ORIGINAL RESEARCH ARTICLE

Circulating microRNA-21-5p expression and serum cytokine profiles in women with recurrent pregnancy loss: A case-control study of inflammatory biomarkers

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Abstract

Recurrent pregnancy loss is associated with immune dysregulation, yet its molecular basis remains poorly understood. This case-control study investigated circulating microRNA-21-5p (miRNA-21-5p) expression and its relationship with serum transforming growth factor-beta 1 (TGF-β1) and interleukin-10 (IL-10) in 100 women during the first trimester (50 with recurrent pregnancy loss and 50 healthy controls). Serum cytokines were measured via ELISA, and miRNA-21-5p expression via RT-qPCR. Results demonstrated significantly lower miRNA-21-5p expression, TGF-β1, and IL-10 levels in the patient group ($p < 0.001$). No significant linear correlation was found between the microRNA and cytokines within groups, reflecting complex regulatory networks. In conclusion, recurrent pregnancy loss is strongly associated with reduced miRNA-21-5p and an attenuated anti-inflammatory cytokine profile. These findings suggest concurrent epigenetic and immune dysregulation as critical components in the breakdown of maternal-fetal tolerance. *(Afr J Reprod Health 2026; 30 [22]: 86-96).*

Keywords: recurrent pregnancy loss; microRNA-21-5p; transforming growth factor-beta 1; interleukin-10; cytokines; epigenetics

Résumé

La perte de grossesse récurrente est associée à un dysfonctionnement immunitaire, mais sa base moléculaire reste mal comprise. Cette étude cas-témoin a examiné l'expression du microARN-21-5p (miARN-21-5p) circulant et sa relation avec le facteur de croissance transformant bêta 1 (TGF-β1) et l'interleukine-10 (IL-10) sériques chez 100 femmes au premier trimestre (50 avec perte de grossesse récurrente et 50 témoins sains). Les cytokines sériques ont été mesurées par ELISA et l'expression du miARN-21-5p par RT-qPCR. Les résultats ont montré une expression du miARN-21-5p, du TGF-β1 et de l'IL-10 significativement plus faible dans le groupe pathologique ($p < 0,001$). Aucune corrélation linéaire significative n'a été trouvée au sein des groupes, reflétant des réseaux de régulation complexes. En conclusion, la perte de grossesse récurrente est fortement associée à une réduction du miARN-21-5p et à un profil de cytokines anti-inflammatoires atténué, suggérant un dysfonctionnement épigénétique et immunitaire concomitant dans la rupture de la tolérance fœto-maternelle. *(Afr J Reprod Health 2026; 30 [22]: 86-96).*

Mots-clés : fausses couches à répétition ; microARN-21-5p ; facteur de croissance transformant bêta 1 ; interleukine-10 ; cytokines ; épigénétique.

Introduction

Recurrent pregnancy loss (RPL), frequently defined as the consecutive loss of two or more pregnancies prior to the 20th week of gestation, represents a significant reproductive challenge with a highly complex etiology. [1] While structural, chromosomal, and systemic endocrine abnormalities account for a portion of clinical cases, a substantial percentage of RPL occurrences remain classified as idiopathic. Emerging immunological paradigms increasingly suggest that disruptions in maternal-fetal tolerance-a meticulously orchestrated process of local and systemic immune adaptation-may represent a

pivotal underlying mechanism in the pathogenesis of these unexplained pregnancy failures.[2,3]

The establishment and successful maintenance of early pregnancy are hypothesized to rely heavily on a dynamic equilibrium within the cytokine network, specifically favoring a tolerogenic and immunoregulatory microenvironment. Key anti-inflammatory mediators, notably Transforming Growth Factor-beta 1 (TGF-β1) and Interleukin-10 (IL-10), are considered essential components in facilitating this adaptation.[4,5]

TGF-β1 is proposed to play a vital role in regulating trophoblast invasion and modulating maternal immune cell activity, whereas IL-10 primarily functions to mitigate excessive inflammatory responses at the maternal-fetal interface.[6] It is postulated that an attenuation in the systemic circulation of these crucial cytokines could contribute to the immunologically hostile environment observed in RPL; however, the precise upstream molecular regulators dictating their expression remain incompletely elucidated.[7]

In recent years, epigenetic modulators, particularly microRNAs (miRNAs), have emerged as critical post-transcriptional regulators of cellular and immunological homeostasis, where miRNAs are short, non-coding RNA molecules that can influence diverse biological pathways, including immune cell polarization and functional signaling.[8] Among the identified profiles, miRNA-21-5p has been recognized for its involvement in cellular proliferation and potential immunomodulatory functions.[9] Although preliminary investigations have explored varied miRNA expressions in different obstetric complications, the specific systemic profile of circulating miRNA-21-5p in the context of RPL requires deeper investigation. Furthermore, current literature indicates a highly intricate, often non-linear, regulatory network between circulating miRNAs and systemic cytokine release, suggesting a need for nuanced exploratory models.[10]

Therefore, the present study aims to comparatively evaluate the relative expression of circulating miRNA-21-5p alongside the systemic concentrations of critical immunoregulatory cytokines (TGF-β1 and IL-10) in women experiencing RPL compared to healthy pregnant controls. By characterizing these specific immunological and molecular parameters, this research seeks to provide targeted insights into the potential systemic dysregulations associated with early pregnancy loss, contributing to a broader understanding of its complex pathophysiology. To our knowledge, this study provides novel insights by simultaneously evaluating epigenetic markers and specific systemic cytokines, a dual approach rarely explored in previous idiopathic RPL research.

Methods

Study design and population

This case-control study was conducted at Bint Al-Huda Maternity Teaching Hospital, affiliated with the Thi-Qar Health Directorate, Iraq, over a period from 10/2025 to 3/2026. The study was designed to evaluate the circulating expression level of miRNA-21-5p and its link with serum levels of IL-6, TGF-β1 and IL-10 in women with RPL. The sample size was determined a priori using statistical power analysis; to achieve a statistical power of 90% at a two-sided alpha level of 0.05 for detecting meaningful biological differences, a minimum of 50 participants per group was required. Consequently, a total of 100 women were enrolled and separated into two groups: 50 women with RPL and a control group of 50 healthy women with at least one successful pregnancy and no history of miscarriage (Figure 1).

Inclusion and exclusion criteria

The study population comprised two distinct groups: women with a confirmed history of Recurrent Pregnancy Loss (RPL), defined as three or more consecutive miscarriages, and a control group of healthy women with at least one successful pregnancy and no history of miscarriage. Cases were actively recruited from patients presenting to the outpatient gynecology department. The control group consisted of healthy pregnant women attending routine antenatal care clinics at the same institution. To minimize confounding variables, controls were frequency-matched to the cases based on maternal age (± 2 years) and geographical location. Participants were excluded from the study if they presented with a history of malignancies, systemic autoimmune diseases, active severe

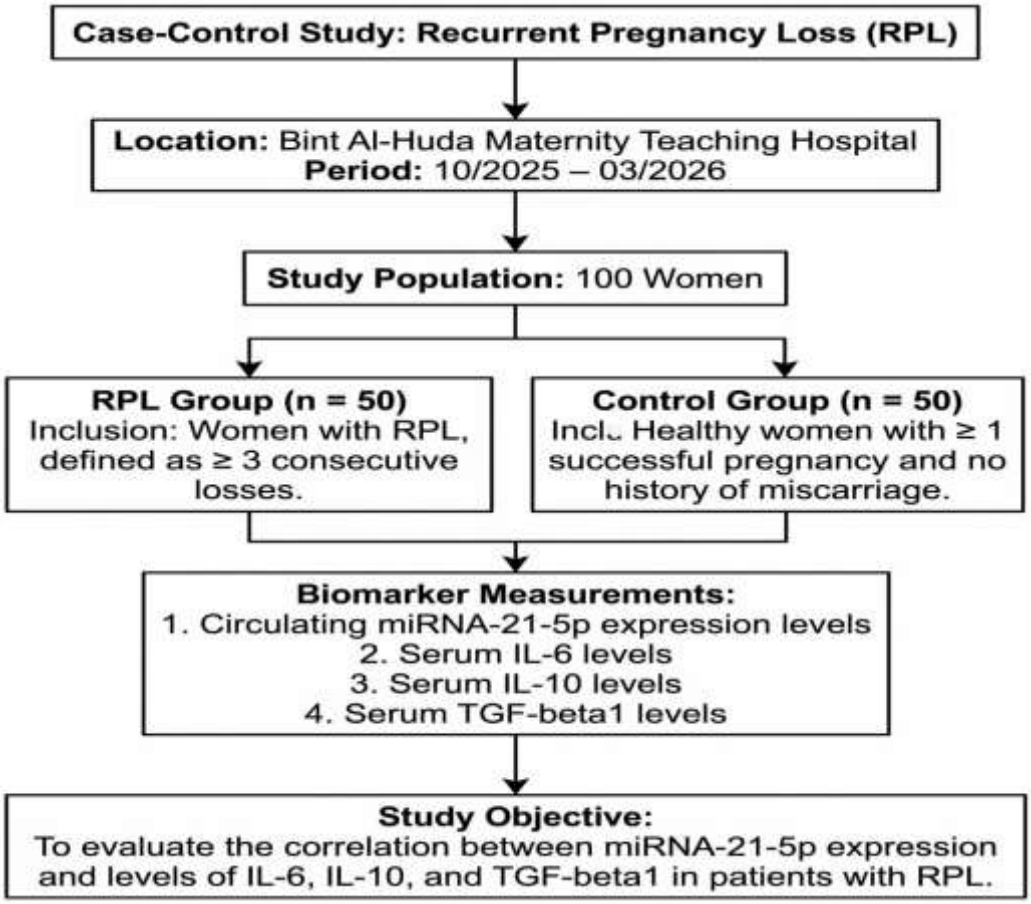

Figure 1: Study design

infections, or chronic viral infections including HBV, HCV, and HIV. Furthermore, individuals currently receiving immunosuppressive therapies or systemic corticosteroids were systematically excluded.

Sample collection

About 5 mL of venous blood was collected from each participant under sterile conditions. Blood samples were transported into plain tubes, allowed to clot at room temperature, and centrifuged at 3000 rpm for 10 minutes to obtain serum. Serum samples were stored at −20°C until analysis.

Measurement of serum cytokines (IL-6, IL-10 and TGF-beta1)

Serum concentrations of IL-6, TGF- β1, and IL-10 were quantified using commercially available enzyme-linked immunosorbent assay (ELISA) kits. Specifically, the Human IL-6 ELISA Kit (Cat. No. ab46027) and the Human TGF-beta1 ELISA Kit (Cat. No. ab100647) supplied by Abcam were utilized. For the detection of IL-10, the Human IL-10 ELISA Kit (Cat. No. E-EL-H0103, Elabscience, USA) was employed. All analyses were performed in strict accordance with the respective manufacturers' guidelines .[11] The optical density (absorbance) of each well was measured using a microplate spectrophotometer at a primary wavelength of 450 nm. Final cytokine concentrations were subsequently calculated by interpolating the measured absorbance values against best-fit standard calibration curves generated for each individual biomarker.

Molecular study

Primer sequences

The qPCR Primers for hsa-miR-21-5p were designed in this study by using Kozomara et al.,

Table 1: Primer sequences used for reverse transcription and quantitative real-time PCR.

Primer	Sequence (5′–3′)
miR-21-5p Forward	TGTTGAGTCGTATCCAGTGCAA
miR-21-5p Reverse	GTATCCAGTGCGTGTGTCGTGG
RT primer (specific) hsa-miR-21-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACCAC
U6 Forward	GTTTTGTAGTTTTTGGAGTTAGTGTTGTGT
U6 Reverse	CTCAACCTACAATCAAAAACAACACAAA

(2019) to select miRNA sequence and using miRNA Primer Design Tool.[12] Whereas qPCR for housekeeping gene U6 were designed in this study by using NCBI-Database. These primers and probe were provided by (Macrogen company, Korea) as shown in the following Table 1

RNA extraction

Total RNA including miRNA was extracted from serum samples using a commercial miRNA extraction kit following the iNtRON, Korea. RNA concentration and purity were assessed using a NanoDrop spectrophotometer.

Reverse transcription

Complementary DNA (cDNA) was synthesized from extracted miRNA using a stem-loop RT primer specific for miRNA-21-5p and reverse transcriptase enzyme.

Quantitative real-time PCR (RT-qPCR)

The expression level of miRNA-21-5p was quantified using SYBR Green based quantitative real-time PCR. U6 small nuclear RNA was used as an endogenous reference gene. Relative expression levels were calculated using the 2 with $-\Delta\Delta Ct$ method.

Statistical analysis

Data management and statistical analyses were performed using appropriate statistical software (GraphPad Prism version 9.0 and SPSS version 26.0). The normality of the data distribution was assessed using the Shapiro-Wilk test. Continuous variables were expressed as mean ± standard deviation (SD), while categorical variables were presented as frequencies and percentages. Comparisons of continuous variables between the two study groups (Control and RPL) were conducted using the independent sample t-test. To determine the magnitude of the differences between the groups, Cohen's d effect sizes were calculated. Pearson correlation analysis was utilized to evaluate the relationships between circulating miRNA-21-5p expression and serum cytokine levels (TGF-β1 and IL-10). Receiver Operating Characteristic (ROC) curve analysis was employed to evaluate the diagnostic accuracy of the measured biomarkers (miRNA-21-5p, TGF-β1, and IL-10). The Area Under the Curve (AUC) with 95% confidence intervals (CI), optimal threshold values, sensitivity, and specificity were calculated to assess the discriminative power of the biomarkers. A two-tailed p-value of less than 0.05 was considered statistically significant for all analyses.

Ethical issues

The study protocol was conducted in strict adherence to the ethical guidelines of the 1975 Declaration of Helsinki. Ethical approval was formally granted by the Institutional Ethics Committee of the College of Applied Medical Science at Dhi-Qar University (Approval Number: 329/1-10-2025). Written informed consent was systematically obtained from all patients and healthy volunteers prior to their enrollment and any sample collection.

Results

The demographic characteristics of the study population (n=100) are detailed in Table 2 Analysis of continuous demographic parameters revealed a statistically significant difference in chronological age, with the RPL group presenting a higher mean age compared to healthy controls (30.6 ± 3.6 years vs. 26.8 ± 3.7 years; $p < 0.001$). Given this variance, age was considered as a potential confounding factor in the interpretation of the systemic immunological profiles. Conversely, the body mass

Table 2: Demographic characteristics of the study population.

Variable	RPL(n=50)	Control (n=50)	P-value
Age (years)	30.6 ± 3.6	26.8 ± 3.7	<0.001
BMI (kg/m^2)	25.4± 4.1	25.0 ± 3.9	0.574

BMI: Body Mass Index; n**:** number of participants. Data are expressed as mean ± standard deviation (SD) or as frequency (%).

Table 3: Comparison of circulating miRNA-21-5p and serum cytokine levels between groups.

Parameter (unit)	RPL (n=50)	Control (n=50)	P-value
miRNA-21-5p (Relative expression)	0.7 ± 0.1	1.0 ± 0.2	<0.001
IL-6 (pg/mL)	6.9 ± 1.3	5.3 ± 0.8	<0.001
TGF-β1 (ng/mL)	11.2 ± 2.0	13.7 ± 1.9	<0.001
IL-10 (pg/mL)	5.0 ± 1.1	6.9 ± 1.3	<0.001

RPL: Recurrent Pregnancy Loss; miRNA-21-5p: microRNA-21-5p; TGF-β1: Transforming Growth Factor beta 1; IL-10: Interleukin 10; n: number of participants.

Table 4: Mean differences, 95% confidence intervals, and effect sizes (Cohen's d).

Parameter	Mean difference (RPL − Control)	95% CI	Cohen's d
miRNA-21-5p (Relative expression)	-0.238	-0.296 to -0.181	-1.642
IL-6 (pg/mL)	1.123	0.695 to 1.551	1.045
TGF-β1 (ng/mL)	-2.502	-3.258 to -1.746	-1.314
IL-10 (pg/mL)	-1.920	-2.403 to -1.438	-1.580

CI: Confidence Interval; RPL: Recurrent Pregnancy Loss; miRNA-21-5p: microRNA-21-5p; TGF-β1: Transforming Growth Factor beta 1; IL-10: Interleukin 10.

Table 5: Pearson correlations (r) between circulating miRNA-21-5p and cytokine levels (overall and stratified).

RPL	R	P-value
IL-6 (pg/mL)	-0.019	0.896
TGF-β1 (ng/mL)	-0.115	0.425
IL-10 (pg/mL)	-0.055	0.704
Control		
IL-6 (pg/mL)	0.031	0.833
TGF-β1 (ng/mL)	-0.182	0.205
IL-10 (pg/mL)	-0.144	0.320

RPL: Recurrent Pregnancy Loss; miRNA-21-5p: microRNA-21-5p; TGF-β1: Transforming Growth Factor beta 1; IL-10: Interleukin 10.

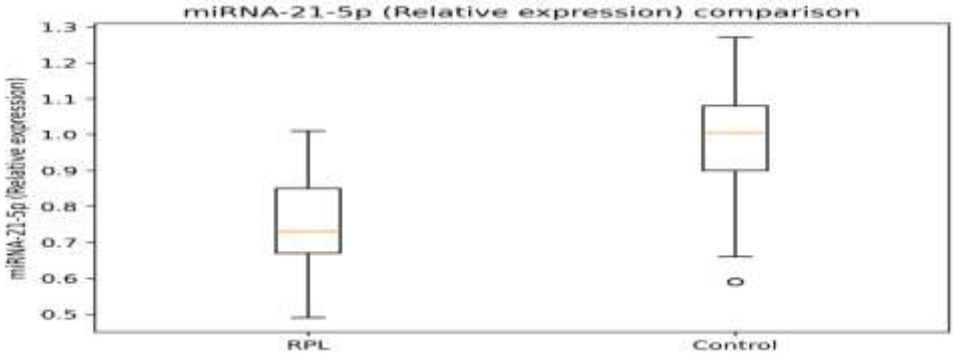

Figure 2: Comparison of circulating miRNA-21-5p expression between women with recurrent pregnancy loss and normal pregnancy.

index (BMI) demonstrated no significant variance between the RPL (25.4 ± 4.1 kg/m²) and control (25.0 ± 3.9 kg/m²) cohorts (p = 0.574), indicating that the groups were well-matched regarding baseline body mass distribution.

Table 3 Molecular and immunological profiling revealed highly significant alterations in the RPL cohort compared to the healthy controls. The relative expression of circulating miRNA-21-5p was significantly downregulated in the RPL group (0.7 ± 0.1) versus the control group (1.0 ± 0.2; p < 0.001). Concurrently, the systemic concentrations of key immunoregulatory cytokines were markedly diminished in the patient cohort. Specifically, both TGF-β1 (11.2 ± 2.0 vs. 13.7 ± 1.9 ng/mL; p < 0.001) and IL-10 (5.0 ± 1.1 vs. 6.9 ± 1.3 pg/mL; p < 0.001) exhibited statistically significant reductions when compared to the control group.

Table 4 Negative mean differences (shown graphically in opposite direction) indicate lower levels in the RPL group, and positive differences indicate higher levels in the RPL group. Cohen's d values of approximately 0.2, 0.5, and 0.8 are frequently used to denote small, medium, and large effects respectively; all Cohen's d values in Table 3 suggest moderate-to-large group separation for the biomarkers assessed.

Correlation between miRNA-21-5p and cytokine levels Pearson correlation analysis was used to examine the correlations between circulating miRNA-21-5p and cytokine (IL-6, TGF-β1 and IL-10) levels. Outcomes are presented for all and stratified by group (Table 4).

Table 5 In the pooled sample, miRNA-21-5p demonstrated a statistically significant negative correlation with IL-6 and positive correlations with TGF-β1 and IL-10. In stratified analyses, correlations within each group were weaker and not statistically significant, suggesting that the overall associations may be driven primarily by between-group differences.

Figure 2 Show that the visual distribution analysis of circulating miRNA-21-5p relative expression demonstrates a distinct downregulation in the RPL cohort compared to normal pregnancies. The box-and-whisker plot illustrates a markedly lower median expression level, alongside a corresponding downward shift in the interquartile range, within the

RPL group. This distribution pattern visually corroborates the statistically significant systemic reduction of miRNA-21-5p associated with the patient cohort.

Figure 3 Show that the visual assessment of the box-and-whisker plot reveals a distinct upward shift in the

median serum concentration within the RPL cohort compared to normal pregnancies. Furthermore, the RPL group exhibits a substantially wider interquartile range, indicating greater data dispersion and pronounced inter-individual variability among the patients relative to the control group.

Figure 4 that the visual distribution analysis of serum TGF-β1 concentrations reveals a distinct downward shift in the RPL cohort relative to healthy controls. The box-and-whisker plot illustrates a visibly lower median value alongside a reduced interquartile range within the patient group, despite the presence of an isolated upper outlier. This graphical representation visually corroborates the statistically significant systemic downregulation of this key immunoregulatory cytokine associated with the studied pathological state.

Figure 5 Show that that visual distribution analysis of serum IL-10 concentrations demonstrates a prominent downward shift in the RPL cohort compared to normal pregnancies. The box-and-whisker plot displays a notably depressed median value and a lower interquartile range within the patient group, contrasting with the higher overall distribution observed in healthy controls. This graphical representation visually reinforces the statistically significant systemic depletion of this crucial anti-inflammatory cytokine associated with the patient population. Figure 6 the bivariate relationship between circulating miRNA-21-5p relative expression and serum IL-6 concentrations. The graphical distribution demonstrates a wide, unpatterned dispersion of data points, lacking any discernible linear trajectory or defined clustering. This visual absence of a correlative trend corroborates the lack of statistical significance in the linear association between these two specific parameters.

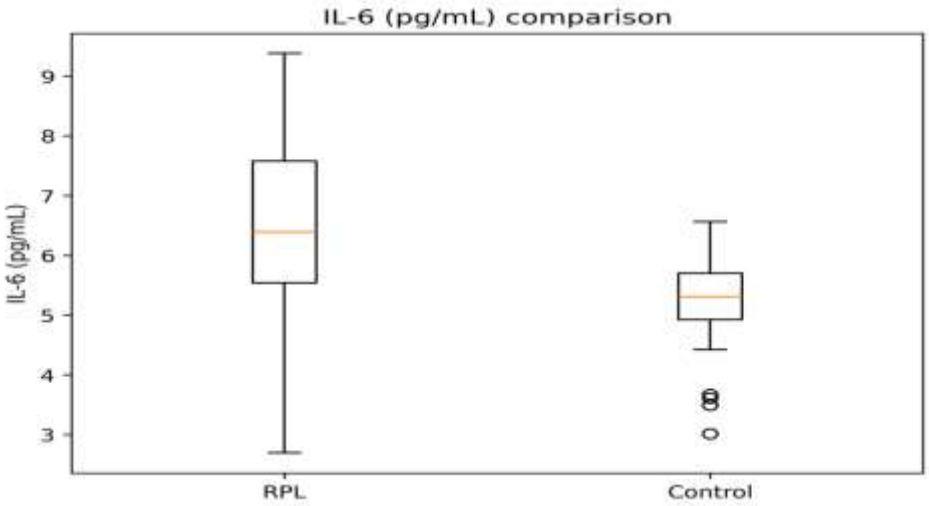

Figure 3: Comparison of IL-6 serum levels between women with recurrent pregnancy loss and normal pregnancy

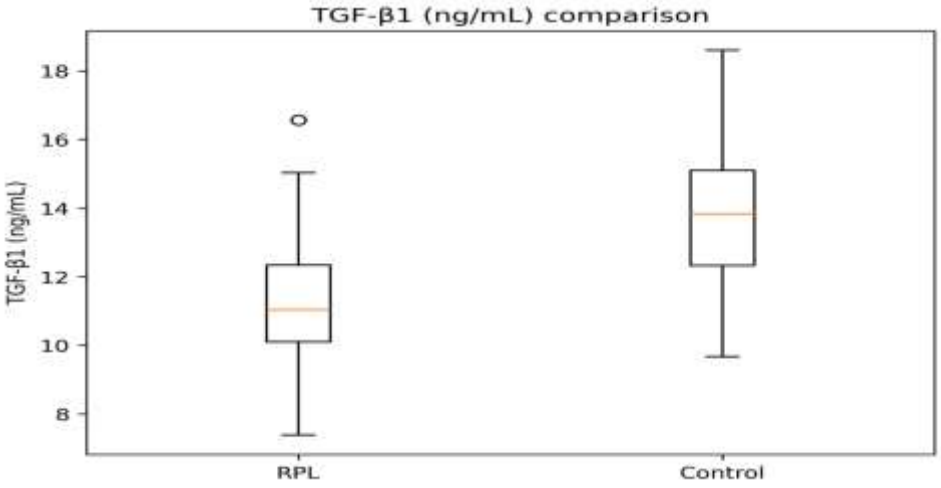

Figure 4: Comparison of TGF-β1 serum levels between women with recurrent pregnancy loss and normal pregnancy.

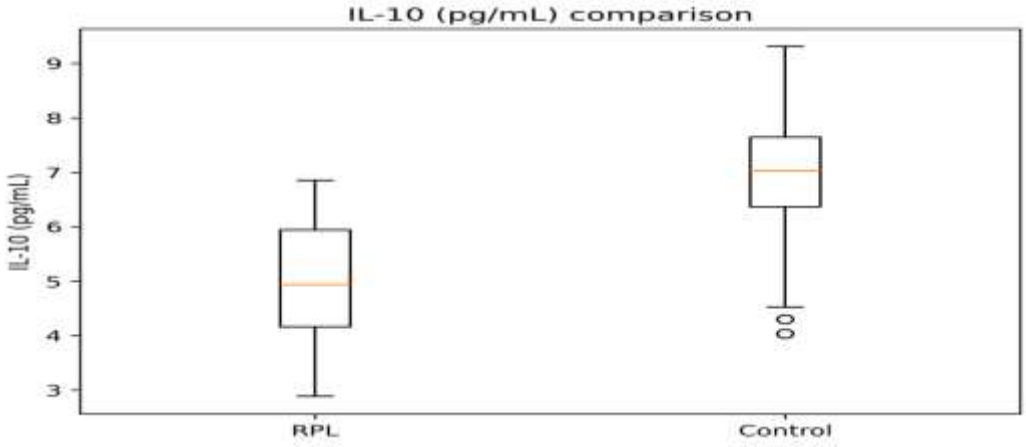

Figure 5: Comparison of IL-10 serum levels between women with recurrent pregnancy loss and normal pregnancy.

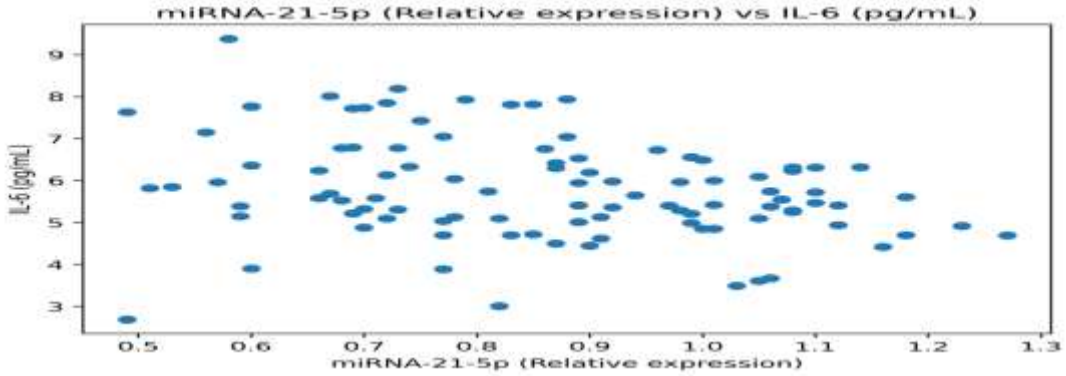

Figure 6: Correlation between circulating miRNA-21-5p expression and IL-6 levels.

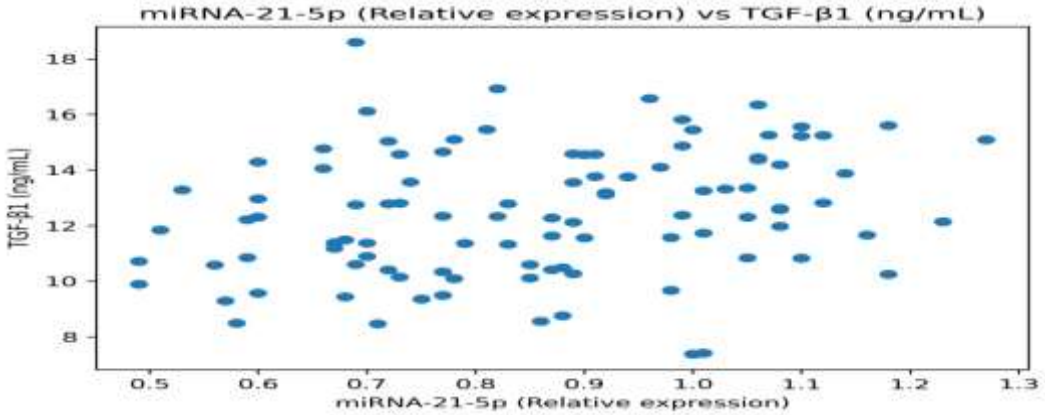

Figure 7: Correlation between circulating miRNA-21-5p expression and TGF-β1 levels.

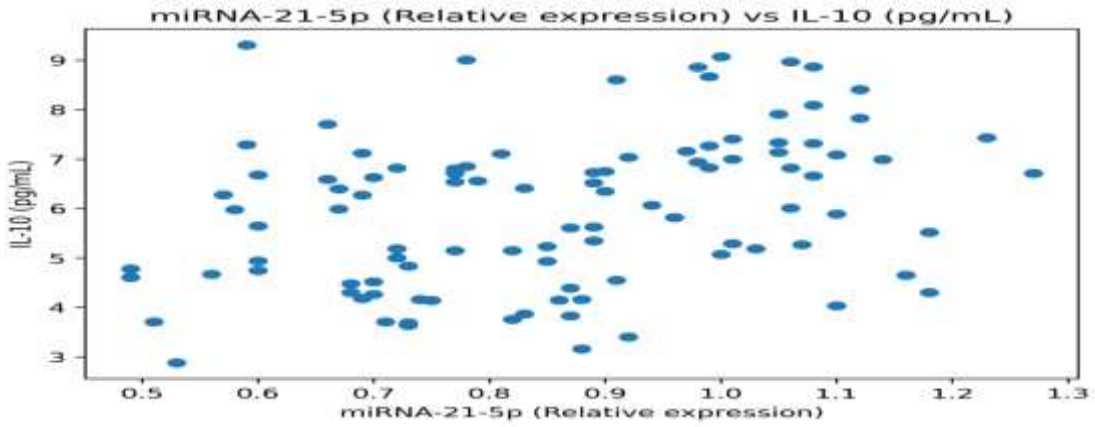

Figure 8: Correlation between circulating miRNA-21-5p expression and IL-10 levels.

Figure 7 illustrates the relationship between circulating miRNA-21-5p relative expression and serum TGF-β1 concentrations. The graphical dispersion of data points illustrates a broad, unpatterned distribution lacking a cohesive linear trajectory or discernible clustering. This visual absence of a definitive trend corresponds with the quantitative analysis, indicating no statistically

significant linear correlation between these specific immunoregulatory parameters across the study cohorts.

Figure 8 illustrates the bivariate relationship between circulating miRNA-21-5p relative expression and serum IL-10 concentrations demonstrates a widespread, unstructured dispersion of data points. The graphical distribution exhibits no discernible linear trajectory, clustering, or definitive trend. This visual representation corroborates the quantitative correlation analysis, confirming the absence of a statistically significant linear association between these two biomarkers within the evaluated study population.

Discussion

The successful establishment of human pregnancy demands a profound immunological paradigm shift, prioritizing fetomaternal tolerance over classical inflammatory responses. The current investigation elucidated the systemic immunological and epigenetic landscape in patients with RPL.[13] Di

These analyses revealed a profound and statistically significant attenuation in the circulating concentrations of crucial anti-inflammatory cytokines, specifically TGF-β1 and IL-10. Biologically, these mediators are indispensable for suppressing alloreactivity and orchestrating regulatory T-cell (Treg) differentiation at the maternal-fetal interface. The substantial depletion of these systemic cytokines strongly implies a compromised tolerogenic network, consequently this state of immunological vulnerability may precipitate the breakdown of maternal tolerance, leading to the immune-mediated rejection of the semi-allogeneic fetus.[14]

In parallel with the cytokine dysregulation, this study demonstrated a highly significant downregulation of circulating miRNA-21-5p relative expression within the RPL cohort. Emerging evidence in reproductive epigenetics indicates that microRNAs are fundamental regulators of trophoblast invasion, endothelial function, and local immune cell polarization.[15] The marked suppression of miRNA-21-5p observed in our patient group suggests an upstream epigenetic aberration.[16] Perhaps this systemic molecular deficit reflects a broader transcriptional dysregulation that adversely affects early placental development or the maintenance of local immune homeostasis, ultimately contributing to the clinical manifestation of repeated pregnancy failure.

Interestingly, despite the concurrent systemic reductions in both the epigenetic modulator and the immunoregulatory cytokines, these analyses revealed an absence of direct linear correlation between miRNA-21-5p expression and either TGF-β1 or IL-10 concentrations.[17] While initially counterintuitive, this lack of a simple linear relationship accurately reflects the inherent biological complexity of post-transcriptional regulatory networks. miRNAs characteristically exert their influence through highly interconnected, multi-target pathways rather than isolated, one-to-one mechanistic axes.[18] Therefore, the simultaneous depletion of these diverse biological markers may signify parallel downstream consequences of an overarching, multi-systemic pathological cascade rather than a direct causative loop. Furthermore, the localized microenvironmental dynamics at the decidua are immensely intricate; perhaps the peripheral circulation solely captures fragmented biological echoes of these localized interactions, masking any direct correlations that exist exclusively at the tissue level.[19,20]

Strengths and limitations of the study

The primary strength of this study lies in its rigorous simultaneous profiling of circulating miRNA-21-5p alongside key immunoregulatory cytokines, providing novel targeted insights into the systemic networks of idiopathic RPL. However, while the present study offers robust effect sizes and focused variable isolation, several limitations require cautious interpretation of the findings. Primarily, a statistically significant age variance existed between the RPL and control cohorts. Although the profound magnitude of the biomarker deviations strongly points toward a pathology-driven etiology, chronological age remains a recognized confounding variable in reproductive immunology that necessitates consideration.

Secondly, the analyses relied exclusively on peripheral blood sampling, which does not entirely mirror the specific immunological and molecular architecture of the localized decidual

microenvironment. Lastly, the cross-sectional methodology captures a singular temporal snapshot, limiting the ability to establish definitive chronological causality between the observed biomarker dysregulation and the onset of pregnancy loss. Future longitudinal investigations utilizing age-matched cohorts and incorporating matched decidual tissue biopsies are recommended to comprehensively validate these systemic findings.

Implications for policy and practice

The findings underscore the potential of circulating miRNA-21-5p as a non-invasive biomarker for the early detection of maternal-fetal immune dysregulation. From a clinical perspective, identifying this epigenetic attenuation could guide future therapeutic strategies and targeted clinical protocols aimed at restoring the tolerogenic cytokine balance, thereby improving pregnancy outcomes and clinical management for women with idiopathic RPL.

Conclusion

This investigation demonstrates a highly significant systemic downregulation of both the epigenetic modulator miRNA-21-5p and the critical anti-inflammatory cytokines TGF-β1 and IL-10 in women experiencing recurrent pregnancy loss. Although a direct linear correlation between the circulating microRNA and cytokine concentrations was not evident-reflecting the multifaceted nature of epigenetic and immunological networks-their concurrent depletion highlights a profound disruption in the maternal tolerogenic environment. These parallel molecular and immunological alterations provide valuable insights into the systemic dysregulation underlying idiopathic pregnancy failure. The findings underscore the potential significance of these biomarkers in understanding the pathogenesis of RPL, warranting future longitudinal and tissue-specific studies to fully elucidate their interactive functional roles at the maternal-fetal interface.

Conflict of interest

The authors declare that they have no relevant financial or non-financial competing interests to disclose.

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Data availability

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

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