

Early Version – Uncorrected

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DOI: <https://dx.doi.org/10.31584/jhsmr.20261480>

Received: 1 March 2026

Revised: 22 March 2026

Accepted: 25 March 2026

Published online: 27 October 2026



Please cite this article as: Khikani ANS, Fırat M, Al-Khafaji ZAI, Alghrairi AH. Expression of HCMV-encoded miR-UL148D and miR-UL36-3p in women with HCMV-associated miscarriage: a case-control study. J Health Sci Med Res doi: <https://dx.doi.org/10.31584/jhsmr.20261480>

Early Version — Uncorrected: This accepted manuscript has not been typeset or proofread.

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Original article

Expression of HCMV-Encoded miR-UL148D and miR-UL36-3P in Women with HCMV-Associated Miscarriage: A Case-Control Study

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Running Title: HCMV miRNAs in Miscarriage

Abstract:

Objectives: To evaluate the expression levels of HCMV-encoded miR-UL148D and miR-UL36-3P in women with a miscarriage associated with HCMV infection.

Materials and Methods: A case-control study was designed to include 50 women who had miscarriages due to active human cytomegalovirus (HCMV) infection, with a high HCMV viral load $>10 \times 10^3$ copies/mL, and a control group consisting of 50 healthy pregnant women who had not previously experienced miscarriages. RNAs were extracted from buffy coat samples to measure the HCMV-miR-UL148D and HCMV-miR-UL36-3P gene expression by the quantitative reverse transcription PCR (qRT-PCR) technique.

Results: Both HCMV-miR-UL148D and HCMV-miR-UL36-3P were significantly upregulated in miscarriage patients (2.66-fold and 21.16-fold changes, respectively; p -value < 0.001). ROC analysis demonstrated strong diagnostic performance. For HCMV-miR-UL148D, sensitivity was 96%, and specificity was 98% (AUC = 0.94). For HCMV-miR-UL36-3P, sensitivity was 91%, and specificity was 87% (AUC = 0.94).

Conclusion: Elevated expressions of HCMV-encoded miR-UL148D and miR-UL36-3P may contribute to HCMV-associated miscarriage and could serve as potential biomarkers for adverse pregnancy outcomes.

Keywords: Human cytomegalovirus, miscarriage, miR-UL148D, miR-UL36-3P, gene expression.

Introduction

Human cytomegalovirus (HCMV), also referred to as the Human betaherpesvirus 5, is a pathogen classified in the Herpesviridae family of viruses. It is also considered the prototype of the Betaherpesvirinae subfamily. It is recognised as the largest genome among the human herpesviruses, with a length of approximately 235 kbp, and its linear, double-stranded deoxyribonucleic acid (DNA) genome is considered one of the largest among other human viruses¹. HCMV is a major etiological agent of intrauterine infection during pregnancy, causing lifelong infection and possible miscarriage, cerebellar deformity, stillbirth, and foetal developmental retardation². Primary maternal HCMV infection is associated with a higher risk of intrauterine transmission and unfavorable pregnancy outcomes³. HCMV is universally recognised as the most

common congenital infection in humans. HCMV infections are known to affect 60-90% of women of childbearing age worldwide, and these infections are acknowledged as endemic due to their prevalence in the majority of human societies; furthermore, the seroprevalence of HCMV is often influenced by the socioeconomic state of the region in question. Consequently, occurrences may reach a maximum of 100% in impoverished regions ⁴. Cytomegalovirus primarily induces an asymptomatic initial infection in immunocompetent individuals but may present as a severe localised illness with diverse clinical manifestations in immunosuppressed people. Similar to other herpesviruses, latency and reactivation are notably evident in instances of HCMV infection. Consequently, enduring lifetime infection with the possibility of reactivation and compromised immune defenses is a significant clinical problem linked to these viruses ⁵. Congenital HCMV infection impacts 0.2-2.2 percent of all live newborns, with 11-12.7 percent exhibiting symptoms such as cerebral and sensorineural impairment ⁶. Primary HCMV infection, occurring in 1% to 4% of pregnant women based on regional seroprevalence, poses significant risks to the foetus if transmitted during the first four months of gestation, a period when the embryo is most susceptible to viral damage ⁷. MicroRNAs (miRNAs) are around 22 nucleotides in length, noncoding RNAs encoded by eukaryotes and viruses, capable of regulating gene expression. In 1993, Lee and coworkers found the first miRNA in *Caenorhabditis elegans* ⁸. miRNAs are small, noncoding RNA molecules that play a crucial role in gene regulation. Recently, circulating cytomegalovirus HCMV-encoded miRNAs have been recognized as valuable biomarkers for diagnosing and distinguishing different diseases; furthermore, their impact on the formation and progression of diseases, both in terms of normal physiological processes and abnormal pathological conditions, has been recently validated ⁹. Therefore, this study aimed to evaluate the expression levels of HCMV-encoded miR-UL148D and miR-UL36-3P in women with HCMV-associated miscarriage and to investigate their potential role as diagnostic biomarkers.

Materials and Methods

Study design and setting:

A case-control study was conducted in Babylon Province between February 2023 and February 2024 in the Babylon Maternity and Children's Teaching Hospital and Al-Hashimiya General Hospital in Babylon Province, Iraq. From the miscarriage group and the control group, healthy

women, 5 mL of fresh venous blood was collected from each. For miRNA extraction, we stored 2 mL in an EDTA tube; for microRNA gene expression, 1 mL of blood was transferred from the EDTA tube to a 3-mL TRIzol tube and stored at -20 °C. The concentration and integrity of RNA were assessed using the A260/280nm ratio by the NanoDrop ND-2000 UV spectrophotometer (Thermo Scientific, USA). The remaining 3 mL of blood was maintained in the gel tube until it clotted. After centrifugation at 2500 rpm for 15 minutes, the serum was collected and stored at -20 °C until it was utilised for HCMV IgM and IgG measurement and HCMV viral load. Those with a copy number $>10 \times 10^3$ copies/mL, thereby suggesting ongoing viral infection, were classified as active samples. The demographic characteristics of participants were recorded. The age of women ranged from 18 to 41 years (mean age 26.4 years). Participants were recruited from Babylon Province, Iraq, including both rural and urban areas, with a higher proportion from rural residences.

Inclusion and exclusion criteria:

Inclusion criteria for the patient group consisted of women within the reproductive age group from 18 to 41 years during the miscarriage stage. **Exclusion** criteria included women with chronic diseases, miscarriage women with low viral load copies ($<10 \times 10^3$ copies/mL), miscarriage women infected with other causative agents, and immunocompromised women.

Estimation of HCMV IgM, IgG, and HCMV viral load

The serological test was done by VIDAS® (Vitek Immunodiagnostic Assay System), manufactured by BioMérieux in France, and was used to identify HCMV IgM and IgG antibodies. The technique behind enzyme-linked fluorescent assays (ELFA) is the foundation of VIDAS. The results: HCMV IgM Positive, if the HCMV IgM result is 0.90 AU/mL or higher. Negative: If the HCMV IgM result is less than 0.70 AU/mL, it is considered negative. Equivocal: If the test result falls within the range of ≥ 0.70 to < 0.90 . The results: HCMV IgG Positive, if the HCMV IgG result is ≥ 6 AU/mL. Negative, if the HCMV IgG result is < 4 AU/mL, and Equivocal: if the test result falls within a specific range, typically ≥ 4 to < 6 AU/mL. The HCMV viral load was detected using the RealBest DNA HCMV viral load kit (Best-Russia) and the SaCycler-96 Real-Time PCR

system (SECASE, Italy). Samples with $>10 \times 10^3$ copies/mL were considered to have active viral infection.

Molecular assays: RNA isolation and complementary deoxyribonucleic acid (cDNA) synthesis

Total RNA from buffy coats was extracted using a customised technique and a TRIzol kit (AccuZolTM, Korea). This work uses a universal stem loop primer (USTLP) in the cDNA synthesis of HCMV-miR-UL148D and HCMV-miR-UL36-3P, as shown in **Table 1**.

Quantitative reverse transcription PCR (qRT-PCR) was carried out with a final reaction volume of 20 μ L using 2 $\times$ PerfectStar® Green qPCR SuperMix. Each reaction consisted of 10 μ L 2 $\times$ SuperMix, 1 μ L forward primer, 1 μ L universal reverse primer, 5 μ L cDNA template, and 3 μ L nuclease-free water. Thermal cycling was conducted by initial denaturation at 94 °C for 10 min, followed by 45 cycles of denaturation at 94 °C for 20 s, annealing at 60 °C for 30 s, and extension at 72 °C. A last extension step was performed at 72 °C for 10 min. Amplification specificity was confirmed by melt curve analysis carried out from 65 °C to 95 °C, which demonstrated a single specific peak for each target.

SYBR Green RT-PCR

The PerfectStar® Green qPCR SuperMix, a Chinese SYBR Green real-time PCR premix, was utilized. The expression levels of HCMV-miR-UL148D, HCMV-miR-UL36-3P, and the control gene (U6) were evaluated using primers designed for this study.

Statistical analysis: Statistical analysis was performed using GraphPad Prism version 9.0 (GraphPad Software, USA) and SPSS software version 16.0 (IBM Corp., Armonk, NY, USA). A p-value < 0.05 was considered statistically significant¹⁰.

Results:

The expression of the HCMV-miR-UL148D gene in the study group

The expression of HCMV-miR-UL148D was **2.66**-fold in the miscarriage group compared to 0.96-fold in the control group. The result revealed that the mean $\pm$ SE (threshold cycle (Ct)) of

HCMV- miR-UL148D gene expression in the miscarriage group was 27.38 ± 2.5 , which showed lower Ct values compared to the control group (27.67 ± 3.6), with a non-significant difference in the p-value of 0.624. The results also revealed that the $2^{-\Delta\Delta CT}$ for the miscarriage group was 2.76 ± 0.44 compared to the control group's 1.172 ± 0.01 , with a significant difference in p-value of 0.041, as shown in **Table 2**.

The expression of the HCMV-miR-UL36-3P gene in the study group

The expression of HCMV-miR-UL36-3P in the miscarriage group showed a **21.16**-fold increase, compared to 0.78-fold in the control group. The result revealed that the mean $\pm$ SE (threshold cycle (Ct)) of HCMV-miR-UL36-3P gene expression in the miscarriage women group was 34.152 ± 4.3 , which showed lower Ct values compared to the control group (38.02 ± 4.1), with a non-significant difference in p-value of 0.12. The results also revealed that the $2^{-\Delta\Delta CT}$ for the miscarriage women group was 19.58 ± 3.7 , compared to the control group's 1.179 ± 0.03 , with a highly significant difference in p-value of < 0.001 , as shown in **Table 3**.

Specificity and sensitivity for HCMV-miR-UL148D and HCMV-miR-UL36-3P

The receiver operating characteristic (ROC) revealed that the HCMV-microRNAs were potentially significant diagnostic markers in the infected group. For the HCMV-miR-UL148D, the sensitivity was 96%, the specificity was 98%, the confidence interval was 0.92 to 1.00, the area under the curve (AUC) $\pm$ SE was 0.94 ± 0.04 , and the cutoff value was 2.0, with a highly significant difference in p-value of < 0.001 . While for the HCMV-miR-UL36-3P, the sensitivity was 91%, the specificity was 87%, the confidence interval was 0.83 to 0.97, the area was 0.94 ± 0.10 , and the cutoff value was 1.3, with a highly significant difference in p-value of < 0.001 , as shown in **Figure 1**; probability values are presented in **Table 4**.

Correlation coefficient (Pearson r correlation matrix) between HCMV antibodies, HCMV viral load, and HCMV-miR-UL148D and HCMV-miR-UL36-3P

The Pearson r correlation matrix showed positive and negative relationships between HCMV IgM, IgG, viral load, and HCMV-miR-UL148D and HCMV-miR-UL36-3P. Group 1: The blue color indicated $r > 0$ (positive correlation), and the red color indicated $r < 0$ (negative correlation),

with a positive correlation (directly proportional). There was a positive correlation between HCMV-miR-UL148D and HCMV-miR-UL36-3P and HCMV viral load (0.47, 0.12), respectively, and p-values (< 0.001 , 0.407), while there was a negative correlation with HCMV IgG and HCMV IgM (-0.03, -0.16), respectively; p-values (0.831, 0.254) were not statistically significant. There was a positive correlation between HCMV-miR-UL36-3P and HCMV-miR-UL148D and HCMV viral load (0.47, 0.07, 0.01), respectively, with p-values (0.001, 0.611, 0.941), while there was a negative correlation with HCMV IgM (-0.25), with a p-value = 0.07, as shown in **Figure 2**. The probability values are presented in **Table 5**.

4. Discussion

In the current study, the expression of HCMV-miR-UL148D showed a 2.66-fold change, and HCMV-miR-UL36-3P showed a 21.16-fold change in the miscarriage group. This suggests that the gene is upregulated in women with HCMV-associated miscarriage, potentially indicating its involvement in the pathogenesis of HCMV infection during pregnancy. Some of the miR-UL148D cellular targets are RANTES (CCL5, which was experimentally confirmed during HCMV lytic infection)¹¹. Previously, it was shown that miR-UL148D is upregulated in chronic hepatitis B¹² and oral lichen planus¹³.

HCMV-miR-UL148D has been reported to interfere with host apoptotic signaling pathways during infection. Previous studies demonstrated that viral miRNAs contribute to the suppression of pro-apoptotic responses and the modulation of endoplasmic reticulum stress-related pathways, thereby promoting viral persistence and immune evasion^{14,15}. This miR-UL148D contributes to immune evasion via targeting the chemokine (C-C Motif) ligand 5 (CCL5) gene and the 3'UTR of RANTES (Regulated on Activation, Normal T Expressed and Secreted) during viral infection¹¹. Furthermore, HCMV-miR-UL148D downregulates the secretion of IL-6 as a response to activin A in latently infected monocytes, which explains its involvement in the replication, infection, and ability to evade the immune system¹⁶. RANTES is a pro-inflammatory chemokine involved in recruiting T cells, basophils, and eosinophils to the site of infection. HCMV virus with mutated miR-UL-148D cannot cause a decrease in RANTES level¹⁷. In the studies by Pan *et al.*^{18,16}, they showed that in infected monocytes, a reduction in IL-6 is related to HCMV-miR-UL-148D through the targeting of the activin A receptor (ACVR1B).¹⁹ Numerous studies have demonstrated the significance of microRNAs in

cytomegalovirus²⁰ and found that HCMV-miR-UL148D, with a fold change of 1.73, could serve as an indicator of adverse pregnancy outcomes (APO) in pregnant women who have contracted HCMV. This result is similar to the results in our study, which showed an increase in gene expression. A study was conducted by W. Zhou *et al.*²¹ on the different expression patterns of microRNAs encoded by human cytomegalovirus in circulation from virus latency to reactivation. Their results showed an increase in gene expression in those infected with cytomegalovirus, which was a 2.87-fold change, and this result is similar to our findings in terms of increased gene expression. Conversely, miR-UL148D showed markedly elevated expression and was inversely correlated with the levels of RANTES. Interestingly, miR-UL148D-1 can inhibit the expression of RANTES during HCMV infection, thereby leading to the marked inhibition of RANTES release from HCMV-infected fibroblast cells and modulating the host immune system¹¹.

In the current study, it was shown that the mean level of HCMV-miR-UL36-3P expression in the miscarriage women group was upregulated. This suggests that the gene is upregulated in women with miscarriage, potentially indicating its involvement in the pathogenesis of HCMV infection during pregnancy. Numerous studies have demonstrated the significance of microRNAs in cytomegalovirus. A study by Z. Gao *et al.*²⁰ found that HCMV-miR-UL36-3P, with a fold change of 1.59, could serve as an indicator of adverse pregnancy outcomes (APO) in pregnant women who have contracted HCMV. Another study indicated that the microRNA-145 gene has a function in the combination of cisplatin and C. fistula extract, which dramatically causes apoptosis²². This result is similar to our findings, as we showed an increase in gene expression. Another study conducted by Khikani *et al.* revealed that the expression of HCMV-miR-UL36-3P in the miscarriage group was 3.11-fold higher than in the control group^{23,9}; this result is similar to our findings. According to a report by Y. Huang *et al.*,²⁴ the prevalence of viral DNA copies post-HCMV infection was measured by real-time PCR at varying time points. The copy numbers of viral DNA in cells transfected with pSilencer-miR-UL36 at 24 HPI were more than 3-fold higher than those in cells transfected with a highly significant difference. CDK6 and FAS are proven targets for miR-UL36-3P, and in this way, miR-UL36-3P can inhibit apoptosis in target cells²⁵. Furthermore, one study demonstrated that miR-UL36-3P has a role in promoting efficient lytic replication by downregulating the expression of the latency-associated protein UL138, and the ectopic expression of miR-UL36 enhanced HCMV DNA synthesis

during the early stages of replication in fibroblasts ²⁶. This result is similar to the findings in our study, which showed an increase in gene expression.

Conclusion: HCMV-miR-UL148D and HCMV-miR-UL36-3P expression levels were significantly higher in women who miscarried due to cytomegalovirus infection than in healthy women. This shows that these microRNAs might play an important role in the pathophysiology of HCMV-related reproductive problems. Further studies are needed to clarify their mechanisms and potential usefulness as biomarkers for predicting miscarriage risk in the setting of HCMV infection.

Study limitations: The present study was conducted using a moderate sample size from a single geographic region. Future multicenter studies with larger sample sizes may further strengthen and generalize the current findings.

Acknowledgements: The authors sincerely acknowledge the laboratory staff of Babylon Maternity and Children's Teaching Hospital and Al-Hashimiya General Hospital for their valuable assistance in sample collection.

Funding Sources: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

Author Contributions: Blind

Ethical Approval: Written informed consent was obtained from all the participants prior to sample collection. The study was approved by the Research Committee of the Babylon Health Directorate, Ministry of Health, Iraq (Approval No. 0139/2022/Babil. Date: 17 January 2023).

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Table 1 The primer sequence of HCMV-miR-UL148D and HCMV-miR-UL36-3P

Target	Sequence	Primer sequence 5' - 3'	Tm(°C)	GC%
UL148D	Stem loop	GTCGTATCCAGTGCAGGGTCCG AG GTATTCGCACTGGATACGACCG	78.3	58
		GTGA		
	F	CACGCATCGTCCTCCCCTT GTCGTATCCAGTGCAGGGTCCG	66	63
		AG		
UL36-3P	Stem loop	GTATTCGCACTGGATACGACGC ACGT	77.5	58
		AG		
	F	CGCGCGTTTCCAGGTGTTT	63.3	58
U6	F	CACTAGGCGCTCACTGTTCTC	62	57
	R	AATCCGTTGACTCCGACCTT	61.4	50
Universal	R	CCAGTGCAGGGTCCGAGGTA	68.2	65
F: Forward, R: Reverse				
U6: Small nuclear RNA used as an internal control				
* Primer design by this study				

Table 2 The expression level of HCMV-miR-UL148D compared between the miscarriage group and the control group

Groups	Mean $\pm$ SE					Fold change
	U6 CT	CT	Δ CT	$\Delta\Delta$ CT	$2^{-\Delta\Delta$ CT}	
Control	20.8 $\pm$ 3.7	27.67 $\pm$ 3.6	5.67 $\pm$ 2.1	-0.033 $\pm$ 0	1.172 $\pm$ 0.01	0.96
Patient	20.28 $\pm$ 2.1	27.38 $\pm$ 2.5	5.796 $\pm$ 1.7	-0.314 $\pm$ 0.01	2.76 $\pm$ 0.44	2.66
p-value	0.891 NS	0.624 NS	0.841 NS	0.441 NS	0.041	< 0.001**

NS: Non-significant; **: Significant p-value < 0.01.

Ct: threshold cycle; Δ Ct: difference between target gene Ct and U6 Ct; $\Delta\Delta$ Ct: difference between patient and control Δ Ct values; fold change calculated using $2^{-\Delta\Delta$ Ct method.

Table 3 The expression level of HCMV-miR-UL36-3P compared between the miscarriage group and the control group

Groups	Mean $\pm$ SE					Fold change
	U6 CT	CT	Δ CT	$\Delta\Delta$ CT	$2^{-\Delta\Delta$ CT}	
Control	20.8 $\pm$ 2.7	38.02 $\pm$ 4.1	15.72 $\pm$ 2.9	0.0199 $\pm$ 0	1.179 $\pm$ 0.03	0.78
Patient	20.28 $\pm$ 2.1	34.152 $\pm$ 4.3	13.768 $\pm$ 2.3	-2.73 $\pm$ 0.2	19.58 $\pm$ 3.7	21.16
p-value	0.82 NS	0.12 NS	0.66 NS	0.001*	< 0.001**	< 0.001**

NS: Non-significant; *: Significant p-value < 0.05; **: significant p-value < 0.01

Ct: threshold cycle; Δ Ct: difference between target gene Ct and U6 Ct; $\Delta\Delta$ Ct: difference between patient and control Δ Ct values; fold change calculated using $2^{-\Delta\Delta$ Ct method.

Table 4 Miscarriage group's ROC curve analysis given by sensitivity, specificity, 95% confidence interval, area (Mean $\pm$ SE), and probability

Analyte	Sensitivity (%)	Specificity (%)	95% CI	AUC $\pm$ SE	Cut-off value	p-value
HCMV-miR-UL148D	96	98	0.92-1.00	0.94 $\pm$ 0.04	2.0	< 0.001
HCMV-miR-UL36-3P	91	87	0.83-0.97	0.94 $\pm$ 0.10	1.3	< 0.001

Table 5 P-values of the Pearson correlation matrix between the antibody CMV and viral load, HCMV-miR-UL148D, and HCMV-miR-UL36-3P

	miR-UL36-3P	miR-UL148D	HCMV viral load	HCMV IgM	HCMV IgG
miR-UL36-3P	1.00	< 0.001**	0.611	0.07	0.941
miR-UL148D		1.00	0.407	0.254	0.831
HCMV viral load			1.00	0.489	0.332
HCMV IgM				1.00	0.054
HCMV IgG					1.00

Non-Ssignificant p-value > 0.05; **: Significant p-value < 0.01

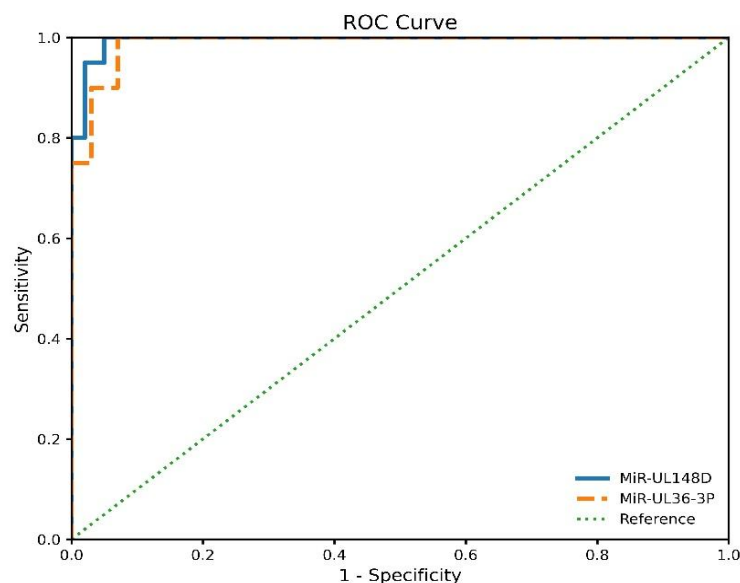


Figure 1 Receiver operating characteristic curve analysis (Wilson/Brown method) Sensitivity% and 1-Specificity %, for HCMV-miR-UL148D and HCMV-miR-UL36-3P.

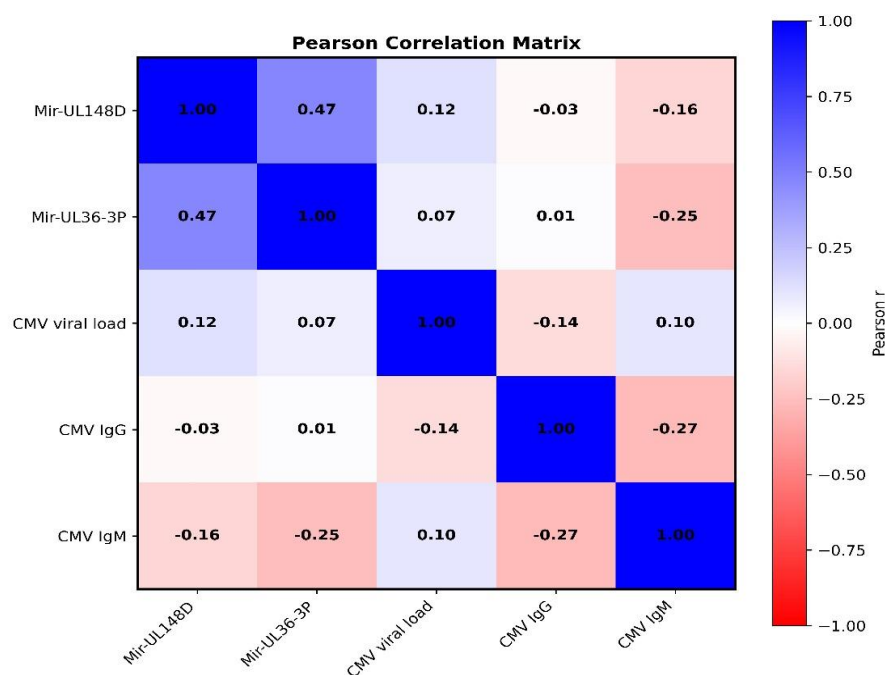


Figure 2 Correlation coefficient (Pearson r correlation matrix of group 1, blue color $r > 0$, positive correlation, red color $r < 0$, negative) between the HCMV antibody and HCMV viral load, and HCMV-miR-UL148D and HCMV-miR-UL36-3P