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SERUM IL-35, PENTRAXIN-3, GALECTIN-3 AND HMGB1 IN RECURRENT PREGNANCY LOSS STRATIFIED BY CYTOMEGALOVIRUS SEROSTATUS: A CASE–CONTROL STUDY OF IRAQI WOMEN

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ABSTRACT

Background: *Recurrent pregnancy loss (RPL) affects roughsly 1–5% of couples, and in about half of cases the cause is never identified. Dysregulated maternal–fetal immune tolerance and chronic low-grade inflammation — to which latent infections such as cytomegalovirus (CMV) may contribute — are increasingly implicated. We compared four immuno-inflammatory biomarkers across RPL stratified by CMV serostatus.*

Methods: *In this case–control study, 210 women of reproductive age were recruited in Tikrit, Iraq (October 2023–September 2024) into three equal groups: parous controls without RPL, RPL with negative CMV serology (RPL CMV−), and RPL with positive CMV serology (RPL CMV+). Serum interleukin-35 (IL-35), pentraxin-3 (PTX-3), galectin-3 and high-mobility-group-box-1 (HMGB1), together with anti-CMV IgG/IgM and a routine laboratory panel, were measured by ELISA and automated analysers. Data were analysed with group comparisons, trend tests, correlation, receiver-operating-characteristic (ROC) analysis and Firth penalized logistic regression.*

Results: *IL-35 and galectin-3 decreased, whereas PTX-3 and HMGB1 increased, in a stepwise manner across control → RPL CMV− → RPL CMV+ (all $P < 0.001$, with significant monotonic trends). C-reactive protein and erythrocyte sedimentation rate rose in parallel. HMGB1 and PTX-3 separated RPL from controls with very high apparent accuracy (area under the curve ≈ 1.00), while IL-35 and galectin-3 were fair to good (0.77–0.88). In adjusted models, HMGB1 and PTX-3 were independently associated with CMV-positive RPL and higher galectin-3 with lower odds.*

Conclusions: *A coordinated shift toward a more pro-inflammatory, less tolerogenic serum profile was associated with RPL and was most marked in CMV-seropositive women. These cross-sectional associations are hypothesis-generating and require prospective external validation before any clinical application.*

KEYWORDS: *recurrent pregnancy loss; cytomegalovirus; interleukin-35; pentraxin-3; galectin-3; HMGB1; maternal–fetal immune tolerance; Iraq.*

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INTRODUCTION

Recurrent pregnancy loss (RPL) is one of the more distressing problems in reproductive medicine. The European Society of Human Reproduction and Embryology (ESHRE) defines it as the loss of two or more pregnancies, and current guidance favours starting investigation and individualised care after two losses rather than waiting for three [1]. Depending on the definition used, RPL affects about 1–5% of couples trying to conceive and carries a substantial psychological and economic burden [1]. Despite a standard work-up that covers anatomical, genetic, endocrine and thrombophilic causes, no explanation is found in roughly half of couples, and these unexplained cases are widely thought to involve disturbed immune regulation at the maternal–fetal interface [1].

A successful pregnancy depends on the maternal immune system tolerating a semi-allogeneic conceptus while still defending against infection. This balance is maintained by regulatory T and B cells, tolerogenic cytokines, and a tightly controlled inflammatory milieu in the decidua. When the balance tips toward excessive or poorly resolved inflammation, trophoblast invasion, spiral-artery remodelling and decidualisation can all be compromised, and the pregnancy may fail. Among the environmental factors capable of perturbing this equilibrium, persistent viral infection has attracted particular attention. Human cytomegalovirus (CMV), a ubiquitous betaherpesvirus that establishes lifelong latency, is one of the most prevalent infections worldwide; its seroprevalence is generally higher in low- and middle-income settings and rises with age [2]. In Iraq, CMV seropositivity among women of childbearing age and among women with a bad obstetric history is consistently high, and several local studies have linked CMV infection to miscarriage and other adverse obstetric outcomes [3,4,5,6].

Four serum mediators sit at the intersection of immune tolerance and inflammation and are therefore plausible candidates for distinguishing women with RPL. Interleukin-35 (IL-35) is an anti-inflammatory cytokine produced by regulatory lymphocytes that supports maternal–fetal tolerance; its serum level is high in healthy pregnancy and is reduced in women with recurrent miscarriage [7,8]. Pentraxin-3 (PTX-3) is a long pentraxin of the innate immune system that is released rapidly at sites of inflammation; it rises physiologically during normal pregnancy but is markedly elevated in women with unexplained RPL, in proportion to the number of previous losses [9,10,11]. Galectin-3 is a chimera-type β-galactoside-binding lectin expressed by trophoblast and decidua that helps regulate apoptosis, implantation and local immune tolerance, and its placental expression is reduced in early pregnancy loss [12]; the related galectin-1 is similarly diminished in the circulation of women with recurrent miscarriage [13]. Finally, high-mobility-group-box-1 (HMGB1) is a prototypical damage-associated molecular pattern (alarmin) that, once released, drives sterile inflammation through RAGE and Toll-like-receptor signalling; it is over-expressed at the maternal–fetal interface and elevated in the serum of women with unexplained recurrent spontaneous abortion [14,15,16].

Although each of these markers has been studied individually, they have rarely been measured together in the same cohort, and — to our knowledge — no study has profiled all four simultaneously in women with RPL while explicitly stratifying by CMV serostatus, particularly in an Iraqi population where CMV exposure is common. This leaves an open question: do these tolerance- and inflammation-related markers change in a coordinated way along a gradient of RPL severity, and is that change accentuated when CMV serology is positive? Addressing this gap, the present case–control study set out to (i) compare serum IL-35, PTX-3, galectin-3 and HMGB1 — alongside a routine haematological, biochemical and hormonal panel — among parous controls, CMV-seronegative RPL and CMV-seropositive RPL women in Amarah, Maysan, Iraq; (ii) test whether these markers follow a monotonic trend across the three groups; and (iii) explore their correlations, discriminative performance and independent association with disease. We frame the findings as associations rather than causal or predictive claims.

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MATERIALS AND METHODS

STUDY DESIGN AND SETTING

This was a hospital- and clinic-based case–control study conducted in Tikrit, Iraq. Participants were recruited from the obstetrics and gynaecology departments of Tikrit Teaching Hospital, Maysan, Al-Zuhour Private Hospital, together with several private gynaecology clinics in the city, over an approximately twelve-month period from October 2023 to September 2024. Reporting follows the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) recommendations for case–control studies [17], and the participant flow is summarised in Figure 1.

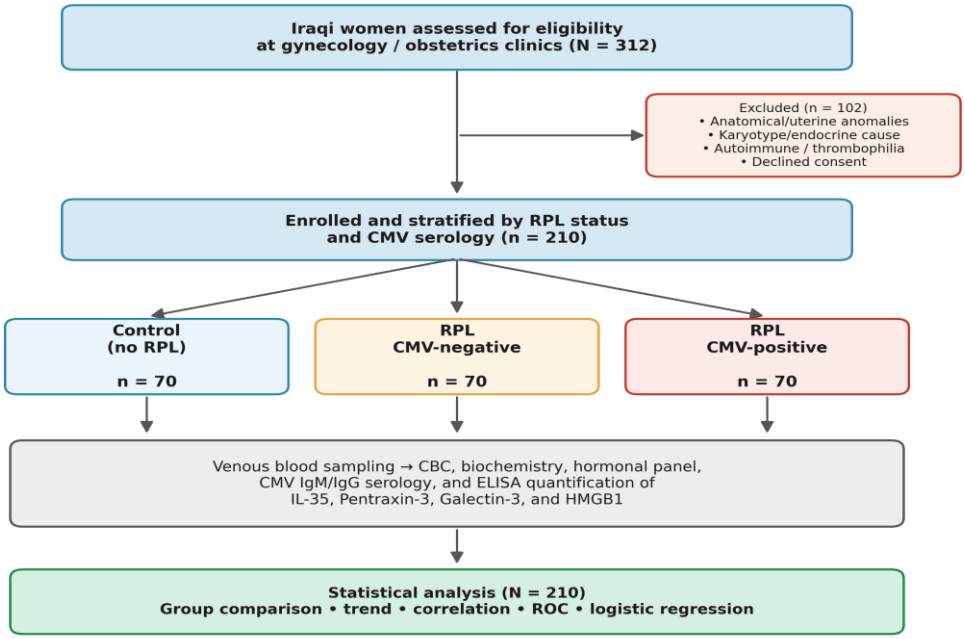

Figure 1: Study flow diagram of participant selection and group allocation, reported according to the STROBE statement. Of 312 women assessed, 102 were excluded for a defined non-immunological cause of pregnancy loss or declined consent, leaving 210 participants allocated to three equal groups (n = 70 each).

PARTICIPANTS, ELIGIBILITY AND GROUPING

Eligible women were of reproductive age (approximately 20–40 years). Cases were women with RPL, defined per the ESHRE 2022/2023 guideline as two or more pregnancy losses before the 20th–24th week of gestation [1]. Controls were parous women with at least one living child and no history of recurrent loss, attending for routine care. To isolate an immunological/inflammatory signal, women were excluded if pregnancy loss could be attributed to a defined non-immunological cause: congenital or acquired uterine/anatomical anomalies, an abnormal parental karyotype, an uncontrolled endocrine disorder (overt thyroid dysfunction, diabetes mellitus or hyperprolactinaemia), a known autoimmune disease or antiphospholipid syndrome, or inherited thrombophilia; women who declined consent were also excluded (Figure 1).

Of 312 women initially assessed, 102 were excluded for one of these reasons or declined to participate, leaving 210 participants. These were allocated to three equal groups of 70: (i) **Control** — parous women without RPL; (ii) **RPL CMV−** — women with RPL and negative CMV serology; and (iii) **RPL CMV+** — women with RPL and positive CMV serology. CMV grouping was based on anti-CMV IgG and/or IgM results (see Section 2.4). The fixed group size of 70 was chosen pragmatically on the basis of patient flow during the recruitment window; with 70 participants per arm the study is well powered to detect the large between-group differences observed for the principal biomarkers, although it is not designed to detect small effects.

CLINICAL DATA AND ANTHROPOMETRY

A structured interviewer-administered questionnaire captured demographic data (age, residence, education, occupation, socioeconomic status, marital duration, consanguinity), a detailed reproductive and obstetric history (gravidity, parity, number and gestational age of miscarriages, previous stillbirth, preterm birth, caesarean section, ectopic pregnancy, menstrual pattern, dysmenorrhoea, vaginal discharge, previous pelvic inflammatory disease, family history of miscarriage and previous CMV infection) and current medication use. Height and weight were measured with participants in light clothing without shoes, and body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). Resting systolic and diastolic blood pressure, pulse rate and axillary body temperature were recorded at enrolment.

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BLOOD SAMPLING AND LABORATORY MEASUREMENTS

After an overnight fast, a single venous blood sample was drawn from each participant at enrolment. An aliquot was collected into EDTA tubes for the complete blood count; the remainder was collected into plain tubes, allowed to clot, and centrifuged to separate serum, which was aliquoted and stored at −20 °C (with longer-term aliquots at −80 °C) until batched analysis, avoiding repeated freeze–thaw cycles.

The complete blood count (haemoglobin, red-cell indices, white-cell differential, platelet count and mean platelet volume) was performed on an automated haematology analyser (Sysmex XN-series; Sysmex Corporation, Kobe, Japan). The erythrocyte sedimentation rate (ESR) was measured by the Westergren method. Routine biochemistry — fasting blood glucose, urea, creatinine, aspartate and alanine aminotransferases, albumin and total protein — was measured on an automated clinical-chemistry analyser (Roche Cobas c311; Roche Diagnostics, Mannheim, Germany), and C-reactive protein (CRP) was quantified on the same platform by a latex-enhanced immunoturbidimetric assay. Hormonal parameters (thyroid-stimulating hormone, free thyroxine, prolactin, follicle-stimulating hormone, luteinizing hormone, estradiol and progesterone) were measured by electrochemiluminescence immunoassay on a Roche Cobas e411 analyser; reproductive hormones were sampled in the early follicular phase where the menstrual history allowed.

Anti-CMV IgG and IgM antibodies were determined by commercial enzyme-linked immunosorbent assay (ELISA) kits (Sunlong Biotech, Hangzhou, China) according to the manufacturer's instructions, with results interpreted as positive or negative against the kit cut-off. Serum IL-35, PTX-3, galectin-3 and HMGB1 were quantified by commercial quantitative sandwich ELISA kits (Sunlong Biotech, Hangzhou, China). In brief, standards and samples were incubated in antibody-coated microwells, followed by enzyme conjugate and substrate; the reaction was stopped and absorbance read at 450 nm on a microplate reader (BioTek ELx800; BioTek Instruments, Winooski, VT, USA). Analyte concentrations were derived from each plate's standard curve. All assays were run in accordance with the kit protocols, with internal controls on every plate; according to the manufacturer the intra- and inter-assay coefficients of variation were below 10% and 12%, respectively. IL-35 is reported in pg/mL and PTX-3, galectin-3 and HMGB1 in ng/mL.

STATISTICAL ANALYSIS

Analyses were performed with SPSS (v26; IBM Corp., Armonk, NY, USA) and R/Python for trend, penalized-regression and ROC procedures. The distribution of each continuous variable was assessed with the Shapiro–Wilk test and homogeneity of variance with Levene's test. Normally distributed variables are presented as mean ± standard deviation and compared across the three groups by one-way analysis of variance (ANOVA), with Welch's ANOVA where variances were unequal and post-hoc Tukey HSD or Games–Howell tests. Non-normal variables are presented as median [interquartile range] and compared by the Kruskal–Wallis H test with Dunn–Bonferroni post-hoc comparisons. Categorical variables are summarised as n (%) and compared by the Pearson χ^2 test, or the Fisher–Freeman–Halton exact test when any expected cell count was below five.

Ordered (monotonic) trends across the three groups, arranged by hypothesised severity (Control → RPL CMV− → RPL CMV+), were tested with the Jonckheere–Terpstra test for continuous variables and the Cochran–Armitage test for binary outcomes. Effect sizes are reported as eta-squared (η^2) for ANOVA, epsilon-squared (ε^2) for Kruskal–Wallis, Cohen's d for pairwise mean differences and Cramér's V for categorical associations. To control the false-discovery rate arising from multiple testing, P values were adjusted within predefined variable families (baseline, reproductive-history and laboratory panels) using the Benjamini–Hochberg procedure [18]; both raw and adjusted P values are reported.

The discriminative performance of each biomarker was assessed by receiver-operating-characteristic (ROC) analysis, with the area under the curve (AUC) and its 95% confidence interval estimated by the DeLong method and correlated AUCs compared by the DeLong test [19]; optimal cut-offs were chosen to maximise the Youden index. Because HMGB1 and PTX-3 produced near-complete separation between groups, crude and adjusted odds ratios were estimated by Firth's penalized-likelihood logistic regression, which reduces the small-sample and separation bias of ordinary maximum likelihood [20]. Adjusted models included the four biomarkers together with age, BMI, consanguinity and low socioeconomic status. All tests were two-sided and $P < 0.05$ was considered statistically significant.

ETHICAL CONSIDERATIONS

The study was approved by the research ethics committee of Department of Biology, College of Science, University of Misan, Amarah, Maysan, Iraq. All procedures were carried out in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from every participant before enrolment, and data were anonymised prior to analysis.

RESULTS

A total of 210 women were analysed in three equal groups of 70. The participant flow and group allocation are shown in Figure 1.

BASELINE CHARACTERISTICS

Baseline demographic and general clinical characteristics are presented in Table 1. The three groups were broadly comparable. Mean age differed modestly across groups (29.2 ± 4.4, 30.5 ± 4.4 and 31.4 ± 4.5 years in the Control, RPL CMV− and RPL CMV+ groups; ANOVA $P = 0.016$, $\eta^2 = 0.039$), but this difference did not survive false-discovery-rate correction ($P\_FDR = 0.147$), and the effect size was small. Height, weight, BMI, blood pressure, pulse and body

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temperature were similar across groups (all P > 0.39). Residence, educational level, occupation, socioeconomic status and consanguinity did not differ significantly after correction (all P_FDR > 0.13). Overall, the groups were well matched on the principal demographic and anthropometric confounders, which strengthens the interpretation of the biomarker comparisons that follow.

Table 1: Baseline demographic and general clinical characteristics of the study groups

Characteristic	Control (n = 70)	RPL CMV− (n = 70)	RPL CMV+ (n = 70)	Test	Statistic	P value	P (FDR)	Effect size
Age, years	29.24 ± 4.37 a	30.54 ± 4.37 ab	31.39 ± 4.49 b	ANOVA	4.20	0.016	0.147	η^2=0.039
Marital duration, years	8.00 [5.00–11.00]	10.00 [7.00–12.00]	8.00 [5.00–11.00]	Kruskal-Wallis	1.57	0.455	0.906	ε^2=0.003
Height, cm	159.85 ± 5.82	159.60 ± 5.89	159.42 ± 5.49	ANOVA	0.10	0.906	0.906	η^2=0.001
Weight, kg	68.95 [63.05–77.15]	69.90 [63.42–76.05]	68.10 [60.02–75.20]	Kruskal-Wallis	1.05	0.591	0.906	ε^2=0.000
BMI, kg/m²	27.35 ± 3.41	27.54 ± 3.66	27.06 ± 3.70	ANOVA	0.33	0.723	0.906	η^2=0.003
Systolic BP, mmHg	114.99 ± 10.79	116.41 ± 10.30	114.97 ± 11.65	ANOVA	0.40	0.669	0.906	η^2=0.004
Diastolic BP, mmHg	75.00 [71.00–79.75]	74.00 [70.00–79.00]	75.00 [71.00–79.00]	Kruskal-Wallis	0.39	0.823	0.906	ε^2=-0.003
Pulse rate, bpm	79.00 [72.00–84.00]	79.50 [70.00–84.75]	76.50 [70.25–83.00]	Kruskal-Wallis	0.85	0.652	0.906	ε^2=-0.001
Body temperature, °C	36.76 ± 0.32	36.69 ± 0.29	36.72 ± 0.28	ANOVA	0.94	0.394	0.906	η^2=0.009
Residence, n (%)				χ^2	7.25	0.027	0.133	0.19
Rural	24 (34.3)	12 (17.1)	25 (35.7)					
Urban	46 (65.7)	58 (82.9)	45 (64.3)					
Educational level, n (%)				χ^2	9.43	0.151	0.252	0.15
Diploma	12 (17.1)	14 (20.0)	11 (15.7)					
Illiterate/Primary	20 (28.6)	12 (17.1)	24 (34.3)					
Secondary	20 (28.6)	22 (31.4)	25 (35.7)					
University/Postgraduate	18 (25.7)	22 (31.4)	10 (14.3)					
Occupation, n (%)				Fisher	6.73	0.346	0.433	0.13
Employed	7 (10.0)	15 (21.4)	9 (12.9)					
Housewife	55 (78.6)	43 (61.4)	47 (67.1)					
Self-employed	5 (7.1)	8 (11.4)	10 (14.3)					
Student	3 (4.3)	4 (5.7)	4 (5.7)					
Socioeconomic status, n (%)				χ^2	9.23	0.056	0.139	0.15
High	12 (17.1)	13 (18.6)	21 (30.0)					
Low	29 (41.4)	17 (24.3)	19 (27.1)					
Middle	29 (41.4)	40 (57.1)	30 (42.9)					
Consanguinity (yes), n (%)	25 (35.7)	20 (28.6)	21 (30.0)	χ^2	0.93	0.629	0.629	0.07

Data are mean ± SD for continuous variables and n (%) for categorical variables. Continuous variables were compared by one-way ANOVA (normally distributed) or the Kruskal–Wallis H test (non-normal); categorical variables by the Pearson χ^2 test or Fisher–Freeman–Halton exact test when ≥1 expected cell <5. Superscript letters (a, b, c) denote post-hoc subsets: groups not sharing a letter differ significantly (Tukey HSD / Games–Howell for ANOVA, Dunn–Bonferroni for Kruskal–Wallis; P<0.05). Effect size: η^2 (eta-squared) for ANOVA, ε^2 (epsilon-squared) for Kruskal–Wallis, Cramérs V for categorical variables. P (FDR) = P value after Benjamini–Hochberg false-discovery-rate correction within the family of baseline variables. RPL, recurrent pregnancy loss; CMV, cytomegalovirus; BMI, body mass index; BP, blood pressure.

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REPRODUCTIVE AND OBSTETRIC HISTORY

Reproductive and obstetric variables are summarised in Table 2. As expected from the case definition, gravidity rose and parity fell across the gradient, while the number of miscarriages increased sharply from a median of 0 in controls to 3 and 4 in the RPL CMV− and RPL CMV+ groups, respectively (Kruskal–Wallis $P < 0.001$; $\varepsilon^2 = 0.72$; significant ordered trend). Among the two RPL groups, the gestational age at the most recent miscarriage was lower in CMV-seropositive women (median 7.95 vs 9.35 weeks; Mann–Whitney $P < 0.001$), indicating earlier losses in this subgroup. Previous stillbirth and preterm birth, menstrual irregularity, dysmenorrhoea and a family history of miscarriage were each more frequent in the RPL groups (all P_FDR ≤ 0.046), whereas previous caesarean section, ectopic pregnancy, abnormal vaginal discharge and pelvic inflammatory disease did not differ significantly. By design, self-reported previous CMV infection tracked closely with serological grouping. These differences reflect the clinical phenotype of RPL rather than independent risk associations and should be read descriptively.

Table 2: Reproductive and obstetric history of the study groups

Variable	Control (n = 70)	RPL CMV− (n = 70)	RPL CMV+ (n = 70)	Test	Statistic	P value	P (FDR)	Effect size	Trend P
Gravidity	3.00 [2.00–3.00] a	4.00 [4.00–5.75] b	5.00 [4.00–6.75] c	Kruskal-Wallis	93.65	<0.001	<0.001	ε^2=0.443	<0.001
Parity	2.00 [1.00–2.00] b	1.00 [0.00–1.00] a	0.00 [0.00–1.00] a	Kruskal-Wallis	74.07	<0.001	<0.001	ε^2=0.350	<0.001
Number of miscarriages	0.00 [0.00–0.00] b	3.00 [3.00–4.00] a	4.00 [3.00–5.00] a	Kruskal-Wallis	152.28	<0.001	<0.001	ε^2=0.724	<0.001
Menstrual cycle length, days	29.00 [27.25–31.00]	28.00 [26.00–31.00]	28.00 [26.00–31.00]	Kruskal-Wallis	3.96	0.138	0.138	ε^2=0.014	0.175
Gestational age at miscarriage, weeks †	N/A	9.35 [8.22–11.75]	7.95 [6.65–9.47]	Mann–Whitney U	3323.50	<0.001	—	d=0.68	—
Previous stillbirth (yes), n (%)	0 (0.0)	8 (11.4)	9 (12.9)	χ^2	9.34	0.009	0.021	0.21	—
Previous preterm birth (yes), n (%)	0 (0.0)	8 (11.4)	9 (12.9)	χ^2	9.34	0.009	0.021	0.21	—
Previous cesarean section (yes), n (%)	9 (12.9)	7 (10.0)	10 (14.3)	χ^2	0.61	0.735	0.735	0.05	—
Previous ectopic pregnancy (yes), n (%)	0 (0.0)	4 (5.7)	2 (2.9)	Fisher	4.12	0.128	0.175	0.14	—
Menstrual irregularity (yes), n (%)	11 (15.7)	27 (38.6)	32 (45.7)	χ^2	15.47	<0.001	0.002	0.27	—
Dysmenorrhea (yes), n (%)	26 (37.1)	35 (50.0)	42 (60.0)	χ^2	7.36	0.025	0.046	0.19	—
Abnormal vaginal discharge, n (%)	33 (47.1)	28 (40.0)	33 (47.1)	χ^2	0.96	0.618	0.703	0.07	—
Previous PID (yes), n (%)	7 (10.0)	4 (5.7)	6 (8.6)	χ^2	0.90	0.639	0.703	0.07	—
Family history of miscarriage (yes), n (%)	8 (11.4)	25 (35.7)	23 (32.9)	χ^2	12.61	0.002	0.007	0.25	—
Previous CMV infection (yes), n (%)	39 (55.7)	0 (0.0)	65 (92.9)	χ^2	122.33	<0.001	<0.001	0.76	—
Medication history, n (%)				Fisher	13.60	0.034	0.054	0.18	—
Hormonal	5 (7.1)	11 (15.7)	9 (12.9)						—
None	50 (71.4)	37 (52.9)	32 (45.7)						—
Other	0 (0.0)	4 (5.7)	3 (4.3)						—
Supplements	15 (21.4)	18 (25.7)	26 (37.1)						—

Data are mean ± SD or median [IQR] for continuous variables and n (%) for categorical variables. Test column indicates the omnibus test applied (ANOVA, Kruskal–Wallis H, χ^2, or Fisher–Freeman–Halton). Trend P is the Jonckheere–Terpstra test for an ordered (monotonic) trend across the three groups (Control → RPL CMV− → RPL CMV+); for binary outcomes the Cochran–Armitage trend test was used. Effect size: η^2/ε^2 for continuous, Cramérs V for categorical. † Gestational age at miscarriage is applicable only to the two RPL groups and was compared by the Mann–Whitney U test; effect size is Cohens d. P (FDR), Benjamini–Hochberg-adjusted P within the reproductive-history family. PID, pelvic inflammatory disease.

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HAEMATOLOGICAL, BIOCHEMICAL AND HORMONAL PANEL

Routine laboratory parameters are shown in Table 3. The complete blood count was essentially uniform across groups: haemoglobin, red-cell indices, the white-cell differential, platelet count and mean platelet volume showed no significant differences (all P_FDR > 0.30), arguing against overt anaemia, leukocytosis or a haematological confounder. Renal and hepatic indices and fasting glucose were likewise comparable. In contrast, the two acute-phase markers rose steeply and monotonically across the groups: CRP increased from a median of 3.10 mg/L in controls to 6.95 and 9.35 mg/L in the RPL CMV− and RPL CMV+ groups (Kruskal–Wallis $P < 0.001$; $\varepsilon^2 = 0.52$), and ESR from 12.9 ± 5.2 to 21.8 ± 6.3 and 28.8 ± 8.3 mm/h (Welch ANOVA $P < 0.001$; $\eta^2 = 0.49$), with all pairwise post-hoc comparisons significant. Within the hormonal profile, TSH was modestly higher and progesterone modestly lower in the RPL groups, while estradiol and prolactin were somewhat higher; free T4, FSH and LH did not differ after correction. The CRP and ESR gradient indicates a graded systemic inflammatory state that parallels disease severity and provides context for the biomarker findings below.

Table 3: Hematological, biochemical, and hormonal laboratory parameters of the study groups

Laboratory parameter	Control (n = 70)	RPL CMV− (n = 70)	RPL CMV+ (n = 70)	Test	Statistic	P value	P (FDR)	Effect size
Complete blood count								
Hemoglobin (g/dL)	13.30 [12.43–13.90]	13.10 [12.60–13.70]	13.10 [12.50–13.80]	Kruskal-Wallis	0.66	0.717	0.855	ε^2=-0.002
RBC Count (×10¹²/L)	4.49 [4.25–4.80]	4.67 [4.42–4.95]	4.58 [4.39–4.75]	Kruskal-Wallis	5.11	0.078	0.301	ε^2=0.020
Hematocrit (%)	40.34 ± 2.55	39.90 ± 2.82	39.80 ± 2.76	ANOVA	0.80	0.451	0.725	η^2=0.008
MCV (fL)	85.11 ± 4.09	84.42 ± 3.93	85.05 ± 5.40	Welch ANOVA	0.59	0.553	0.746	η^2=0.005
MCH (pg)	27.15 ± 2.28	27.03 ± 2.19	27.22 ± 2.17	ANOVA	0.14	0.873	0.912	η^2=0.001
MCHC (g/dL)	31.97 ± 1.05	31.91 ± 1.04	32.16 ± 0.96	ANOVA	1.18	0.309	0.639	η^2=0.011
RDW (%)	14.45 [13.70–15.75]	14.55 [13.53–15.30]	14.50 [13.62–15.20]	Kruskal-Wallis	0.04	0.978	0.978	ε^2=-0.005
WBC Count (×10⁹/L)	6.70 [6.10–7.50]	6.95 [6.00–7.70]	7.10 [5.93–7.80]	Kruskal-Wallis	1.36	0.508	0.743	ε^2=0.002
Neutrophils (%)	54.84 ± 6.28	54.50 ± 7.18	53.79 ± 7.16	ANOVA	0.43	0.651	0.808	η^2=0.004
Lymphocytes (%)	31.14 ± 5.74	31.91 ± 6.08	31.96 ± 5.45	ANOVA	0.44	0.643	0.808	η^2=0.004
Monocytes (%)	6.00 [5.00–7.00]	5.00 [5.00–7.00]	6.00 [5.00–7.00]	Kruskal-Wallis	1.52	0.467	0.725	ε^2=0.002
Eosinophils (%)	3.00 [2.00–4.00]	3.00 [3.00–4.00]	3.00 [2.00–4.00]	Kruskal-Wallis	2.17	0.337	0.653	ε^2=0.006
Basophils (%)	0.50 [0.30–0.70]	0.50 [0.40–0.70]	0.45 [0.30–0.60]	Kruskal-Wallis	3.25	0.197	0.546	ε^2=0.011
Platelet Count (×10⁹/L)	255.91 ± 46.64	260.13 ± 49.27	257.51 ± 54.88	ANOVA	0.12	0.883	0.912	η^2=0.001
MPV (fL)	8.68 ± 0.85	8.44 ± 0.79	8.58 ± 0.82	ANOVA	1.47	0.231	0.551	η^2=0.014
Biochemistry & inflammation								
Fasting Blood Glucose (mg/dL)	86.00 [82.25–91.00]	86.00 [80.00–92.00]	85.00 [80.00–90.00]	Kruskal-Wallis	2.61	0.271	0.599	ε^2=0.008
Urea (mg/dL)	30.87 ± 6.14	28.94 ± 6.69	30.06 ± 6.57	ANOVA	1.57	0.211	0.546	η^2=0.015
Creatinine (mg/dL)	0.79 ± 0.13	0.78 ± 0.12	0.78 ± 0.12	ANOVA	0.15	0.859	0.912	η^2=0.001
AST (U/L)	25.73 ± 7.89	26.70 ± 7.35	24.36 ± 7.79	ANOVA	1.64	0.196	0.546	η^2=0.016
ALT (U/L)	20.00 [17.00–23.00]	20.00 [16.00–24.00]	20.00 [16.00–23.00]	Kruskal-Wallis	0.50	0.777	0.893	ε^2=-0.002
Albumin (g/dL)	4.25 [4.03–4.47]	4.30 [4.10–4.57]	4.30 [4.10–4.57]	Kruskal-Wallis	1.28	0.527	0.743	ε^2=0.001

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Total Protein (g/dL)	7.50 [7.12–7.90]	7.40 [7.10–7.70]	7.45 [7.20–7.70]	Kruskal-Wallis	2.03	0.363	0.662	ε^2=0.005
CRP (mg/L)	3.10 [2.30–4.55] a	6.95 [4.65–9.67] b	9.35 [7.00–11.55] c	Kruskal-Wallis	109.47	<0.001	<0.001	ε^2=0.519
ESR (mm/h)	12.90 ± 5.23 a	21.80 ± 6.29 b	28.79 ± 8.30 c	Welch ANOVA	102.74	<0.001	<0.001	η^2=0.487
Hormonal profile								
TSH (mIU/L)	2.16 ± 0.78 b	2.54 ± 0.90 a	2.88 ± 0.96 a	ANOVA	11.49	<0.001	<0.001	η^2=0.100
Free T4 (ng/dL)	1.19 [1.08–1.39]	1.14 [0.96–1.30]	1.14 [1.02–1.35]	Kruskal-Wallis	5.96	0.051	0.225	ε^2=0.024
Prolactin (ng/mL)	13.70 [9.95–17.98] a	16.50 [13.20–20.18] ab	17.05 [12.40–22.15] b	Kruskal-Wallis	9.03	0.011	0.056	ε^2=0.038
FSH (mIU/mL)	5.85 [5.00–7.20]	6.30 [5.20–7.60]	6.45 [5.00–8.00]	Kruskal-Wallis	1.55	0.460	0.725	ε^2=0.003
LH (mIU/mL)	5.45 [3.82–6.78]	5.80 [4.20–7.67]	5.75 [4.43–7.97]	Kruskal-Wallis	4.02	0.134	0.461	ε^2=0.014
Estradiol (pg/mL)	64.75 [48.55–80.35] b	79.00 [58.27–98.70] a	86.55 [57.88–111.38] a	Kruskal-Wallis	14.02	<0.001	0.006	ε^2=0.062
Progesterone (ng/mL)	9.30 [7.72–11.53] b	7.35 [6.22–9.20] a	7.00 [5.90–8.93] a	Kruskal-Wallis	26.57	<0.001	<0.001	ε^2=0.122

Data are mean ± SD (normally distributed) or median [IQR] (non-normal). Normality was assessed by the Shapiro–Wilk test and homogeneity of variance by Levenes test. Normally distributed parameters were compared by one-way ANOVA (post-hoc Tukey HSD; Games–Howell where variances were unequal); non-normal parameters by the Kruskal–Wallis H test (post-hoc Dunn–Bonferroni). Superscript letters (a, b, c): groups not sharing a letter differ significantly (P<0.05). Effect size: η^2/ε^2. P (FDR): Benjamini–Hochberg-adjusted P within the laboratory-parameter family (31 tests). RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red-cell distribution width; WBC, white blood cell; MPV, mean platelet volume; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; TSH, thyroid-stimulating hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

THE FOUR IMMUNO-INFLAMMATORY BIOMARKERS

Serum concentrations of the four target biomarkers are detailed in Table 4 and displayed in Figures 2 and 3. All four differed highly significantly across the three groups (all P < 0.001) and showed a significant monotonic trend. Two markers fell with increasing disease severity: IL-35 declined from 184.8 ± 38.2 pg/mL in controls to 147.3 ± 36.6 and 123.8 ± 33.5 pg/mL in the RPL CMV− and RPL CMV+ groups (η^2 = 0.33), and galectin-3 from 11.8 ± 3.1 to 9.6 ± 2.6 and 8.0 ± 2.0 ng/mL (η^2 = 0.27). Two markers rose: PTX-3 increased from 2.27 ± 0.77 to 8.01 ± 2.53 and 11.60 ± 3.31 ng/mL (η^2 = 0.72), and HMGB1 from 4.47 ± 1.40 to 47.12 ± 11.76 and 72.39 ± 14.49 ng/mL (η^2 = 0.87). Post-hoc testing confirmed that every pairwise contrast was significant for all four markers, including the comparison between the two RPL groups, and the pairwise effect sizes were large (for example, Cohen's d = 1.70 for IL-35 and −6.60 for HMGB1 in the Control-versus-CMV+ contrast). The violin and box plots in Figure 2 illustrate the separation of the distributions, and the mean-with-confidence-interval plot on a logarithmic axis in Figure 3 shows the consistent stepwise gradient for all four markers across Control → RPL CMV− → RPL CMV+.

Table 4: Serum concentrations of the four immuno-inflammatory biomarkers across the study groups, with post-hoc and trend analyses

Biomarker	Control (n = 70)	RPL CMV− (n = 70)	RPL CMV+ (n = 70)	F	P value	η^2	Control vs CMV−	Control vs CMV+	CMV− vs CMV+	Trend P
IL-35, pg/mL	184.78 ± 38.18	147.27 ± 36.58	123.80 ± 33.49	50.73	**<0.001**	0.329	<0.001 (d=1.00)	<0.001 (d=1.70)	<0.001 (d=0.67)	**<0.001**
Pentraxin-3, ng/mL	2.27 ± 0.77	8.01 ± 2.53	11.60 ± 3.31	401.25	**<0.001**	0.715	0.001 (d=-3.07)	0.001 (d=-3.88)	0.001 (d=-1.22)	**<0.001**
Galectin-3, ng/mL	11.77 ± 3.07	9.61 ± 2.56	7.96 ± 1.96	39.16	**<0.001**	0.272	0.001 (d=0.76)	0.001 (d=1.48)	0.001 (d=0.72)	**<0.001**
HMGB1, ng/mL	4.47 ± 1.40	47.12 ± 11.76	72.39 ± 14.49	1194.08	**<0.001**	0.872	0.001 (d=-5.09)	0.001 (d=-6.60)	0.001 (d=-1.91)	**<0.001**

Data are mean ± SD (pg/mL for IL-35; ng/mL for Pentraxin-3, Galectin-3, and HMGB1). All four biomarkers were normally distributed within each group (Shapiro–Wilk P>0.05). The omnibus F is from one-way ANOVA (IL-35) or Welchs ANOVA where variances were unequal (Pentraxin-3, Galectin-3, HMGB1). η^2 = eta-squared effect size (0.01

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small, 0.06 medium, 0.14 large). Pairwise columns give the post-hoc P value (Tukey HSD or Games–Howell) with Cohens d in parentheses. Trend P is the Jonckheere–Terpstra test for a monotonic ordered trend across Control → RPL CMV− → RPL CMV+. IL-35 and Galectin-3 decreased, whereas Pentraxin-3 and HMGB1 increased, across the disease-severity gradient.

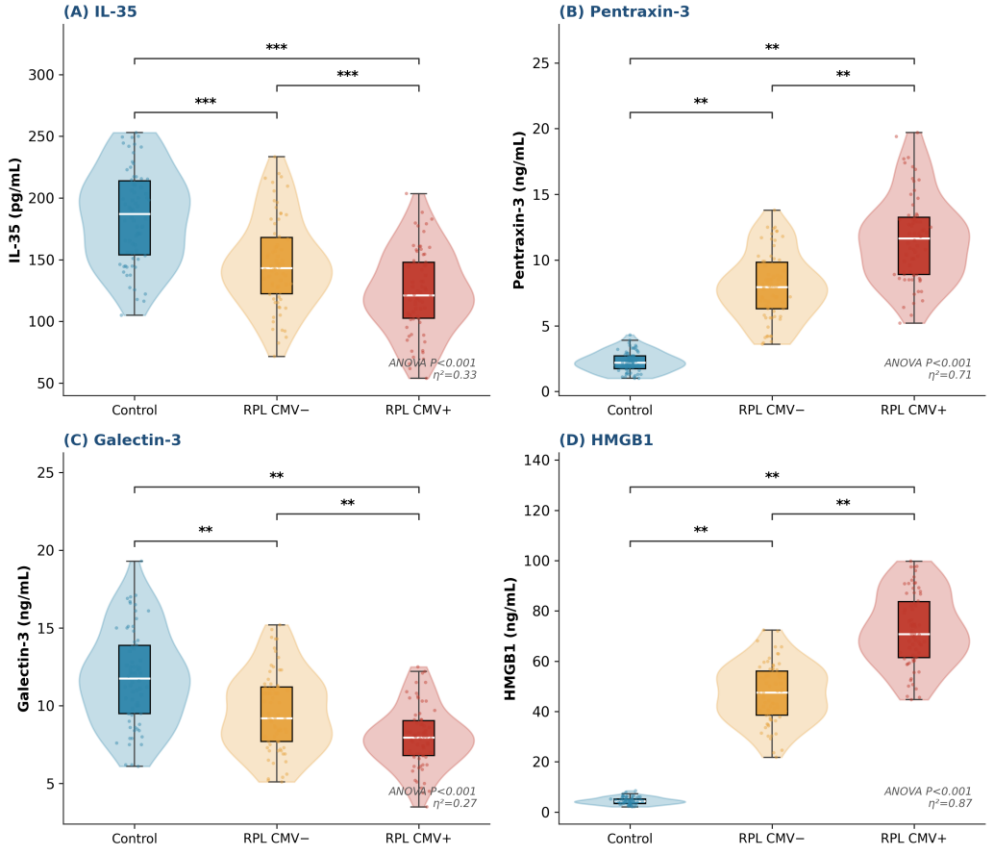

Figure 2: Serum concentrations of the four immuno-inflammatory biomarkers across the three study groups. Violin plots show the full data distribution; the superimposed box plots show the median and interquartile range, and dots are individual participants. Horizontal brackets denote post-hoc pairwise comparisons (Tukey HSD or Games–Howell): *P<0.05, **P<0.01, ***P<0.001. η² is the one-way ANOVA effect size. IL-35 and Galectin-3 decreased, whereas Pentraxin-3 and HMGB1 increased, with disease severity.

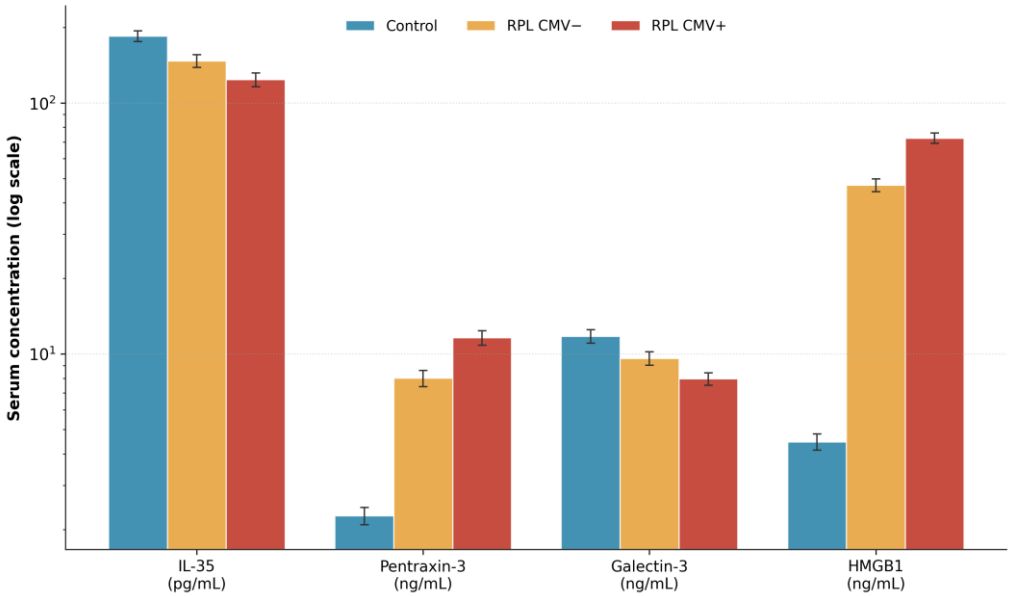

Figure 3: Mean (±95% confidence interval) serum concentrations of the four biomarkers by study group, displayed on a logarithmic y-axis to accommodate the wide dynamic range of HMGB1 and Pentraxin-3. The monotonic gradient across Control → RPL CMV− → RPL CMV+ is evident for all four markers.

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CMV SEROLOGY AND BIOMARKER CONCENTRATIONS BY SEROSTATUS

CMV serological results and biomarker concentrations stratified by IgG serostatus are given in Table 5. Anti-CMV IgG positivity was 55.7% in controls, 0% in the RPL CMV− group (by definition) and 92.9% in the RPL CMV+ group, while IgM positivity was confined to the RPL CMV+ group (15.7%; 11 women) and absent elsewhere (Table 5A). It is worth noting that more than half of the controls were IgG-seropositive, reflecting the high background CMV exposure typical of the region; the groups therefore contrast RPL status and the CMV-defined subgroup rather than the simple presence or absence of past CMV exposure. When the whole sample (N = 210) was dichotomised by IgG serostatus irrespective of group (Table 5B), IgG-positive women had modestly lower IL-35 and galectin-3 and modestly higher PTX-3 and HMGB1 than IgG-negative women (all $P < 0.05$), consistent in direction with the group-level findings, though the differences were small at the level of the whole sample.

Table 5: CMV serological status across groups and serum biomarker concentrations stratified by CMV-IgG status

(A) CMV serology by study group

Serological marker	Control (n = 70)	RPL CMV− (n = 70)	RPL CMV+ (n = 70)	Test	Statistic	P value
CMV IgG positive, n (%)	39 (55.7)	0 (0.0)	65 (92.9)	χ^2	122.33	**<0.001**
CMV IgM positive, n (%)	0 (0.0)	0 (0.0)	11 (15.7)	Fisher–Freeman–Halton	23.22	**<0.001**

(B) Biomarker concentrations by CMV-IgG serostatus (whole sample, N = 210)

Biomarker	CMV-IgG positive	CMV-IgG negative	Test	Statistic	P value
IL-35, pg/mL	145.00 ± 44.00	158.77 ± 42.92	t	-2.30	**0.023**
Pentraxin-3, ng/mL	8.17 ± 5.33	6.43 ± 3.47	U	6533.00	**0.020**
Galectin-3, ng/mL	9.44 ± 3.15	10.11 ± 2.81	U	4585.50	**0.035**
HMGB1, ng/mL	47.30 ± 35.36	35.47 ± 22.47	U	6972.50	**<0.001**

Panel A: data are n (%) of seropositive women; groups compared by the Pearson χ^2 or Fisher–Freeman–Halton exact test. By design, CMV-IgM positivity was confined to a subset of the RPL CMV+ group, mandating the exact test. Panel B: data are mean ± SD; CMV-IgG-positive versus -negative women compared by the independent-samples t test (Welch) or Mann–Whitney U test according to normality. CMV, cytomegalovirus; IgG/IgM, immunoglobulin G/M.

CORRELATIONS AMONG BIOMARKERS, INFLAMMATION AND MISCARRIAGE BURDEN

The correlation matrix is presented in Table 6 and visualised in Figure 5. The two tolerogenic markers behaved oppositely to the two pro-inflammatory markers. IL-35 correlated positively with galectin-3 (r = +0.31) and negatively with PTX-3 (r = −0.50), HMGB1 (r = −0.51), CRP (r = −0.35) and the number of miscarriages (r = −0.49). PTX-3 and HMGB1 were strongly positively correlated with each other (r = +0.78) and each correlated positively with CRP (r = +0.63 and +0.65) and with the number of miscarriages (r = +0.72 and +0.74). CMV IgG and IgM showed weak but mostly significant correlations with the pro-inflammatory markers (for example, HMGB1 with IgG r = +0.23 and with IgM r = +0.27). These associations are internally coherent — inflammation and miscarriage burden track together and inversely with the tolerogenic markers — but, being cross-sectional correlations, they cannot establish the direction of any effect.

Table 6: Correlation matrix among the immuno-inflammatory biomarkers, CMV serology, inflammation, and miscarriage burden

Variable	IL-35	PTX-3	Galectin-3	HMGB1	CRP	No. miscarriages	CMV IgG	CMV IgM
IL-35	1	**-0.50*** **	**+0.31*** **	**-0.51*** **	**-0.35*** **	**-0.49*** **	**-0.15***	**-0.17***
PTX-3	**-0.50*** **	1	**-0.45*** **	**+0.78*** **	**+0.63*** **	**+0.72*** **	**+0.16***	**+0.21****
Galectin-3	**+0.31*** **	**-0.45*** **	1	**-0.43*** **	**-0.35*** **	**-0.41*** **	**-0.15***	-0.06
HMGB1	**-0.51*** **	**+0.78*** **	**-0.43*** **	1	**+0.65*** **	**+0.74*** **	**+0.23*** **	**+0.27*** **
CRP	**-0.35*** **	**+0.63*** **	**-0.35*** **	**+0.65*** **	1	**+0.56*** **	+0.07	**+0.20****
No. miscarriages	**-0.49*** **	**+0.72*** **	**-0.41*** **	**+0.74*** **	**+0.56*** **	1	+0.01	**+0.17***
CMV IgG	**-0.15***	**+0.16***	**-0.15***	**+0.23*** **	+0.07	+0.01	1	**+0.24*** **
CMV IgM	**-0.17***	**+0.21****	-0.06	**+0.27*** **	**+0.20****	**+0.17***	**+0.24*** **	1

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Values are correlation coefficients: Pearsons r for pairs in which both variables were normally distributed and Spearmans ρ otherwise. CMV IgG and IgM were coded 1 = positive, 0 = negative (point-biserial/rank). Shaded cells are statistically significant (orange = positive, blue = negative). *P<0.05, **P<0.01, ***P<0.001. PTX-3, pentraxin-3; CRP, C-reactive protein; CMV, cytomegalovirus.

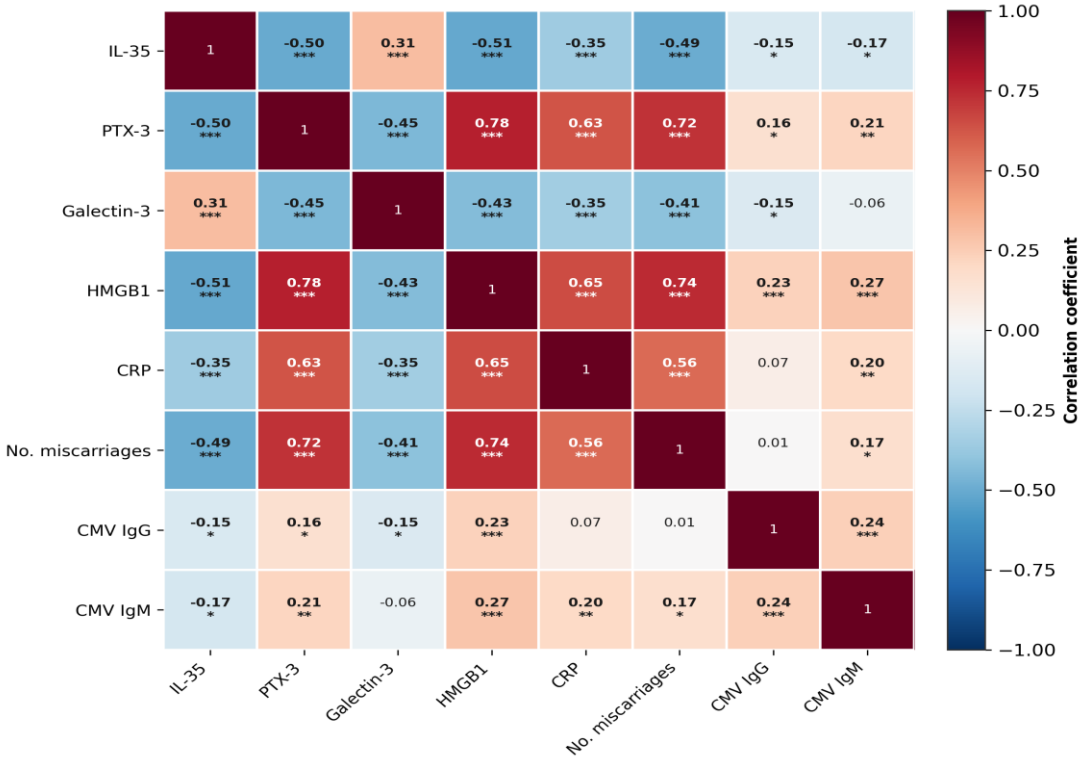

Figure 5: Correlation matrix among the biomarkers, CMV serology, C-reactive protein, and number of miscarriages. Cell values are Pearsons r or Spearmans ρ according to the distribution of each pair; color encodes the coefficient (red positive, blue negative). *P<0.05, **P<0.01, ***P<0.001.

DIAGNOSTIC PERFORMANCE (ROC ANALYSIS)

ROC results for discriminating RPL from controls are reported in Table 7 and plotted in Figure 4. For RPL (any) versus controls (Table 7A), HMGB1 and PTX-3 separated the groups almost completely (AUC = 1.000 and 0.999), whereas IL-35 and galectin-3 were good to fair (AUC = 0.818 and 0.774). The pattern was similar for CMV-positive RPL versus controls (Table 7B; HMGB1 and PTX-3 AUC = 1.000, IL-35 = 0.878, galectin-3 = 0.850). The DeLong test confirmed that HMGB1 and PTX-3 outperformed IL-35 and galectin-3 (all P < 0.001). The near-perfect AUCs for HMGB1 and PTX-3 are striking and indicate very large group separation in this sample; however, such values can also reflect an idealised separation that may not generalise. The data-derived cut-offs reported in Table 7 should therefore be regarded as exploratory and require prospective, external validation before any diagnostic use.

Table 7: Diagnostic performance (ROC analysis) of the four biomarkers for discriminating recurrent pregnancy loss

(A) RPL (any) versus Control

Biomarker	AUC	95% CI	Optimal cut-off	Sensitivity %	Specificity %	PPV %	NPV %	Youden J
IL-35, pg/mL	**0.818**	0.760–0.877	≤162.00	81.4	70.0	84.4	65.3	0.514
Pentraxin-3, ng/mL	**0.999**	0.997–1.000	≥4.10	97.9	98.6	99.3	95.8	0.964
Galectin-3, ng/mL	**0.774**	0.707–0.842	≤9.20	65.0	77.1	85.0	52.4	0.421
HMGB1, ng/mL	**1.000**	1.000–1.000	≥21.80	100.0	100.0	100.0	100.0	1.000

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(B) CMV-positive RPL versus Control

Biomarker	AUC	95% CI	Optimal cut-off	Sensitivity %	Specificity %	PPV %	NPV %	Youden J
IL-35, pg/mL	**0.878**	0.823–0.932	≤161.50	90.0	70.0	75.0	87.5	0.600
Pentraxin-3, ng/mL	**1.000**	1.000–1.000	≥5.20	100.0	100.0	100.0	100.0	1.000
Galectin-3, ng/mL	**0.850**	0.787–0.912	≤9.10	78.6	77.1	77.5	78.3	0.557
HMGB1, ng/mL	**1.000**	1.000–1.000	≥44.70	100.0	100.0	100.0	100.0	1.000

AUC, area under the receiver-operating-characteristic curve, with 95% confidence interval estimated by the DeLong method. The optimal cut-off maximizes the Youden index (J = sensitivity + specificity − 1); the direction (≥ or ≤) reflects whether the biomarker increases or decreases with disease. PPV, positive predictive value; NPV, negative predictive value, computed at the observed group prevalence. Pairwise comparison of correlated AUCs (DeLong test) confirmed that HMGB1 and Pentraxin-3 significantly out-performed IL-35 and Galectin-3 (all P<0.001). Cut-offs are data-derived and require external validation. AUC interpretation: >0.90 excellent, 0.80–0.90 good, 0.70–0.80 fair.

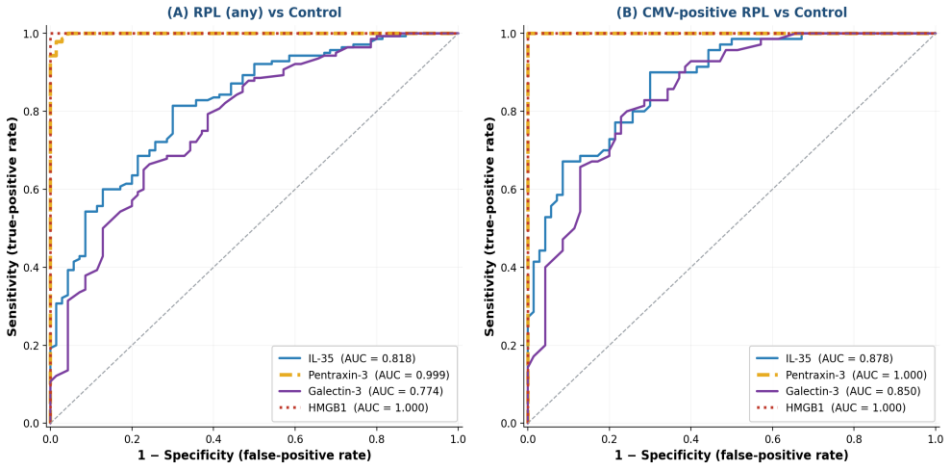

Figure 4: Receiver-operating-characteristic (ROC) curves of the four biomarkers for discriminating (A) recurrent pregnancy loss (any) versus controls and (B) CMV-positive RPL versus controls. Areas under the curve (AUC) are shown in the legend; the diagonal dashed line denotes no discrimination. HMGB1 and Pentraxin-3 achieved near-perfect separation in both contrasts.

INDEPENDENT ASSOCIATIONS (FIRTH PENALIZED REGRESSION)

Crude and adjusted odds ratios from Firth penalized logistic regression are presented in Table 8 and the adjusted CMV-positive model is summarised in the forest plot in Figure 6. In the RPL-versus-Control model (Table 8A), HMGB1 and PTX-3 produced complete separation, so their crude odds ratios are not reliably estimable (reported as > 1000); in the adjusted model HMGB1 remained strongly associated with disease while the other markers were attenuated. The better-behaved CMV-positive model (Table 8B; discrimination AUC = 0.983, Hosmer–Lemeshow P = 0.971) is the primary multivariable analysis: per one-standard-deviation increase, HMGB1 (adjusted OR 76.3, 95% CI 13.4–434.9) and PTX-3 (adjusted OR 7.22, 95% CI 2.26–23.1) were independently associated with higher odds of CMV-positive RPL, whereas higher galectin-3 was associated with lower odds (adjusted OR 0.35, 95% CI 0.15–0.85); IL-35 was not independently significant after adjustment, and age, BMI, consanguinity and low socioeconomic status were not associated with the outcome. The very wide confidence interval for HMGB1 reflects the near-separation in the data and counsels caution in interpreting the point estimate.

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Table 8: Firth penalized logistic regression: crude and adjusted odds ratios for the biomarkers as predictors of disease

(A) Outcome: RPL (any) versus Control

Predictor	Crude OR (95% CI)	P value	Adjusted OR (95% CI)	P value
IL-35 (per 1 SD)	0.24 (0.16–0.37)	<0.001	**0.81 (0.27–2.45)**	**0.709**
Pentraxin-3 (per 1 SD)	>1000 ‡	0.001	**4.42 (0.45–43.11)**	**0.201**
Galectin-3 (per 1 SD)	0.32 (0.22–0.46)	<0.001	**1.00 (0.35–2.87)**	**1.000**
HMGB1 (per 1 SD)	>1000 ‡	<0.001	**77.29 (6.09–981.75)**	**<0.001**
Age (per 1 SD)	—	—	**0.82 (0.26–2.61)**	**0.743**
BMI (per 1 SD)	—	—	**0.69 (0.23–2.02)**	**0.495**
Consanguinity (yes vs no)	—	—	**0.66 (0.07–6.40)**	**0.723**
Low SES (yes vs no)	—	—	**0.40 (0.03–4.86)**	**0.470**

Model fit: discrimination AUC = 1.000; Hosmer–Lemeshow P = 0.958; events = 140; events-per-variable = 8.8.

(B) Outcome: CMV-positive RPL versus all others

Predictor	Crude OR (95% CI)	P value	Adjusted OR (95% CI)	P value
IL-35 (per 1 SD)	0.29 (0.19–0.44)	<0.001	**0.60 (0.28–1.28)**	**0.186**
Pentraxin-3 (per 1 SD)	9.55 (5.15–17.69)	<0.001	**7.22 (2.26–23.05)**	**<0.001**
Galectin-3 (per 1 SD)	0.29 (0.19–0.45)	<0.001	**0.35 (0.15–0.85)**	**0.021**
HMGB1 (per 1 SD)	83.31 (19.82–350.25)	<0.001	**76.30 (13.39–434.87)**	**<0.001**
Age (per 1 SD)	—	—	**1.27 (0.66–2.45)**	**0.475**
BMI (per 1 SD)	—	—	**1.01 (0.58–1.77)**	**0.968**
Consanguinity (yes vs no)	—	—	**0.77 (0.19–3.15)**	**0.715**
Low SES (yes vs no)	—	—	**0.86 (0.25–2.95)**	**0.807**

Model fit: discrimination AUC = 0.983; Hosmer–Lemeshow P = 0.971; events = 70; events-per-variable = 8.8.

Odds ratios were estimated by Firths penalized-likelihood logistic regression, which corrects the small-sample and separation bias arising because HMGB1 and Pentraxin-3 are near-perfect discriminators. Continuous predictors are standardized: OR expresses the change in odds per 1-SD increase. The adjusted model includes all four biomarkers plus age, BMI, consanguinity, and socioeconomic status. With ~70 events per model the events-per-variable ratio (≈8.8) approaches the recommended threshold of 10; Firths method was used specifically to mitigate this. ‡ In the RPL-versus-Control contrast (panel A) HMGB1 and Pentraxin-3 produced complete separation, so the crude OR is not reliably estimable and is reported as ">1000"; the well-behaved CMV-positive contrast (panel B, AUC = 0.983) is the primary multivariable model. OR, odds ratio; CI, confidence interval; SD, standard deviation; BMI, body mass index; SES, socioeconomic status.

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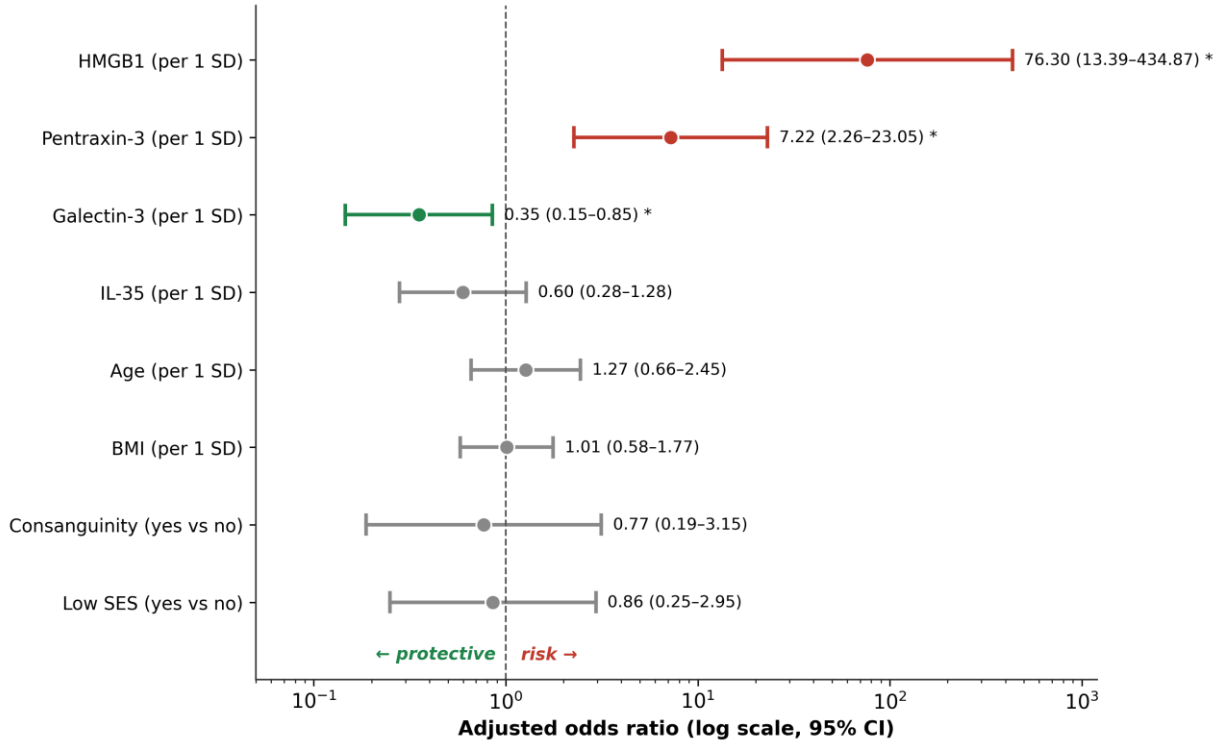

Figure 6: Forest plot of adjusted odds ratios from the Firth penalized multivariable logistic-regression model for CMV-positive recurrent pregnancy loss. For continuous predictors the odds ratio expresses the change in odds per 1-SD increase; bars are 95% confidence intervals on a logarithmic scale, and the dashed line marks the null value (OR = 1). HMGB1 and Pentraxin-3 were independent risk markers, whereas higher Galectin-3 was protective.

DISCUSSION

In this case–control study of 210 Iraqi women, four serum mediators of immune tolerance and inflammation changed in a coordinated and graded fashion across parous controls, CMV-seronegative RPL and CMV-seropositive RPL. The two tolerogenic markers, IL-35 and galectin-3, fell progressively, while the two pro-inflammatory markers, PTX-3 and the alarmin HMGB1, rose progressively; the shift was greatest in the CMV-seropositive subgroup and was mirrored by a parallel rise in CRP and ESR. Taken together, the pattern is best read as a coordinated tilt away from a tolerogenic, anti-inflammatory serum profile toward a pro-inflammatory one in association with RPL, accentuated where CMV serology was positive.

COMPARISON WITH THE BIOMARKER LITERATURE

Our IL-35 findings agree with earlier work showing that this regulatory cytokine is elevated in healthy pregnancy and reduced in recurrent miscarriage [7], and that components of the IL-35 axis are altered in the immunopathology of RPL [8]. The progressive fall in galectin-3 is consistent with reports that galectin-3 expression in placental villi is down-regulated in early pregnancy loss [12] and with the broader observation that tolerogenic galectins, including the closely related galectin-1, are diminished in the circulation of women with recurrent miscarriage [13]. For PTX-3, our results echo the case–control data of Ibrahim and colleagues, who found markedly higher first-trimester serum PTX-3 in women with primary unexplained RPL than in controls and a positive correlation with the number of previous losses [10]; the role of pentraxins as early sensors of innate immune activation at implantation provides a coherent mechanistic backdrop [9], although PTX-3 also rises during uncomplicated pregnancy and must be interpreted against gestational reference values [11]. The steep elevation of HMGB1 fits a growing body of evidence that this alarmin is over-expressed at the maternal–fetal interface and elevated in the serum of women with unexplained recurrent spontaneous abortion, where it drives sterile inflammation and pyroptosis through RAGE/Toll-like-receptor–NF-κB signalling [14,15,16]. The very high discriminative performance of HMGB1 and PTX-3 in our ROC analysis is therefore directionally consistent with prior studies, even if the magnitude of separation in our sample is unusually large and warrants cautious interpretation.

COMPARISON WITH IRAQI AND REGIONAL STUDIES

Our work sits alongside a substantial Iraqi literature on CMV and pregnancy loss. In Erbil, Inaam and colleagues reported a high CMV-IgG seroprevalence among women with recurrent pregnancy loss and linked specific viral genes to miscarriage, underlining the relevance of CMV to RPL in the Iraqi context [3]. In Kirkuk, Aljumaili and co-workers found higher CMV IgG and IgM seroprevalence in women with a bad obstetric history than in women with normal pregnancies, with seropositivity influenced by age, residence and education [4]. A case–control study from Erbil similarly associated CMV serology with abortion and intrauterine death [5]. More mechanistically, Khamees and Al-

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Ouqaili examined chromosomal and immunological factors, including antiphospholipid antibodies and IL-6, in Iraqi couples with RPL, reinforcing the importance of immune dysregulation in this population [6]. Our findings extend this body of work by profiling four tolerance- and inflammation-related serum markers simultaneously and stratifying explicitly by CMV serostatus. One notable difference is that CMV-IgG positivity in our controls (55.7%) was lower than the very high rates (often 90–100%) reported in some Iraqi bad-obstetric-history series [4,5]; this likely reflects differences in the study population (parous controls without RPL versus symptomatic or antenatal cohorts), in the assay platform and cut-off, and in regional exposure, and it cautions against direct numerical comparison across studies. Internationally, our acute-phase and biomarker gradients are in line with the general view that subclinical inflammation and disturbed maternal–fetal tolerance contribute to RPL [1].

POSSIBLE MECHANISMS

A plausible, though unproven, mechanistic narrative ties these observations together. Healthy pregnancy depends on a tolerogenic decidual environment maintained by regulatory lymphocytes and mediators such as IL-35 and galectins, which restrain effector responses and support trophoblast survival and invasion. A reduction in IL-35 and galectin-3 would be expected to weaken this tolerance, while elevated PTX-3 and HMGB1 indicate heightened innate-immune and alarmin signalling. HMGB1, once released from stressed or dying cells, engages RAGE and Toll-like receptors to activate NF-κB and inflammasome-driven pyroptosis at the maternal–fetal interface, processes implicated experimentally in recurrent spontaneous abortion [14,15]. In this setting, latent CMV is biologically relevant because reactivation or low-grade persistence can amplify local and systemic inflammation and promote DAMP release; the modest but consistent correlations of CMV serology with HMGB1 and PTX-3, and the more extreme biomarker profile in the CMV-seropositive subgroup, are compatible with such an amplifying role. The accompanying rise in CRP and ESR supports a graded systemic inflammatory state. We emphasise that this remains a hypothesis: a cross-sectional design cannot establish whether these changes precede, accompany or follow pregnancy loss.

CLINICAL IMPLICATIONS

If confirmed prospectively, a compact panel anchored on HMGB1 and PTX-3, complemented by IL-35 and galectin-3, could in principle help characterise an inflammatory endotype of RPL and identify women in whom CMV-related inflammation is prominent. Such information might eventually inform risk stratification or the selection of patients for anti-inflammatory or immunomodulatory strategies. At present, however, these are exploratory associations measured at a single time point and largely outside pregnancy; the data-derived cut-offs are not validated, and the markers should not be used clinically for diagnosis or prognosis on the strength of this study alone. The findings are best viewed as a rationale for larger, prospective and mechanistic investigation rather than as a ready clinical tool.

STRENGTHS AND LIMITATIONS

The main strengths of this study are its balanced three-group design with well-matched demographic and anthropometric characteristics, the simultaneous measurement of four complementary biomarkers alongside a full routine panel, explicit stratification by CMV serostatus, and a rigorous analytical approach that included trend testing, false-discovery-rate control, ROC analysis with DeLong comparison, and Firth penalized regression to handle near-separation.

Several limitations temper the conclusions. First, the case–control design precludes any inference about causation or temporal sequence; the biomarkers reflect the women's state at enrolment, generally outside an index pregnancy, and cannot be interpreted as validated predictors of future loss. Second, CMV grouping rested on serology: IgG positivity reflects past or latent infection rather than active disease, only a minority of CMV-positive women were IgM-positive, and no IgG-avidity testing, CMV DNA PCR or viral-load measurement was performed, so active versus past infection could not be distinguished. Third, more than half of the controls were CMV-IgG positive, so the contrasts concern RPL status and a serologically defined subgroup rather than CMV exposure per se. Fourth, the near-perfect AUCs and very large odds ratios for HMGB1 and PTX-3, with their wide confidence intervals, raise the possibility of idealised separation and overfitting; these estimates need external validation and should not be over-interpreted. Fifth, recruitment was confined to Maysan, and used pragmatic, clinic-based sampling, which may limit generalisability and introduce selection effects; biomarkers were measured by research-use ELISA at a single time point; and residual confounding by unmeasured factors cannot be excluded.

FUTURE DIRECTIONS

Prospective cohort studies that measure these markers before and during pregnancy, ideally with serial sampling, are needed to clarify temporal relationships and any predictive value for live birth. CMV status should be characterised more completely using IgG avidity and quantitative PCR to separate latent from active or reactivated infection. External, multi-centre validation in independent Iraqi and international populations is essential before any cut-off is adopted, and mechanistic studies of decidual and trophoblast tissue could test whether the serum changes reflect events at the maternal–fetal interface. Finally, interventional work could explore whether modulating the HMGB1/PTX-3 axis or restoring tolerogenic signalling affects outcomes in carefully selected women.

CONCLUSION

In Iraqi women with recurrent pregnancy loss, serum IL-35 and galectin-3 were lower and PTX-3 and HMGB1 were higher than in parous controls, in a graded manner that was most pronounced in CMV-seropositive women and was accompanied by rising CRP and ESR. HMGB1 and PTX-3 showed the strongest associations with disease and the

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highest discriminative performance, while higher galectin-3 was independently associated with lower odds of CMV-positive RPL. These cross-sectional findings point to a coordinated shift toward a more inflammatory, less tolerogenic serum profile in association with RPL and CMV seropositivity. They are hypothesis-generating rather than definitive and require prospective, externally validated studies before any clinical application.

DECLARATIONS

Ethics approval and consent to participate. The study was approved by the research ethics committee of Natural Resources Research Center, and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

Consent for publication. Not applicable; the manuscript contains no individually identifiable data.

Availability of data and materials. The de-identified dataset supporting the findings of this study is available from the corresponding author on reasonable request.

Competing interests. The authors declare that they have no competing interests.

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Authors' contributions. All authors contributed to the study conception and design, data collection, analysis and interpretation, drafting and critical revision of the manuscript, and approved the final version.

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