Characteristic Curve).

### 3.2.2 miR-19 Expression Patterns

In contrast to miR-21, miR-19 showed a modest but consistent downward trend across study groups. BA patients exhibited 0.47-fold lower expression compared to controls ( $p = 0.36$ ) (Table 4), though this reduction failed to reach statistical significance (Figure 2). Unlike miR-21, miR-19 levels showed no response to surgical intervention (pre- vs. post-Kasai  $p > 0.99$ ) and demonstrated poor discriminatory capacity between BA and PFIC cases ( $p = 0.92$ ). The lack of significant variation in miR-19 expression across all comparison groups (including PFIC vs. controls,  $p = 0.22$ ) suggests it may have limited utility as a BA biomarker.

**Table 4** The miR-19 expression in the BA patients and the control groups.

| Comparison (Case vs. Reference)        | Mean $\Delta Ct$<br>(Case Group) | Mean $\Delta Ct$<br>(Reference Group) | Fold Change<br>( $2^{-\Delta\Delta Ct}$ ) | 95% CI<br>( $2^{-\Delta\Delta Ct}$ ) | p-value | Sig. |
|----------------------------------------|----------------------------------|---------------------------------------|-------------------------------------------|--------------------------------------|---------|------|
| PFIC vs. Normal                        | 5.12 $\pm$ 0.63                  | 4.90 $\pm$ 0.52                       | 2.285                                     | -0.9171 to 5.487                     | 0.22    | ns   |
| BA (with Kasai) vs. Normal             | 5.21 $\pm$ 0.72                  | 4.90 $\pm$ 0.52                       | 1.571                                     | -1.044 to 4.185                      | 0.36    | ns   |
| BA (without Kasai) vs. Normal          | 5.17 $\pm$ 0.68                  | 4.90 $\pm$ 0.52                       | 1.579                                     | -1.035 to 4.193                      | 0.36    | ns   |
| BA (with Kasai) vs. PFIC               | 5.21 $\pm$ 0.72                  | 5.12 $\pm$ 0.63                       | -0.7140                                   | -3.916 to 2.488                      | 0.92    | ns   |
| BA (without Kasai) vs. PFIC            | 5.13 $\pm$ 0.65                  | 5.12 $\pm$ 0.63                       | -0.7057                                   | -3.908 to 2.496                      | 0.92    | ns   |
| BA (without Kasai) vs. BA (with Kasai) | 5.22 $\pm$ 0.74                  | 5.21 $\pm$ 0.72                       | 0.008388                                  | -2.606 to 2.623                      | >0.99   | ns   |

### 3.2.3 Comparative Analysis and Clinical Correlations

The differential behavior of these two miRNAs following the Kasai procedure was particularly revealing. While miR-21 expression normalized post-operatively, mirroring improvements in clinical parameters, miR-19 remained stable regardless of surgical intervention or disease state. This dichotomy suggests distinct roles in BA pathophysiology - with miR-21 potentially linked to reversible inflammatory processes and miR-19 possibly associated with more fixed aspects of disease progression.

## 4. Discussion

In this study, we aimed to investigate miR-21 and miR-19 expression levels in BA children in an Iranian population and to determine whether circulating miRNAs are also of diagnostic value in pediatric liver disease. miR-21 and miR-19 were chosen because they have previously been described to be linked to liver fibrosis [15, 20].

MicroRNAs are small endogenous non-coding RNAs that are recognized as regulators of biological processes in liver diseases [21]. They regulate the levels of specific mRNAs and their posttranscriptional process, so they have an essential role in the pathogenesis of nonalcoholic fatty liver disease, viral hepatitis, steatohepatitis, fibrogenesis, and hepatocellular carcinoma [22].

Several studies on chronic liver disease with fibrosis have shown significant correlations between specific miR expression patterns and disease progression/prognostic outcomes as well as responses to drug treatments [23, 24]. In children, while liver biopsy is used to diagnose liver disease and hepatic fibrosis, it is an invasive procedure, and due to the small sample size, it is very prone to error (24). Therefore, a non-invasive diagnostic alternative for BA is ideal. Recently, the study of microRNAs has increased as biomarkers that can aid in the prognosis, diagnosis, or treatment of diseases, including liver disease.

The present study investigated the expression levels of miR-21 and miR-19 in children with biliary atresia (BA) within an Iranian population, aiming to evaluate their potential as non-invasive diagnostic biomarkers for pediatric liver disease. Our findings contribute to the growing body of evidence suggesting that circulating miRNAs, particularly miR-21, may play a significant role in BA pathogenesis and diagnosis.

Our results demonstrated a 5-fold increase in miR-21 expression in BA patients compared to healthy controls, aligning with previous studies. Makhmudi et al. reported an 18-fold rise in miR-21 in Indonesian BA patients, further supporting its association with liver fibrosis [15]. Additionally, Loyer et al. found that miR-21 inhibition reduces liver injury and fibrosis, suggesting its role in disease progression [25]. Shen et al. also identified miR-21 as a promoter of fibrotic pathways in BA, reinforcing its potential as a therapeutic target [26].

To strengthen our analysis, we included a second control group consisting of children with progressive familial intrahepatic cholestasis (PFIC). While miR-21 levels were elevated in PFIC patients, the increase was significantly lower than in BA cases, implying that miR-21 upregulation may be more specifically linked to BA than to general liver fibrosis.

Notably, we observed a significant decrease in miR-21 expression following Kasai surgery, suggesting that miR-21 levels could serve as an indicator of postoperative recovery. This finding supports the hypothesis that miR-21 is dynamically involved in BA progression and may help monitor treatment response.

In contrast to miR-21, our study did not detect statistically significant changes in miR-19 expression between BA patients and healthy controls. Although miR-19 levels were slightly lower in BA cases, the difference was not important, nor was there a notable change post-Kasai surgery.

Previous studies on miR-19 have yielded mixed results. Dong Zhao et al. suggested that miR-19b contributes to fibrogenesis in BA [16]. At the same time, Huang et al. proposed an anti-fibrotic role for miR-19 via TGF- $\beta$  suppression [27]. The lack of significant findings in our study may be due to a small sample size or population-specific factors. Further research with larger cohorts is necessary to clarify the role of miR-19 in BA.

The strong correlation between miR-21 and BA highlights its potential as a non-invasive diagnostic biomarker, reducing reliance on liver biopsies. Given its decline after Kasai surgery, miR-21 could also aid in postoperative monitoring. However, miR-19 requires further investigation to determine its relevance in BA.

Our findings of sustained miR-21 overexpression in pre-Kasai livers, despite the relatively short half-lives of individual miRNAs, provide a crucial molecular perspective on the pathogenesis of BA. This persistent elevation reflects continuous production, driven by ongoing inflammatory and fibrotic signaling within the liver microenvironment, underscoring that BA is a dynamic and progressive inflammatory cholangiopathy rather than a static malformation. The significant downregulation of these miRNAs post-Kasai further confirms that alleviating the cholestatic insult can interrupt this vicious cycle. While the primary drivers in BA are likely distinct, the broader principle that diverse insults, including viral, toxic, or inflammatory insults, can converge on common pathways, such as miR-21 dysregulation to sustain fibrosis, is recognized [28]. Our data firmly place BA within this paradigm of a self-perpetuating inflammatory process, culminating in accelerated fibrosis.

#### **4.1 Future Perspectives and Limitations**

Identifying the miRNAs with the greatest impact on liver fibrogenesis remains both a challenge and an exciting frontier in hepatology research. Pinpointing these key miRNAs—along with their molecular targets—could unlock novel diagnostic biomarkers and therapeutic strategies for biliary atresia (BA) in the coming years [27-29].

The identification of miRNAs with the most significant impact on liver fibrogenesis remains both a challenge and an exciting frontier in hepatology research. Pinpointing these key miRNAs—along with their molecular targets—could unlock novel diagnostic biomarkers and therapeutic strategies for biliary atresia (BA) in the coming years [29-31]. Our study, in line with previous research, underscores the strong diagnostic potential of circulating miR-21 in pediatric BA. The consistent upregulation of miR-21 in BA patients, along with its decline post-Kasai surgery, suggests its utility not only for non-invasive diagnosis but also for monitoring disease progression and treatment response.

In contrast, the role of miR-19 in BA remains inconclusive. While some studies propose its involvement in fibrogenic pathways [16, 26], our findings did not reveal a statistically significant association. This discrepancy may stem from sample size limitations or population-specific factors, necessitating further investigation in larger, multi-center cohorts to clarify miR-19's diagnostic relevance.

Moving forward, research should focus on:

- Validating miR-21 as a robust clinical biomarker for BA,
- Exploring miR-21-targeted therapies, given its pro-fibrotic role,
- Expanding miRNA profiling to uncover additional biomarkers with diagnostic and prognostic value.

The growing evidence on circulating miRNAs, particularly miR-21, paves the way for less invasive, more precise diagnostic tools in BA, potentially transforming clinical management for affected children.

## 5. Conclusion

Our findings support the diagnostic value of miR-21 in BA, reinforcing its role in disease progression and postoperative recovery. In contrast, miR-19 did not show a significant association with BA in this cohort, warranting further research. Circulating miRNAs, particularly miR-21, represent a promising non-invasive tool for BA diagnosis and management, potentially improving clinical outcomes for pediatric patients.

## Abbreviation List

|             |                                                          |
|-------------|----------------------------------------------------------|
| BA          | Biliary atresia                                          |
| miRNAs      | microRNAs                                                |
| SPLCCS      | Shiraz Pediatric Liver Cirrhosis Cohort Study            |
| CMV         | cytomegalovirus                                          |
| PTEN        | phosphatase and tensin homolog deleted on chromosome ten |
| UTR         | untranslated region                                      |
| PELD scores | Pediatric End-Stage Liver Disease scores                 |
| PFIC        | progressive familial intrahepatic cholestasis            |
| ALP         | alkaline phosphatase                                     |
| ALT         | alanine aminotransferase                                 |
| AST         | aspartate aminotransferase                               |
| GGT         | Gamma-Glutamyl Transferase                               |
| ROC         | Receiver Operating Characteristic Curve                  |

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## Author Contributions

All authors made significant contributions to the study conceptualization and design. The preparation of materials, as well as data collection and analysis, was carried out by M.D., N.A., N.M., M.H-A., S-M.D., B.G., and E.E. The first draft of the manuscript was authored by M.D. All authors reviewed and approved the final version of the manuscript.

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## Competing Interests

The authors declare that they have no competing interests.

## Data Availability Statement

The data sets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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