Original
ISSN 2413-0516

Association of Cytomegalovirus Load, miR-135a-5p Expression, and C-Reactive Protein in Women with Recurrent Pregnancy Loss: A Case–Control Study

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(Submitted: 13 October 2025 – Revised version received: 21 October 2025 – Accepted: 24 October 2025 – Published online: 26 October 2025)

Abstract

Objective: To assess the association between CMV viral load, serum CRP levels, and miR-135a-5p expression in women with RPL compared to healthy pregnant controls.

Methods: A case–control study was conducted involving 60 women with ≥ 3 first-trimester losses and 30 healthy pregnant women. Blood samples were analyzed for CMV DNA via quantitative polymerase chain reaction (PCR), miR-135a-5p expression via RT-qPCR, and CRP levels via ELISA. Statistical correlations were evaluated.

Results: CMV DNA was detected in **81.7%** of RPL patients vs. **23.3%** of controls ($P < 0.0001$). Mean CMV load, CRP levels, and miR-135a-5p expression were significantly higher in the RPL group. Strong positive correlation was observed among the three biomarkers ($r > 0.6$, $P < 0.0001$).

Conclusion: Elevated CMV load, CRP levels, and miR-135a-5p expression are significantly associated with RPL. These markers may reflect a common pathogenic pathway involving infection-induced inflammation and epigenetic regulation.

Keywords: Abortion, habitual, cytomegalovirus, C-reactive protein, microRNAs, polymerase chain reaction

Introduction

Recurrent pregnancy loss (RPL) is traditionally defined as the occurrence of two or more consecutive miscarriages before the 20th week of gestation, although some clinical guidelines still refer to the older definition requiring three or more losses.[1] RPL affects approximately 1–2% of couples attempting conception and is considered a challenging clinical condition due to its multifactorial etiology and the emotional burden it imposes.[2,3] Despite comprehensive clinical, genetic, anatomical, and hormonal evaluations, nearly 40–50% of RPL cases remain unexplained.[4,5]

Among the potential contributors to unexplained RPL, infection agent-specific viruses have drawn increasing attention. Cytomegalovirus (CMV), a member of the Betaherpesvirinae subfamily, is a ubiquitous virus capable of establishing lifelong latency after primary infection and reactivating under immune-compromised conditions, such as pregnancy.[6] Cytomegalovirus seroprevalence exceeds 90% in many low and middle-income countries, and the virus is a leading cause of congenital infections worldwide.[7] Several studies have suggested an association between CMV reactivation and adverse pregnancy outcomes, including miscarriage, intrauterine growth restriction, and preterm birth, although the precise mechanisms remain under investigation.[8,9]

Cytomegalovirus targets cells of the placenta, including cytotrophoblasts and decidual stromal cells, interfering with trophoblast differentiation, immune tolerance, and spiral artery remodeling. These disturbances can impair placental perfusion and fetal development.[10,11] Moreover, CMV infection is known to provoke systemic and local inflammation through upregulation of cytokines such as IL-6, TNF-α, and interferon, which may further disrupt maternal-fetal immune balance.[12]

A growing body of evidence points to microRNAs (miRNAs) as key in the regulation of immune tolerance, implantation, and placental development. These small non-coding RNAs modulate gene expression post-transcriptionally and are increasingly recognized for their roles in reproductive health and disease. One such miRNA, miR-135a-5p, has been implicated in endometrial receptivity and decidualization. Aberrant expression of miR-135a-5p has been observed in women with unexplained infertility and RPL, suggesting it may contribute to an altered uterine environment or implantation failure.[13–15]

Interestingly, CMV infection itself has been shown to modulate host miRNA expression CMV encodes several miRNAs that interact with host cell pathways, and the virus can also influence the transcription of host miRNAs such as the miR-135 family, linking viral pathogenesis to epigenetic regulation.[16] This interaction may further explain the virus's ability to subvert immune surveillance and promote persistent infection, potentially contributing to pregnancy complications.

In addition to viral infection and epigenetic factors, systemic inflammation is another hallmark of RPL. C-reactive protein, an acute-phase protein produced by the liver in response to pro-inflammatory cytokines, serves as a non-specific but sensitive biomarker of inflammation. Elevated maternal CRP levels have been reported in women with early pregnancy loss, preeclampsia, and other obstetric complications.[17] Inflammation-driven disruptions at the maternal-fetal interface can lead to defective implantation, inadequate vascular remodeling, and pregnancy failure. Given that CMV infection can stimulate CRP production via cytokine release, its presence may serve as both a trigger and a biomarker for inflammatory-mediated RPL.[18]

Taken together, CMV infection, altered miRNA expression, and systemic inflammation may represent interconnected pathways contributing to the pathogenesis of RPL.

Therefore, we hypothesize that active CMV infection in women with recurrent pregnancy loss promotes dysregulation of miR-135a-5p, which in turn contributes to impaired trophoblast function and inflammatory activation, ultimately reflected in elevated CRP levels. In this model, CMV acts as an upstream trigger, altering miRNA-mediated gene regulation at the maternal–fetal interface, while CRP represents a systemic downstream marker of the resulting inflammatory response. By evaluating CMV viral load alongside miR-135a-5p expression and CRP concentration, this study aims to clarify whether these biomarkers are mechanistically linked components of a shared pathological pathway in RPL.

This study aims to investigate the correlation between CMV viral load, miR-135a-5p expression, and CRP level in women with a history of RPL, compared to healthy pregnant controls. Understanding the interaction among viral replication, inflammatory markers, and epigenetic regulation may offer new insights into the molecular mechanisms underlying RPL and guide future diagnostic and therapeutic strategies. To our knowledge this is the first study of this kind and of this objectives to be achieved in our region aiming to explore the relations of MicroRNAs with repeated abortion.

Material and Methods

Study Protocol

This was a case-control study, which was conducted from July 2024 to April 2025 at Erbil Maternity Teaching Hospital in Erbil city, and the study population included women with a history of recurrent pregnancy loss (RPL) and healthy pregnant women without a history of abortion.

Ethical Considerations

The study received approval from the Ethics Committee of the College of Medicine at Hawler Medical University and the Erbil Health Directorate. Written informed consent was obtained from each participant as well as from their husbands or relatives to enrollment. Before collecting blood samples, the procedure was carefully explained to all participants to ensure full understanding. The women and their relatives were made aware that opting out of the study would carry no prejudice or penalty.

Study Population

The study's included a total of 90 participants, comprising 60 women with RPL and 30 age-matched healthy pregnant controls. The sample size was determined based on previously published case–control studies investigating CMV infection and molecular biomarkers in pregnancy complications. Prior research assessing CMV viral load and miRNA expression in reproductive disorders has typically utilized sample sizes ranging from 40 to 120 participants, which were shown to be sufficient to detect statistically meaningful differences between groups with acceptable power (≥80%) and alpha (0.05).[19,20] Additionally, given the estimated prevalence of CMV reactivation among women with RPL (approximately 50–80%) and among healthy pregnant controls (20–30%), a minimum case-to-control ratio of 2:1 was considered appropriate to enhance statistical robustness while maintaining feasibility. Thus, the chosen cohort size in the present study is methodologically consistent with published research and is sufficient to detect clinically relevant differences in CMV viral load, miR-135a-5p expression, and CRP levels between groups. Participants were selected based on rigorous inclusion and exclusion criteria. The inclusion criteria for the RPL group were as follows: women aged 20–40 years with a history of three or more consecutive spontaneous pregnancy losses before 12 weeks of gestation. The control group consisted of healthy women with at least one full-term live birth and no history of participants. The exclusion criteria for both groups encompassed a range of conditions, including chronic systemic disease such as diabetes mellitus, hypertension, and autoimmune disorder; known genetic or anatomical abnormalities; recent viral or bacterial infections; and the use of immunosuppressive or antiviral medications.

Analytical Methods

Blood samples were collected from all participants under aseptic conditions for the assessment of cytomegalovirus (CMV) viral load, miR-135a-5p expression, and CRP levels. Two blood samples were collected from participants for analysis: one in EDTA tubes (5 ml) for molecular analysis and the second in gel clot activator tubes (5 ml) for serum separation. Blood samples were transported immediately on ice to the molecular biology laboratory at the Public Health Laboratory Management. Plasma was separated from blood in EDTA tubes by centrifugation twice at 3,000 rpm for 15 minutes at 4°, aliquoted, and stored at –80°C for further analysis. Serum was obtained by centrifuging the gel tubes at 3,000 rpm for 10 minutes at room temperature. The separated serum was stored at –20°C until analysis.

Viral DNA Extraction

Extraction of viral nucleic acids from blood samples was carried out using the QIAamp DNA Blood Mini Kit (QIAGEN, Germany; Cat. No. 51104), following the manufacturer's instructions. Briefly, a 200 µL blood sample was first mixed with lysis buffer and proteinase K and incubated at room temperature for 10 minutes to break down viral particles and release nucleic acids. Followed by adding ethanol to facilitate the binding of nucleic acids to the silica membrane within the spin column. The mixture was then loaded onto the column and subjected to two wash steps to eliminate proteins and other impurities. Subsequently purified viral DNA was eluted in 200 µL elution buffer and stored at –20°C for future use. The concentration and purity of the extracted DNA were measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

Real-Time PCR for CMV DNA Detection

Quantification of CMV DNA was performed using the CMV RG PCR Kit (QIAGEN, Germany; Cat. No. 4503265) on the Rotor-Gene Q real-time PCR system, following the manufacturer's instructions. Each PCR reaction included 30 µL of master mix and 20 µL of extracted DNA, for a total volume of 50 µL. The kit includes internal control and quantitation standards for accurate viral load determination. The thermal cycling conditions and fluorescence detection were performed

as specified in the kit protocol. Data were analyzed using Rotor-Gene Q software, and CMV DNA quantification was based on comparison with provided standards.

MicroRNA Extraction and Quantification

RNA Extraction

Total RNA, including microRNAs (miRNAs), was isolated from 200 µL of EDTA-treated plasma using the miRNeasy Serum/plasma Advanced kit (QIAGEN, Germany; Cat. No. 217204), following the manufacturer's instructions. Initially, plasma samples were mixed with Buffer RPL and Buffer RPP to achieve complete lysis and protein precipitation. During the lysis step, UniSp6 synthetic spike-in RNA (Qiagen) was added to each sample as an internal control to monitor the efficiency of RNA extraction and subsequent reverse transcription. Following centrifugation, the RNA-containing supernatant was collected, mixed with isopropanol, and applied to a RNeasy UCP MinElute spin column, and subjected to centrifugation to allow the binding of RNA to the silica membrane. Following a series of washes with the supplied buffers, RNA was eluted in 20 µL of RNase-free water. The extracted RNA was stored at –80°C until further use. RNA purity and concentration were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

cDNA Synthesis

Complementary DNA (cDNA) was generated from plasma RNA using the miRCURY LNA RT Kit (QIAGEN, Germany; Cat. No. 339340) according to the manufacturer's protocol. In brief, 4 µL of template RNA was mixed with miRCURY RT Reaction Buffer and miRCURY RT Enzyme Mix, bringing the final reaction volume to 10 µL. The reverse transcription was carried out at 42°C for 60 minutes, followed by an enzyme inactivation step at 95°C for 5 minutes. The synthesized cDNA was then diluted appropriately and stored at –20°C until used for quantitative PCR (qPCR) analysis.

Quantitative Real-Time PCR

Quantitative real-time PCR (qPCR) was conducted using the miRCURY LNA miRNA Probe PCR Kit (QIAGEN, Germany; Cat. No. 339371) with LNA-enhanced primers specific for hsa-miR-135a-5p (target) and hsa-miR-191-5p (reference control), following the manufacturer's instructions. Reactions were performed on the Rotor-Gene Q real-time PCR system (QIAGEN, Germany; Cat. No. R1018175 and R100972) under these thermal cycling conditions: an initial heat activation at 95°C for 2 minutes, then 40 cycles of a two-step protocol consisting of denaturation at 95°C for 5 seconds and combined annealing/extension at 56°C for 30 seconds. The relative expression of miR-135a-5p was calculated using the 2^ΔΔCT method, normalized to miR-191-5p as the endogenous control.

C-Reactive Protein

C-reactive protein (CRP) levels were measured using a high-sensitivity ELISA kit (DRG International, Inc., Cat. No. EIA-3954, USA), following the manufacturer's instructions. Serum samples and standards were added to microplate wells that had been pre-coated with anti-CRP antibodies, followed by an incubation period to allow binding. After washing to remove unbound substances, an enzyme-labeled antibody was added, followed by a substrate solution. The reaction was stopped, and absorbance was measured at 450 nm using a BioTek microplate reader with Gen5 software. The concentration of CRP was determined by interpolation from a standard curve generated using known calibrators.

Statistical Analysis

All data were analyzed using IBM SPSS software, version 26.0 (IBM, USA). Prior to applying parametric tests, the distribution of continuous variables was assessed for normality using the Shapiro-Wilk test. Variables that met the normality assumption were analyzed using the Student's *t*-test for comparisons between groups, while non-normally distributed data were analyzed using the Mann-Whitney U Test Continuous variables were expressed as mean ± standard deviation (SD) and compared using the student's *t*-test as appropriate categorical variables was compared using the chi-square test. Pearson or Spearman correlation coefficients were used to evaluate association between CMV load, miR-135a-5p expression, and CRP levels. A *P*-value <0.05 was considered statistically significant.

Results

1. Comparison between RPL group and controls (Table 1).

Recurrent pregnancy loss patients showed a highly significant increase in all three biomarkers compared to healthy controls. The mean CMV viral load was markedly elevated in the RPL group (1009.48 ± 857.26) compared with controls (92.22 ± 75.8; $P < 0.0001$). Similarity, miR-135a-5p expression levels were significantly upregulated in RPL patients (4.08 ± 0.84) versus controls (1.62 ± 0.5; $P < 0.0001$). Serum CRP concentration were also higher in RPL cases (4.8 ± 1.01) compared with the controls group (1.78 ± 0.6; $P < 0.0001$). These results demonstrate that women with RPL experience increased viral activity, altered miRNA expression, and a heightened inflammatory state.

2. Comparison within RPL group (CMV-positive vs. CMV-negative) (Table 2).

Table 1. **CMV viral load, miR-135a-5p expression and CRP levels in RPL patients vs. controls**

Marker	RPL ($n = 60$)	Control ($n = 30$)	*P*-value
CMV viral load	1009.48 ± 857.26	92.22 ± 75.8	<0.0001
miR-135a-5p (ΔΔCt ± SD)	4.08 ± 0.84	1.62 ± 0.5	<0.0001
CRP (mg/L ± SD)	4.8 ± 1.01	1.78 ± 0.6	<0.0001

Table 2. **Comparison of biomarkers between CMV-positive and CMV-negative women in the RPL group**

Marker	CMV-positive RPL ($n = 51$)	CMV-negative ($n = 9$)	*P*-value
miR-135a-5p (ΔΔCt ± SD)	4.28 ± 0.7	2.93 ± 0.69	<0.0001
CRP (mg/L ± SD)	4.97 ± 0.98	3.82 ± 0.5	= 0.0011

Table 3. **Correlation among CMV viral load, miR-135a-5p, and CRP in RPL patients**

Variables	*r*	*P*-value
CMV load vs. miR-135a-5p	0.4336	<0.01
CMV load vs. CRP	0.2331	NS
miR-135a-5p vs. CRP	0.2386	NS

Subgroup analysis patients revealed significant differences based on CMV infection status. CMV-positive women ($n = 51$) displayed higher miR-135a-5p expression (4.28 ± 0.7) than CMV-negative women ($n = 9$; 2.93 ± 0.69; $P < 0.0001$). Additionally, serum CRP levels were significantly higher among CMV-positive cases (4.97 ± 0.98) compared to CMV-negative cases (3.82 ± 0.5; $P = 0.0011$). These findings suggest that active CMV infection in RPL patients intensifies both miRNA dysregulation and inflammatory response.

3. Correlation analysis (RPL group, $n = 60$) (Table 3).

The Correlation analysis revealed a significant positive relationship between CMV viral load and miR-135a-5p expression ($r = 0.4336$, $P < 0.01$), indicating that increased viral replication is associated with greater miRNA upregulation. In contrast, CMV viral load showed only a weak and non-significant correlation with CRP levels ($r = 0.2331$). Similarly, the correlation between miR-135a-5p expression and CRP was weak and statistically non-significant ($r = 0.2386$). These results suggest that CMV infection exerts its pathogenic influence in RPL primarily through modulation of miRNA pathway rather than systemic inflammatory markers.

Discussion

Recurrent pregnancy loss remains a complex obstetric disorder, often multifactorial in origin and incompletely understood. In this study, we demonstrated that women with RPL had significantly elevated CMV viral load, increased expression of miR-135a-5p, and higher serum CRP levels compared with healthy controls. Moreover, CMV-positive RPL patient exhibited greater miRNA dysregulation and inflammatory activation than CMV-negative women. Importantly, correlation analysis revealed a moderate, statistically significant relationship between CMV viral load and miR-135a-5p, while correlations with CRP were weak and non-significant.

These finding highlight the potential mechanistic role of CMV and epigenetic regulation via microRNAs in the pathogenesis of RPL.

Role of CMV in RPL

The present data are consistent with studies reporting an association between CMV and miscarriage. Inaam et al. identified CMV genes UL146 and US28 in women with recurrent miscarriage, suggesting viral persistence may contribute to pregnancy loss.[21]

Similarly, Mocanu et al, reviewed latent CMV infection and highlighted reports of increased CMV DNA in women with adverse pregnancy outcomes.[22] However, some studies relying on serology rather than viral load have failed to demonstrate such associations, underscoring methodological variability. An Iraqi case-control study using PCR also found higher IgM and IgG prevalence among miscarriage cases compared with controls, but lacked quantitation of viral burden.[23] Our results therefor strengthen the argument by providing quantitative viral evidence.

MicroRNA Dysregulation

The upregulation of miR-135a-5p in RPL, particularly in CMV-positive women, align with reports that this microRNA impairs trophoblast function. Lu et al. demonstrated that, miR-135a-5p overexpression in trophoblast cells inhibited proliferation, migration, invasion, and angiogenesis, and was elevated in villous tissue from spontaneous abortion cases.[19] By contrast, Wu et al. reported that miR-135a-5p promoted trophoblast invasion in preeclampsia models.[24] This apparent contradiction highlights the context-dependent role of miRNAs: while in preeclampsia miR-135a-5p may act as a compensatory factor, in RPL it appears pathogenic. Global reviews of miRNA expression in RPL emphasize alterations in miR-125a, miR-146a, miR-155, and let-7 family,[20,25] making our finding on miR-135a-5p relatively novel.

Systemic Inflammation

C-reactive protein levels were significantly elevated in our RPL patients, supporting the role of systemic inflammation in reproductive failure. Similar trends have been described in immunological studies of RPL, which emphasize aberrant inflammatory signaling, complement activation, and cytokines imbalance.[26] However, the lack of significant correlation between CRP and either CMV load or miR-135a-5p suggest that systemic inflammation, as captured by CRP, may not directly mirror local placental or uterine events. Instead, it likely represents a downstream effect rather than a sensitive mechanistic biomarker.

Correlation Finding

The moderate positive correlation between CMV load and miR-135a-5p ($r = 0.43$, $P < 0.01$) suggests that viral activity may party drive miRNA dysregulation. This quantitative link strengthens the plausibility of a viral-miRNA axis in RPL pathogenesis. The absence of correlation with CRP highlights dissociation between systemic and local processes. Similar patterns, where miRNAs show moderate correlation with pathogenic triggers but weak links with systemic markers, have been noted in other reproductive pathologies.[20] Thus, our findings support a model in which CMV infection contributes directly to miRNA deregulation, while systemic inflammation is a secondary phenomenon.

Conclusion

This study demonstrates that women with recurrent pregnancy loss exhibit significantly higher CMV viral load, increased expression of miR-135a-5p, and elevated CRP levels compared to healthy pregnant controls. The positive correlation identified between CMV activity and miR-135a-5p expression suggests that viral reactivation may influence epigenetic regulation at the maternal–fetal interface, potentially contributing to impaired trophoblast function and implantation failure. In contrast, the weaker association between CMV or miRNA levels and CRP indicates that systemic inflammation may

represent a downstream or secondary phenomenon rather than a primary driver in this pathway.

These findings provide translational relevance, suggesting that miR-135a-5p could serve as a novel biomarker of CMV-mediated reproductive pathology and may have value in early risk stratification among women with unexplained RPL. Furthermore, quantifying CMV viral load in conjunction with miRNA profiling could improve diagnostic precision beyond routine serology alone. From a therapeutic standpoint, strategies aimed at controlling CMV reactivation, along with emerging miRNA-targeted modulators, may offer new avenues for intervention to restore immune tolerance and placental function in future pregnancies.

Further large-scale, multicenter, and longitudinal studies—including placental tissue analysis—are warranted to validate the CMV–miR-135a-5p axis and to explore its feasibility as a clinical screening tool or therapeutic target in the management of recurrent pregnancy loss.

Strengths and Limitations

Strengths

To our knowledge, this is the first study to simultaneously assess CMV viral load, CRP, and miR-135a-5p in RPL using validated molecular and immunoassay techniques in a well-matched case-control design to reduce confounding.

Limitation

Single-center study limits generalizability. Lack of follow-up data on pregnancy outcomes after sampling. The study did not assess local immune markers (e.g., decidual NK cells, cytokines) which may further clarify mechanisms.

Recommendations

Further studies are warranted to:

- Confirm these findings in larger, multicenter cohorts.
- Explore longitudinal trends in CMV, CRP, and miRNAs across gestation.
- Investigate endometrial or placental tissue expression of miR-135a-5p and inflammatory markers.

Acknowledgments

The authors would like to express their sincere appreciation to the Health and Education Directorates of Erbil for their support in facilitating this study. Special thanks are extended to all the women who generously participated in the research. The authors are also grateful to the nurses and laboratory technicians at Erbil Maternity Teaching Hospital in Erbil, who provided valuable assistance during sample collection.

Financial Support and Sponsorship

Nil.

Conflicts of Interest

There are no conflicts of interest. ■

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