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SERUM INTERLEUKIN-35, PENTRAXIN-3, GALECTIN-3 AND HMGB1 IN BRUCELLA-ASSOCIATED RECURRENT PREGNANCY LOSS: A HOSPITAL-BASED CASE–CONTROL STUDY FROM MAYSAN, SOUTHERN IRAQ

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ABSTRACT

Background. *Brucellosis is endemic in Iraq and has been associated with adverse pregnancy outcomes, yet the serum immunological profile of Brucella-associated recurrent pregnancy loss (RPL) remains poorly characterised. We evaluated four candidate biomarkers — interleukin-35 (IL-35), pentraxin-3 (PTX3), galectin-3 and high-mobility group box 1 (HMGB1) — together with a panel of inflammatory mediators in women with and without Brucella infection.*

Methods. *In this hospital-based case–control study, 210 women aged 20–40 years were enrolled into three groups of 70: healthy fertile controls (G1), Brucella-negative RPL (G2) and Brucella-positive RPL (G3). Serum biomarkers were measured by enzyme-linked immunosorbent assay (ELISA). Brucella infection was confirmed by the Rose Bengal test and IgG/IgM ELISA. Groups were compared by one-way ANOVA or Kruskal–Wallis tests with post-hoc correction; diagnostic performance was assessed by receiver operating characteristic (ROC) analysis.*

Results. *From G1 to G3, IL-35 decreased progressively (217.3 → 150.2 → 109.0 pg/mL), whereas PTX3 (2.6 → 8.4 → 14.2 ng/mL), galectin-3 (10.3 → 14.8 → 19.4 ng/mL) and HMGB1 (87.4 → 140.7 → 209.4 ng/mL) increased (all $P < 0.001$). IL-10 and VEGF declined, while TNF-α, IL-6, TGF-β1, MMP-9, CRP and ESR rose. HMGB1 and PTX3 separated Brucella-positive RPL from controls with the highest accuracy.*

Conclusions. *Brucella-positive RPL was characterised by a distinct serum signature combining suppressed immunoregulatory cytokines with heightened innate-immune and alarmin activity. These biomarkers merit prospective validation as candidate indicators of infection-associated pregnancy loss in endemic settings.*

KEYWORDS: *Brucellosis; Recurrent Pregnancy Loss; Interleukin-35; Pentraxin-3; Galectin-3; Hmgb1; Iraq*

INTRODUCTION

Human brucellosis remains one of the most widespread bacterial zoonoses worldwide and continues to impose a substantial burden across the Middle East, where consumption of unpasteurised dairy products and close contact with livestock sustain transmission [1]. Iraq is among the countries with persistently high disease activity. A recent national synthesis reported thousands of human cases over the past four decades, with an overall human seroprevalence well above regional averages and a clear predominance among women, while surveillance data covering 2007–2018 identified recurring spring and summer peaks and pronounced geographical clustering linked to locally produced fresh dairy [2,3]. Southern provinces, including Maysan, are recognised foci where agricultural livelihoods and dietary habits favour continued exposure.

Beyond its classical rheumatological and febrile manifestations, brucellosis has long been implicated in adverse reproductive outcomes. A systematic review of the obstetric literature concluded that maternal brucellosis almost certainly precipitates spontaneous abortion, with reported loss rates of roughly one quarter to one third of affected pregnancies — several-fold higher than background population rates — alongside increased risks of intrauterine fetal death and preterm delivery [4]. Clinical series and multicentre cohorts have reinforced this association and have repeatedly recommended that unexplained pregnancy loss in endemic regions be investigated for Brucella infection [5–7], although a minority of seroprevalence surveys have found no significant excess, underscoring residual uncertainty [8]. Mechanistically, Brucella species are facultative intracellular pathogens with marked tropism for placental trophoblasts; intracellular persistence, local placentitis and a strongly polarised T-helper-1 (Th1) response are thought to disrupt the finely balanced immune environment required to sustain pregnancy [9]. Comparative case–control data from neighbouring countries, including markedly elevated Brucella seropositivity among women with spontaneous abortion and a strong link to raw-milk consumption, lend further weight to a causal contribution [10,11].

Recurrent pregnancy loss — conventionally defined as three or more consecutive spontaneous abortions before 20 weeks of gestation — affects a meaningful proportion of couples and remains unexplained in up to half of cases after standard evaluation. A large body of evidence implicates dysregulated maternal–fetal immune tolerance, in which a shift away from regulatory T-cell and Th2 activity towards Th1 and Th17 predominance creates a hostile implantation milieu [12]. The net balance between pro-inflammatory and anti-inflammatory mediators, rather than any single cytokine, appears to determine whether pregnancy is maintained or lost [13].

Within this framework, four serum proteins are of particular interest. Interleukin-35 (IL-35) is an immunoregulatory cytokine of the IL-12 family that supports maternal–fetal tolerance and is reduced in recurrent spontaneous abortion [14,15]; notably, IL-35 is also suppressed in brucellosis, providing a plausible point of convergence between infection and pregnancy loss [16]. Pentraxin-3 (PTX3) is an acute-phase protein of the innate immune system expressed at the maternal–fetal interface, and its placental and circulating levels are increased in unexplained recurrent pregnancy loss [17,18]. Galectin-3 is a β-galactoside-binding lectin involved in trophoblast function, angiogenesis and immune regulation, whose dysregulation compromises placentation [19,20]. Finally, high-mobility group box 1 (HMGB1) is a prototypical damage-associated molecular pattern (alarmin) that is over-expressed at the maternal–fetal interface in recurrent abortion, where it drives sterile inflammation, and is likewise elevated in the circulation during active brucellosis [21–23].

Despite this mechanistic rationale, the serum behaviour of these biomarkers in Brucella-associated pregnancy loss has not, to our knowledge, been characterised in any Iraqi or regional population. Iraqi investigations of recurrent miscarriage have so far focused mainly on chromosomal abnormalities, antiphospholipid antibodies and a limited set of cytokines, without an integrated infection-stratified biomarker panel [24,25]. The present study was therefore designed to compare serum IL-35, PTX3, galectin-3 and HMGB1 — together with a broader panel of inflammatory mediators and routine laboratory parameters — among healthy fertile women, women with Brucella-negative RPL and women with Brucella-positive RPL in Maysan, southern Iraq, and to explore the diagnostic relevance and interrelationships of these markers.

MATERIALS AND METHODS

STUDY DESIGN AND SETTING

This analytical case–control study with prospective recruitment was conducted over an 18-month period, between January 2023 and June 2024, at Al-Sadr Teaching Hospital and Al-Hakeem General Hospital, together with affiliated private obstetrics and gynaecology clinics, in Maysan Governorate, southern Iraq. The study followed the principles of the Declaration of Helsinki and is reported in line with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) recommendations for case–control studies.

ETHICAL APPROVAL

The study protocol was reviewed and approved by the Research Ethics Committee of the College of Science, University of Misan, Maysan, Iraq. Written informed consent was obtained from every participant before enrolment, after the nature and purpose of the study had been explained. Participation was voluntary, and all data were anonymised and handled confidentially.

PARTICIPANTS AND GROUPING

Women aged 20–40 years attending the participating obstetrics and gynaecology services were screened for eligibility. A total of 250 women were assessed; 40 were excluded because of incomplete clinical or laboratory data or because they declined to participate or were lost to follow-up, leaving 210 participants who were allocated to three equal groups of 70 (Figure 1). Group 1 (G1) comprised healthy fertile controls with at least one previous live birth and no history of pregnancy loss. Group 2 (G2) and Group 3 (G3) comprised women with recurrent pregnancy loss, defined as three or more consecutive spontaneous abortions before 20 weeks of gestation, who were subsequently stratified by Brucella status into Brucella-negative (G2) and Brucella-positive (G3) groups on the basis of serological testing. Women with known chromosomal abnormalities, uterine anatomical anomalies, overt thyroid or other endocrine disease, diabetes mellitus, antiphospholipid syndrome, or other recognised systemic or autoimmune causes of pregnancy loss were excluded, as were women with current pregnancy, malignancy, or chronic hepatic or renal disease.

DATA COLLECTION

A structured interviewer-administered questionnaire was used to record sociodemographic characteristics (age, residence, educational level, occupation and socioeconomic status), a detailed obstetric and gynaecological history (marital duration, gravidity, parity, number of miscarriages, number of live births and menstrual pattern), and recognised brucellosis risk factors, including contact with animals and consumption of unpasteurised (raw) milk and its products.

ANTHROPOMETRIC AND CLINICAL MEASUREMENTS

Body weight and height were measured with participants in light clothing and without shoes using a calibrated clinical scale and stadiometer; body mass index (BMI) was calculated as weight in kilograms divided by the square of height in metres. Resting blood pressure, pulse rate and body temperature were recorded at enrolment.

BLOOD SAMPLING AND PROCESSING

After an overnight fast, approximately 8 mL of venous blood was collected from each participant. Aliquots for haematological analysis were placed in dipotassium EDTA tubes, and the remainder was allowed to clot and centrifuged at 3000 × g for 10 minutes; serum was separated, divided into aliquots and stored at −80 °C until analysis. Repeated freeze–thaw cycles were avoided.

BRUCELLA SEROLOGY

Brucella infection was screened using the Rose Bengal plate agglutination test with commercial stained Brucella antigen (Spinreact, Girona, Spain) according to the manufacturer's instructions, in keeping with internationally recommended diagnostic practice [26]. Anti-Brucella IgG and IgM antibodies were quantified by ELISA (IBL International GmbH, Hamburg, Germany); results were interpreted using the manufacturer's cut-offs (<8 U/mL negative, 8–12 U/mL equivocal, >12 U/mL positive). Brucella-positive status (G3) required a positive Rose Bengal test together with a positive IgG and/or IgM ELISA result.

BIOMARKER QUANTIFICATION

Serum concentrations of the four primary biomarkers — IL-35, PTX3, galectin-3 and HMGB1 — and of the supplementary inflammatory mediators IL-10, tumour necrosis factor-α (TNF-α), IL-6, transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9) — were determined by quantitative sandwich ELISA using commercial human-specific kits (CUSABIO Technology LLC, Houston, TX, USA / Wuhan, China); the IL-35 assay used kit CSB-EL012393HU (analytical sensitivity 15.6 pg/mL; detection range 31.2–2000 pg/mL). All assays were performed strictly according to the manufacturer's protocols. Optical density was read at 450 nm on an automated microplate reader (BioTek ELx800, BioTek Instruments, Winooski, VT, USA) coupled with an automated microplate washer, and concentrations were derived from standard curves constructed with each assay run. Samples were assayed in duplicate, and values falling above the upper limit of detection were reassayed after dilution.

ROUTINE HAEMATOLOGY AND BIOCHEMISTRY

Complete blood count and haematological indices were measured on an automated haematology analyser (Mindray BC-5150, Shenzhen Mindray Bio-Medical Electronics, Shenzhen, China). Liver function tests (aspartate aminotransferase [AST], alanine aminotransferase [ALT], albumin), renal function tests (urea, creatinine) and fasting blood glucose were measured on an automated clinical chemistry analyser (Roche Cobas c311, Roche Diagnostics, Mannheim, Germany) using the manufacturer's reagents. C-reactive protein (CRP) was measured by a latex-enhanced immunoturbidimetric assay on the same analyser, and the erythrocyte sedimentation rate (ESR) was determined by the Westergren method. Reference ranges followed standard laboratory guidelines.

STATISTICAL ANALYSIS

Data were analysed using Python 3 (SciPy, NumPy, Pandas, scikit-learn and statsmodels libraries). Continuous variables are presented as mean ± standard deviation (SD) and categorical variables as number (percentage). The normality of distributions was assessed using the Shapiro–Wilk test and homogeneity of variance using Levene's test. Normally distributed continuous variables were compared across the three groups by one-way analysis of variance (ANOVA) with the Tukey honestly significant difference post-hoc test; non-normally distributed variables were compared by the Kruskal–Wallis H test with Dunn's post-hoc test and Bonferroni adjustment. Categorical variables were compared using the Pearson chi-square test, or Fisher's exact test when expected cell counts were below five. Associations between

biomarkers and clinical parameters were examined using the Spearman rank correlation coefficient. Diagnostic performance was evaluated by ROC curve analysis, with the area under the curve (AUC) and 95% confidence intervals (CIs) estimated by bootstrap resampling; optimal cut-off values were derived from the Youden index, and the corresponding sensitivity, specificity, and positive and negative predictive values were calculated. Univariable binary logistic regression was used to estimate odds ratios (ORs) for predictors of Brucella-positive RPL. A two-tailed P value below 0.05 was considered statistically significant.

RESULTS

PARTICIPANT CHARACTERISTICS

Of 250 women screened, 210 met the eligibility criteria and were allocated to the three study groups (Figure 1). The groups were broadly comparable for most baseline characteristics (Table 1; Figure 7). Mean age differed significantly across groups (27.4 ± 4.0, 30.5 ± 5.1 and 31.6 ± 4.7 years in G1, G2 and G3, respectively; $P < 0.001$), the difference being driven by the contrast between controls and the two RPL groups, which did not differ from one another on post-hoc testing ($P = 0.53$). Body mass index, residence, educational level, occupation, socioeconomic status and consanguinity did not differ significantly between groups (all $P > 0.05$), indicating adequate matching. Consistent with the infectious nature of the exposure, reported animal contact (20.0%, 40.0% and 62.9% in G1, G2 and G3; $P < 0.001$) and raw-milk consumption (30.0%, 37.1% and 57.1%; $P = 0.003$) increased markedly across the groups.

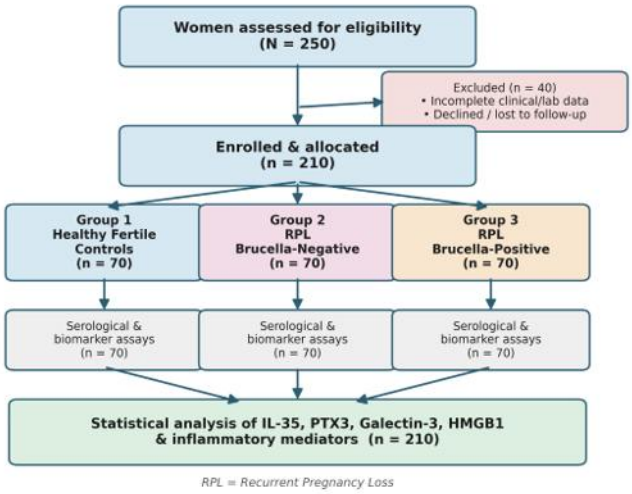

Figure 1: Study flow diagram

Of 250 women screened, 40 were excluded for incomplete data or non-participation, leaving 210 women allocated equally to healthy fertile controls (G1), Brucella-negative recurrent pregnancy loss (G2) and Brucella-positive recurrent pregnancy loss (G3). RPL, recurrent pregnancy loss.

Table 1: Demographic and socioeconomic characteristics of the study population

Variable	Control (G1) (n=70)	RPL-Negative (G2) (n=70)	RPL-Positive (G3) (n=70)	P-value
Age (years)	27.41 ± 4.01	30.47 ± 5.12	31.60 ± 4.67	<0.001
BMI (kg/m²)	25.43 ± 3.64	25.97 ± 3.80	26.43 ± 3.71	0.281
Residence – Urban, n (%)	47 (67.1)	46 (65.7)	50 (71.4)	0.752
Education (Illit/Prim/Sec/Coll/PG)	11/13/29/13/4	10/20/21/15/4	13/17/23/13/4	0.898
Occupation – Employed, n (%)	15 (21.4)	15 (21.4)	18 (25.7)	0.225
Socioeconomic status – High, n (%)	15 (21.4)	13 (18.6)	10 (14.3)	0.807
Consanguinity – Yes, n (%)	31 (44.3)	25 (35.7)	22 (31.4)	0.277
Animal contact – Yes, n (%)	14 (20.0)	28 (40.0)	44 (62.9)	<0.001
Raw-milk consumption – Yes, n (%)	21 (30.0)	26 (37.1)	40 (57.1)	0.003

Data are mean ± SD or n (%). P-values from one-way ANOVA (continuous) or chi-square test (categorical). Education categories: Illit, illiterate; Prim, primary; Sec, secondary; Coll, college/university; PG, postgraduate. Groups were comparable except for age and the brucellosis risk factors of animal contact and raw-milk consumption.

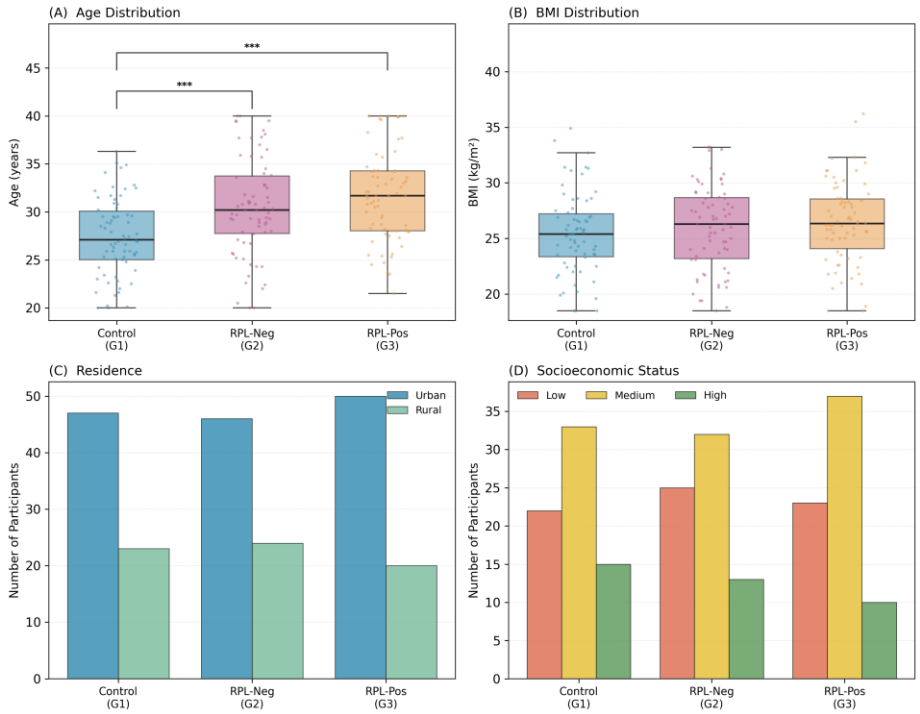

Figure 7: Demographic characteristics across study groups

Age (A) was higher in the two RPL groups than in controls, whereas body mass index (B), residence (C) and socioeconomic status (D) did not differ significantly between groups. Boxes show medians and interquartile ranges; bars show participant counts. ***P < 0.001.

OBSTETRIC AND GYNAECOLOGICAL PROFILE

Reproductive parameters differed as expected between fertile controls and women with RPL (Table 2). Gravidity was higher in G2 and G3 than in G1 (P < 0.001), while parity and the number of live births were lower (P = 0.002). By definition, controls had no miscarriages, whereas G2 and G3 reported 3.0 ± 1.1 and 3.5 ± 1.3 losses, respectively; the modest excess in the Brucella-positive group did not reach significance on post-hoc comparison (P = 0.08). Marital duration was longer in both RPL groups than in controls (P < 0.001). Irregular menstrual cycles were more frequent in the RPL groups (P = 0.016), whereas mean cycle length did not differ significantly (P = 0.34).

Table 2: Obstetric and gynaecological characteristics

Variable	Control (G1)	RPL-Negative (G2)	RPL-Positive (G3)	P-value
Marital duration (years)	5.93 ± 3.06	8.13 ± 3.32	7.26 ± 3.41	<0.001
Gravidity	1.99 ± 0.99	4.20 ± 1.37	4.54 ± 1.48	<0.001
Parity	1.99 ± 0.99	1.44 ± 1.36	1.40 ± 1.55	0.002
Number of miscarriages	0.00 ± 0.00	3.03 ± 1.08	3.50 ± 1.29	<0.001
Number of live births	1.99 ± 0.99	1.44 ± 1.36	1.40 ± 1.55	0.002
Menstrual cycle length (days)	27.50 ± 3.00	28.23 ± 3.21	28.03 ± 2.80	0.337
Irregular cycles, n (%)	11 (15.7)	26 (37.1)	20 (28.6)	0.016

Data are mean ± SD or n (%). P-values from one-way ANOVA or Kruskal–Wallis test (continuous) and chi-square test (categorical). RPL groups showed higher gravidity and lower parity/live births than controls, as expected by design.

PRIMARY BIOMARKERS

The four primary biomarkers showed a clear and graded relationship with disease status (Table 3; Figure 2). Serum IL-35 fell progressively from controls to Brucella-negative RPL to Brucella-positive RPL (217.3 ± 49.1, 150.2 ± 38.6 and 109.0 ± 37.8 pg/mL), whereas PTX3 (2.6 ± 0.9, 8.4 ± 2.8 and 14.2 ± 3.5 ng/mL), galectin-3 (10.3 ± 2.5, 14.8 ± 3.0 and 19.4 ± 3.7 ng/mL) and HMGB1 (87.4 ± 20.7, 140.7 ± 37.9 and 209.4 ± 49.0 ng/mL) rose progressively. All overall comparisons were highly significant (P < 0.001), and every pairwise post-hoc contrast — including the comparison between the two RPL groups — remained significant (P < 0.001), indicating that Brucella status added incremental change beyond RPL alone.

Table 3: Serum concentrations of the four primary biomarkers across study groups

Biomarker	Control (G1)	RPL-Negative (G2)	RPL-Positive (G3)	P-value	Test
IL-35 (pg/mL)	217.28 ± 49.05	150.17 ± 38.56	109.01 ± 37.81	<0.001	ANOVA
PTX3 (ng/mL)	2.59 ± 0.94	8.40 ± 2.77	14.22 ± 3.52	<0.001	KW
Galectin-3 (ng/mL)	10.27 ± 2.45	14.76 ± 3.03	19.38 ± 3.72	<0.001	ANOVA
HMGB1 (ng/mL)	87.39 ± 20.67	140.72 ± 37.90	209.40 ± 48.95	<0.001	KW

Data are mean ± SD. ANOVA, one-way analysis of variance; KW, Kruskal–Wallis H test. All pairwise post-hoc comparisons (including G2 vs G3) were significant at $P < 0.001$. IL-35 decreased while PTX3, galectin-3 and HMGB1 increased progressively from G1 to G3.

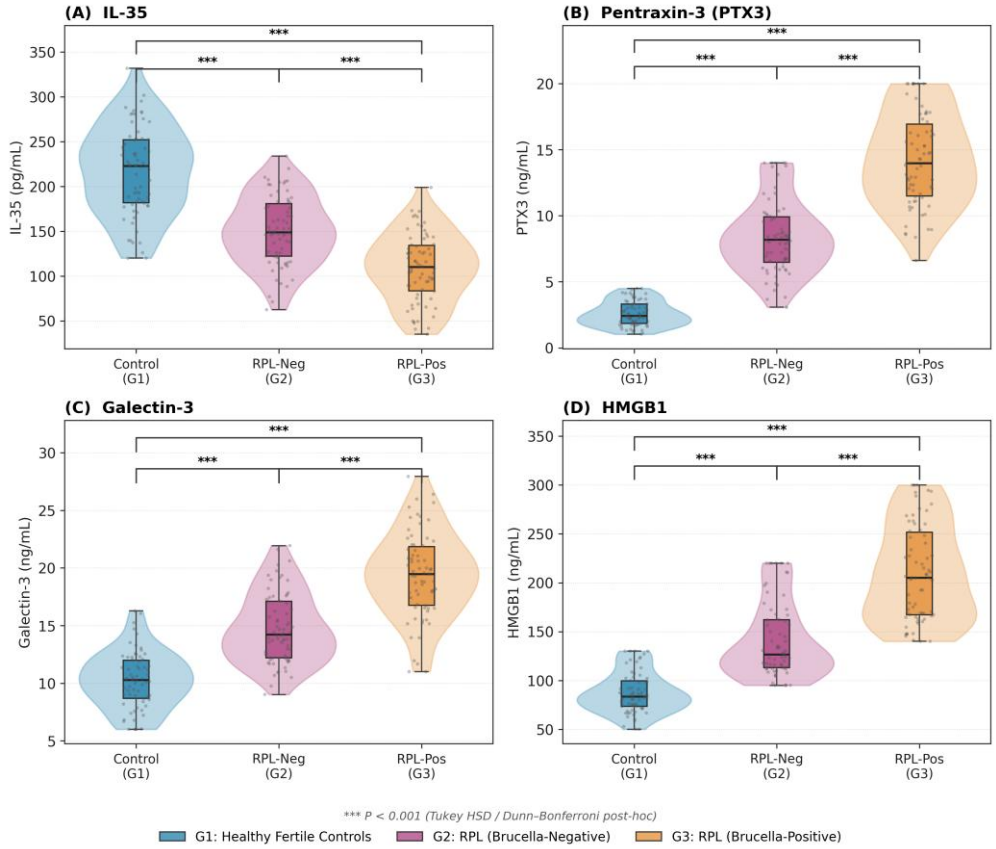

Figure 2: Serum concentrations of the four primary biomarkers across study groups

IL-35 (A) decreased progressively, whereas PTX3 (B), galectin-3 (C) and HMGB1 (D) increased from G1 to G3. Boxes show medians and interquartile ranges; violins show the kernel density of the distribution; points are individual values. ***$P < 0.001$ for all pairwise post-hoc comparisons.

INFLAMMATORY AND ANGIOGENIC MEDIATORS

The supplementary mediators displayed two opposing patterns (Table 4; Figure 5). The anti-inflammatory cytokine IL-10 declined progressively across groups (6.4 ± 1.5, 4.7 ± 1.1 and 3.3 ± 1.2 pg/mL; $P < 0.001$), and VEGF likewise decreased (179.6 ± 52.6, 126.8 ± 43.7 and 93.6 ± 41.0 pg/mL; $P < 0.001$). In contrast, the pro-inflammatory and tissue-remodelling mediators TNF-α, IL-6, TGF-β1 and MMP-9 all increased progressively from G1 to G3 (all $P < 0.001$). This combination — falling IL-10 and VEGF alongside rising TNF-α, IL-6, TGF-β1 and MMP-9 — indicates a shift towards a pro-inflammatory, immunoregulation-deficient state that was most pronounced in Brucella-positive women.

Table 4: Supplementary inflammatory and angiogenic mediators

Mediator	Control (G1)	RPL-Negative (G2)	RPL-Positive (G3)	P-value
IL-10 (pg/mL)	6.40 ± 1.45	4.66 ± 1.05	3.33 ± 1.24	<0.001
TNF-α (pg/mL)	12.18 ± 3.69	20.39 ± 5.84	28.80 ± 8.17	<0.001
IL-6 (pg/mL)	7.68 ± 3.20	15.78 ± 4.91	29.63 ± 8.61	<0.001
TGF-β1 (pg/mL)	209.12 ± 68.34	335.17 ± 104.07	563.86 ± 153.30	<0.001
VEGF (pg/mL)	179.59 ± 52.56	126.77 ± 43.74	93.62 ± 40.99	<0.001
MMP-9 (ng/mL)	84.08 ± 23.01	138.05 ± 31.87	170.67 ± 45.32	<0.001

Data are mean ± SD; P-values from one-way ANOVA. IL-10 and VEGF decreased across groups, whereas TNF-α, IL-6, TGF-β1 and MMP-9 increased, indicating a pro-inflammatory, immunoregulation-deficient profile most marked in Brucella-positive women.

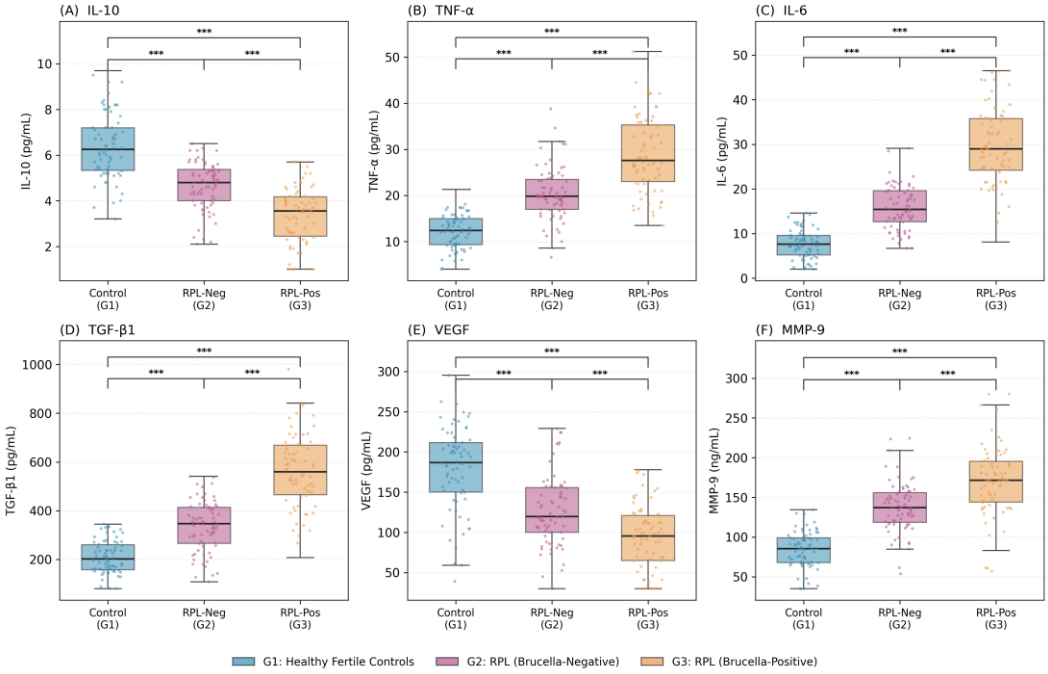

Figure 5: Supplementary inflammatory and angiogenic mediators across study groups

The anti-inflammatory cytokine IL-10 (A) and VEGF (E) decreased across groups, whereas TNF-α (B), IL-6 (C), TGF-β1 (D) and MMP-9 (F) increased. Boxes show medians and interquartile ranges. ***P < 0.001.

BRUCELLA SEROLOGY

Serological results confirmed the group definitions (Table 5). All 70 women in G3 were positive on both the Rose Bengal test and Brucella IgG ELISA, whereas all participants in G1 and G2 were negative on both. IgM positivity was present in 23 of 70 G3 women (32.9%), consistent with a recent or active phase of infection in a subset. Mean anti-Brucella IgG (24.2 ± 12.6 U/mL) and IgM (14.1 ± 14.9 U/mL) titres in G3 were several-fold higher than the negative-range values seen in G1 and G2 (P < 0.001), with no significant difference between G1 and G2.

Table 5: Brucella serological test results

Test parameter	Control (G1)	RPL-Negative (G2)	RPL-Positive (G3)	P-value
Rose Bengal test positive, n (%)	0 (0)	0 (0)	70 (100)	<0.001
Brucella IgG positive, n (%)	0 (0)	0 (0)	70 (100)	<0.001
Brucella IgM positive, n (%)	0 (0)	0 (0)	23 (32.9)	<0.001
Brucella IgG (U/mL)	4.70 ± 1.41	4.84 ± 1.55	24.21 ± 12.60	<0.001
Brucella IgM (U/mL)	3.67 ± 1.39	3.74 ± 1.78	14.08 ± 14.87	<0.001

Categorical data as n (%); antibody levels as mean ± SD. All G3 women were Rose Bengal- and IgG-positive; IgM positivity in one third indicates a recent/active phase in a subset. G1 and G2 did not differ in antibody titres.

HAEMATOLOGICAL AND INFLAMMATORY PARAMETERS

Most haematological indices remained within reference ranges (Table 6; Figure 8). Haemoglobin was modestly lower in Brucella-positive women (13.2 ± 1.2, 13.1 ± 1.3 and 12.6 ± 1.5 g/dL; P = 0.031), as was haematocrit (P = 0.036), suggesting mild anaemia of chronic infection. Contrary to what might be expected, the total white-cell count did not differ significantly between groups (P = 0.54), nor did the red-cell count or platelet count (P > 0.05). By contrast, the acute-phase markers CRP (0.9 ± 0.7, 2.1 ± 1.8 and 4.9 ± 4.5 mg/L) and ESR (12.2 ± 6.4, 20.7 ± 7.4 and 37.6 ± 12.9 mm/h) rose sharply and progressively across groups (both P < 0.001), confirming a systemic inflammatory response that was greatest in infected women.

Table 6: Complete blood count and inflammatory markers

Parameter	Control (G1)	RPL-Negative (G2)	RPL-Positive (G3)	Ref. range	P-value
Haemoglobin (g/dL)	13.19 ± 1.19	13.09 ± 1.29	12.62 ± 1.51	12.0–16.0	0.031
RBC (×10¹²/L)	4.38 ± 0.35	4.43 ± 0.38	4.44 ± 0.40	4.0–5.5	0.591
WBC (×10⁹/L)	6.84 ± 1.41	6.66 ± 1.44	6.58 ± 1.39	4.0–11.0	0.542
Platelets (×10⁹/L)	261.94 ± 54.57	261.71 ± 53.65	272.66 ± 51.21	150–400	0.381
Haematocrit (%)	39.40 ± 3.72	39.32 ± 4.03	37.81 ± 4.39	36.0–46.0	0.036
CRP (mg/L)	0.87 ± 0.73	2.05 ± 1.83	4.93 ± 4.47	<10	<0.001
ESR (mm/h)	12.21 ± 6.38	20.68 ± 7.41	37.57 ± 12.98	<20	<0.001

Data are mean ± SD; P-values from one-way ANOVA (CBC) or Kruskal–Wallis test (CRP, ESR). Haemoglobin and haematocrit were modestly lower in G3, whereas WBC, RBC and platelets did not differ significantly. CRP and ESR rose sharply across groups. RBC, red blood cell; WBC, white blood cell.

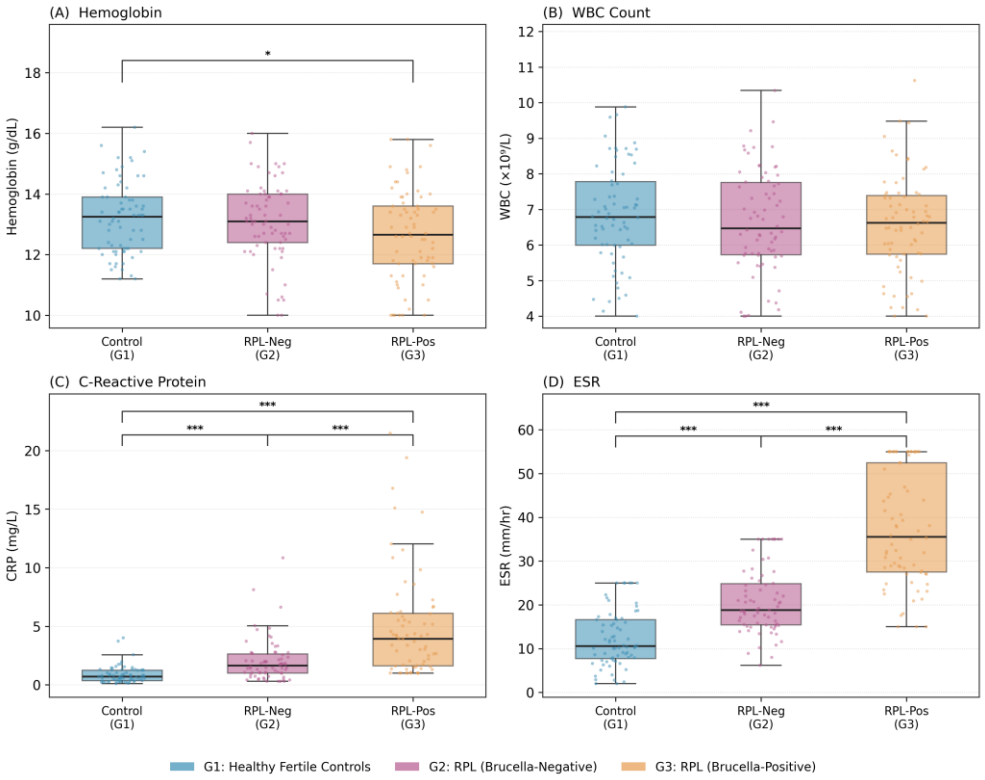

Figure 8: Haematological and inflammatory markers across study groups

Haemoglobin (A) was modestly lower in Brucella-positive women, the white-cell count (B) did not differ significantly between groups, whereas CRP (C) and ESR (D) increased progressively. ***P < 0.001; *P < 0.05; the absence of a bracket denotes a non-significant comparison. CRP, C-reactive protein; ESR, erythrocyte sedimentation rate.

HEPATIC AND RENAL FUNCTION

Renal function was preserved, with no significant difference in serum urea or creatinine across groups (Table 7; P > 0.05). Hepatic parameters, however, showed mild changes in the Brucella-positive group: AST (P = 0.002) and ALT (P < 0.001) were modestly higher and serum albumin slightly lower (P < 0.001) in G3 than in the other groups, although group mean values remained close to or within reference limits. These findings are compatible with the subclinical hepatic involvement frequently described in brucellosis rather than with overt organ dysfunction.

Table 7: Hepatic and renal function tests

Parameter	Control (G1)	RPL-Negative (G2)	RPL-Positive (G3)	Ref. range	P-value
AST (U/L)	23.82 ± 5.59	23.64 ± 6.09	27.76 ± 10.47	10–40	0.002
ALT (U/L)	19.00 ± 6.85	17.81 ± 6.86	26.98 ± 9.55	7–56	<0.001
Albumin (g/dL)	4.11 ± 0.28	4.14 ± 0.28	3.83 ± 0.32	3.5–5.0	<0.001
Urea (mg/dL)	15.08 ± 3.50	14.49 ± 3.89	14.75 ± 3.96	7–20	0.652
Creatinine (mg/dL)	0.82 ± 0.09	0.82 ± 0.10	0.82 ± 0.09	0.6–1.2	0.964

Data are mean ± SD; P-values from one-way ANOVA. AST and ALT were modestly higher and albumin slightly lower in G3, consistent with mild hepatic involvement of brucellosis, while group means remained close to reference limits. Renal function was preserved. AST, aspartate aminotransferase; ALT, alanine aminotransferase.

DIAGNOSTIC PERFORMANCE

ROC analysis showed that the four biomarkers discriminated Brucella-positive RPL from healthy controls with high accuracy (Table 8; Figure 3A). HMGB1 and PTX3 achieved complete separation in this sample (AUC = 1.000), followed by galectin-3 (AUC = 0.976; 95% CI 0.952–0.994) and IL-35 (AUC = 0.960; 95% CI 0.929–0.984). Discrimination between the two RPL groups (G3 versus G2), which isolates the contribution of Brucella infection from RPL itself, was more modest but still good for PTX3 (AUC = 0.902), HMGB1 (AUC = 0.869) and galectin-3 (AUC = 0.830), and acceptable for IL-35 (AUC = 0.770) (Table 8; Figure 3B). The near-perfect separation against healthy controls reflects the wide biological gap between uninfected and infected women in this cohort and should be interpreted with caution pending external validation (see Limitations).

Table 8: ROC analysis of the four primary biomarkers

Biomarker	Comparison	AUC (95% CI)	Cut-off	Sens (%)	Spec (%)	PPV (%)	NPV (%)
IL-35	G3 vs G1	0.960 (0.929–0.984)	167.9	95.7	84.3	85.9	95.2
PTX3	G3 vs G1	1.000 (1.000–1.000)	6.61	100	100	100	100
Galectin-3	G3 vs G1	0.976 (0.952–0.994)	15.16	91.4	95.7	95.5	91.8
HMGB1	G3 vs G1	1.000 (1.000–1.000)	140.0	100	100	100	100
IL-35	G3 vs G2	0.770 (0.695–0.845)	135.3	77.1	65.7	69.2	74.2
PTX3	G3 vs G2	0.902 (0.849–0.948)	10.62	85.7	82.9	83.3	85.3
Galectin-3	G3 vs G2	0.830 (0.760–0.895)	17.91	68.6	84.3	81.4	72.8
HMGB1	G3 vs G2	0.869 (0.808–0.921)	145.4	95.7	65.7	73.6	93.9

AUC, area under the ROC curve (95% CI by bootstrap); cut-off by Youden index. Sens, sensitivity; Spec, specificity; PPV/NPV, positive/negative predictive value. Near-perfect separation of G3 from G1 reflects the wide biological gap between uninfected and infected women and should be validated externally.

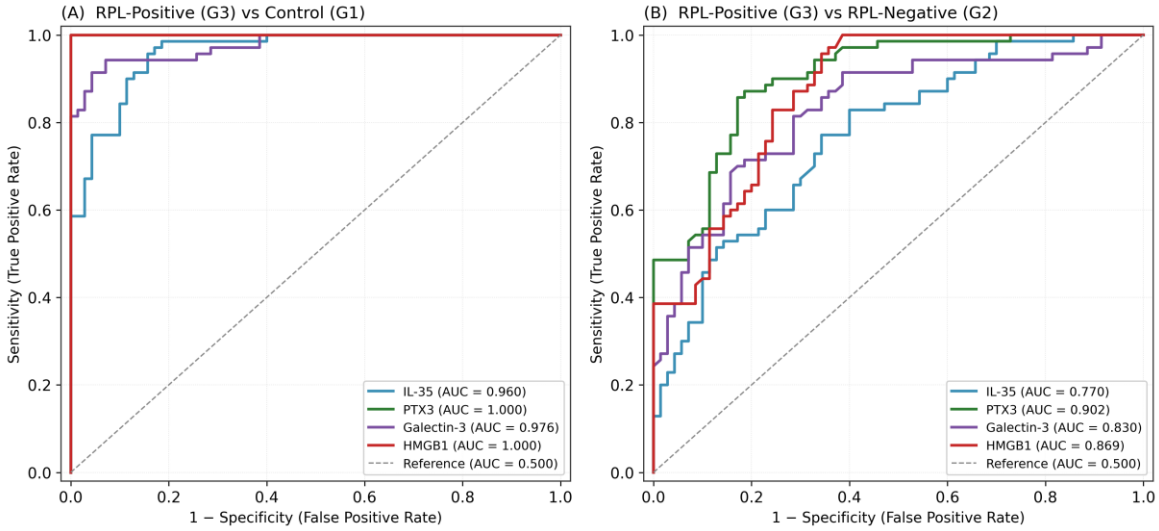

Figure 3: Receiver operating characteristic (ROC) curves for the four biomarkers

(A) Discrimination of Brucella-positive RPL (G3) from healthy controls (G1); (B) discrimination of G3 from Brucella-negative RPL (G2). Areas under the curve are shown in the legend; the diagonal denotes a non-informative test. The near-complete separation in panel A should be interpreted with caution and validated externally (see Discussion).

CORRELATIONS AND PREDICTORS

Spearman analysis revealed a coherent web of associations (Table 9; Figure 4). IL-35 correlated inversely with HMGB1 (ρ = −0.62), Brucella IgG (ρ = −0.54) and the number of miscarriages (ρ = −0.61), whereas PTX3, galectin-3 and HMGB1 correlated positively with Brucella IgG (ρ = 0.63, 0.54 and 0.59, respectively) and with markers of systemic inflammation such as CRP and ESR (all P < 0.001). PTX3 and HMGB1 showed the strongest associations with the number of miscarriages (ρ = 0.75 and 0.68). The relationships between Brucella IgG, HMGB1, PTX3 and IL-35 are illustrated in Figure 6, which shows distinct clustering of Brucella-positive women at the high-inflammation, low-IL-35 end of each distribution. In univariable logistic regression restricted to women with RPL, animal contact (OR = 2.54; 95% CI 1.29–5.02) and raw-milk consumption (OR = 2.26; 95% CI 1.15–4.44) were associated with Brucella-positive status, and each biomarker was an independent statistical predictor of Brucella-positive RPL (all P < 0.001).

Table 9: Spearman rank correlations between biomarkers and clinical parameters

Variable 1	Variable 2	Spearman ρ	P-value	Direction
IL-35	Brucella IgG	−0.537	<0.001	Negative
IL-35	HMGB1	−0.620	<0.001	Negative
IL-35	Number of miscarriages	−0.614	<0.001	Negative
PTX3	Brucella IgG	+0.625	<0.001	Positive
PTX3	HMGB1	+0.736	<0.001	Positive
PTX3	Number of miscarriages	+0.749	<0.001	Positive
Galectin-3	Brucella IgG	+0.542	<0.001	Positive
HMGB1	Brucella IgG	+0.588	<0.001	Positive
HMGB1	ESR	+0.632	<0.001	Positive
HMGB1	Number of miscarriages	+0.683	<0.001	Positive

Spearman rank correlation. Inflammatory biomarkers (PTX3, galectin-3, HMGB1) correlated positively with Brucella IgG and inflammation, whereas IL-35 correlated inversely; PTX3 and HMGB1 showed the strongest associations with miscarriage number. CRP, C-reactive protein; ESR, erythrocyte sedimentation rate.

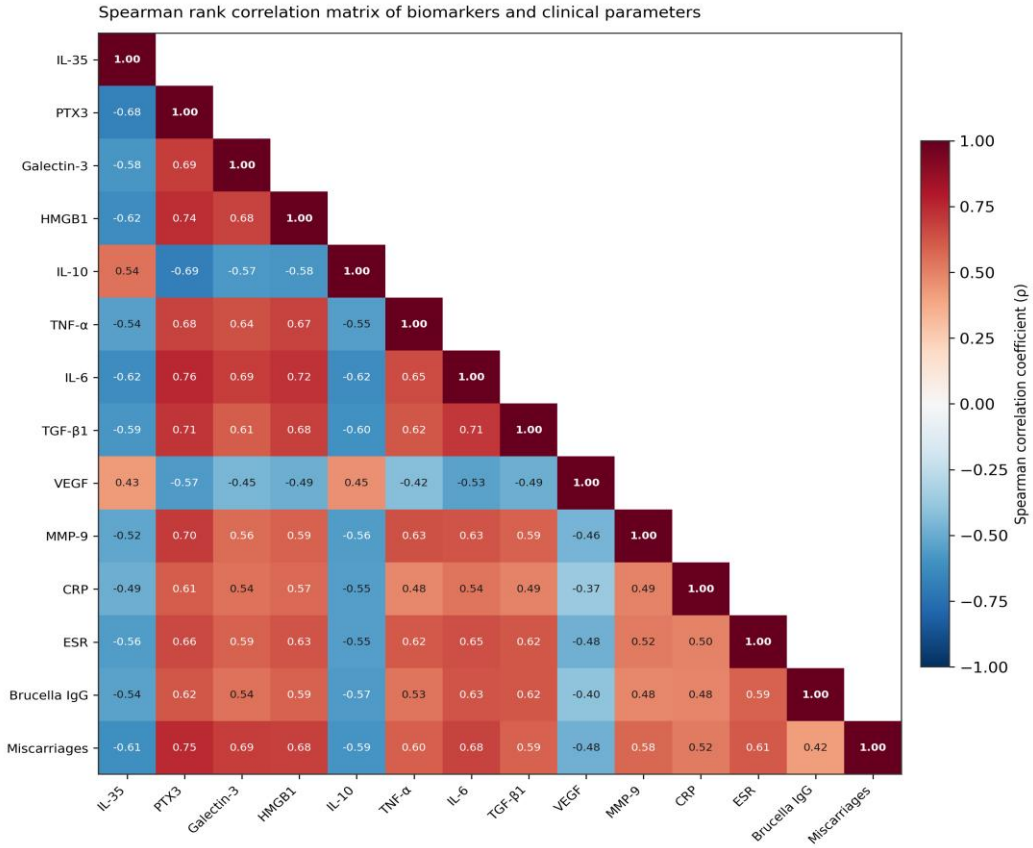

Figure 4: Spearman correlation matrix of biomarkers and clinical parameters

Warm colours indicate positive and cool colours negative correlations; the colour scale gives the Spearman coefficient (ρ). IL-35 correlated inversely with the inflammatory markers and with Brucella IgG, whereas PTX3, galectin-3 and HMGB1 correlated positively with infection and inflammation markers.

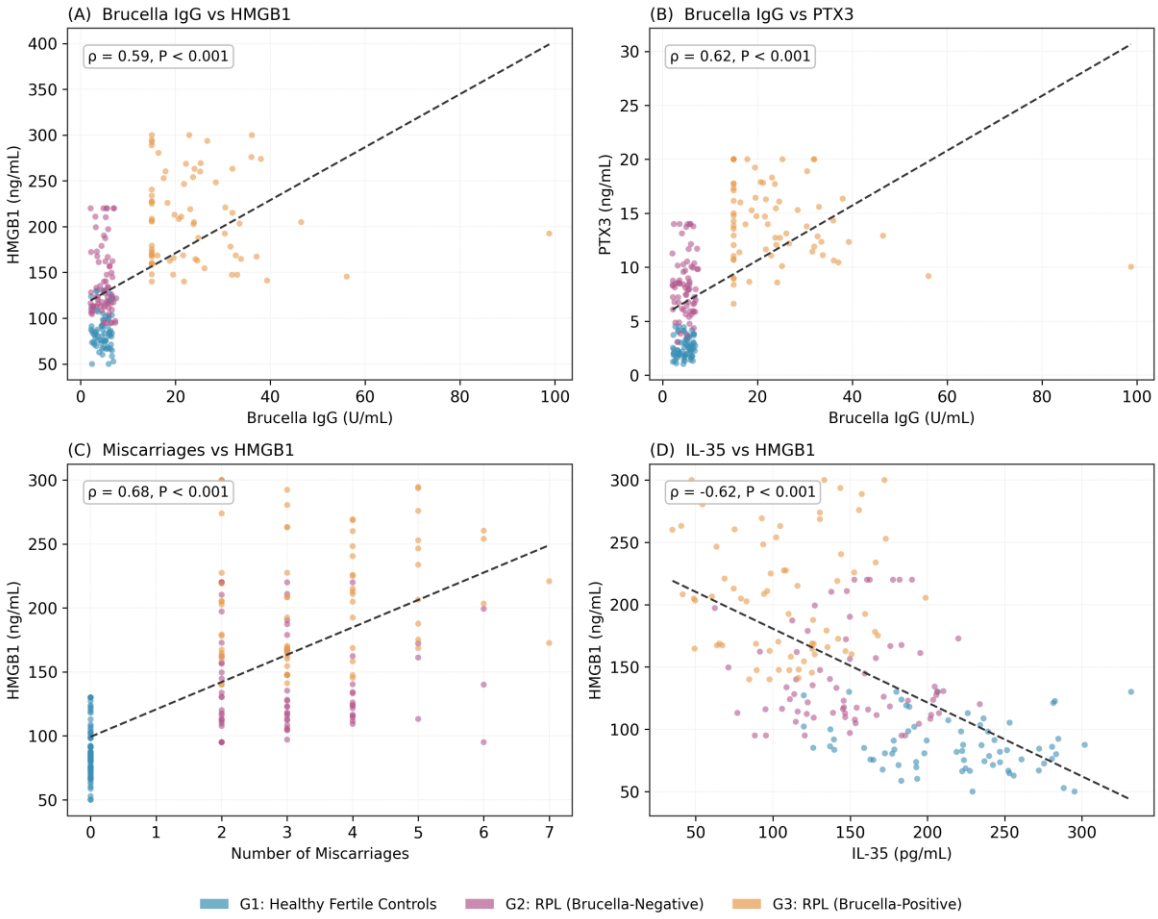

Figure 6: Bivariate relationships between Brucella IgG, biomarkers and miscarriage number

Brucella IgG correlated positively with HMGB1 (A) and PTX3 (B); the number of miscarriages correlated positively with HMGB1 (C); and IL-35 correlated inversely with HMGB1 (D). Dashed lines are linear fits and ρ values are Spearman coefficients; colours denote study groups.

DISCUSSION

In this case–control study of women from Maysan, southern Iraq, Brucella-associated recurrent pregnancy loss was distinguished from both healthy fertility and Brucella-negative RPL by a consistent serum signature: progressively lower IL-35 set against progressively higher PTX3, galectin-3 and HMGB1, accompanied by falling IL-10 and VEGF and rising TNF-α, IL-6, TGF-β1, MMP-9, CRP and ESR. The graded pattern across the three groups, in which every primary biomarker differed significantly even between the two RPL groups, suggests that Brucella infection contributes an additional immunological perturbation beyond that associated with recurrent loss alone.

IL-35 AND THE LOSS OF IMMUNOREGULATION

The progressive decline in IL-35 is biologically coherent. IL-35 is produced largely by regulatory T cells and supports maternal–fetal tolerance; Yue and colleagues showed that serum IL-35 is markedly elevated in normal pregnancy and significantly reduced in recurrent spontaneous abortion [14], and Ozkan et al. similarly reported lower IL-35 in idiopathic RPL [15]. Our data extend these observations by showing that the lowest IL-35 concentrations occur specifically in infected women. This is consistent with evidence that brucellosis itself suppresses IL-35: Ellergezen et al. found significantly reduced serum IL-35 in patients with acute and chronic brucellosis compared with healthy controls [16]. The convergence of these two literatures supports the interpretation that Brucella infection compounds the immunoregulatory deficit characteristic of RPL.

PTX3 AS A MARKER OF INNATE-IMMUNE ACTIVATION

The stepwise rise in PTX3 accords with its role as an acute-phase mediator of innate immunity at the maternal–fetal interface. Zeybek et al. demonstrated increased PTX3 expression in placental tissue from women with unexplained recurrent pregnancy loss [17], and an earlier serum study reported substantially higher first-trimester PTX3 in such women, although that report has since been the subject of an editorial Expression of Concern and its exact magnitudes should be treated with caution [18]. Our finding that PTX3 was highest in Brucella-positive women, and correlated strongly with Brucella IgG, CRP and the number of miscarriages, is congruent with an exaggerated innate-immune response to intracellular infection superimposed on the inflammatory background of RPL.

GALECTIN-3 AT THE MATERNAL–FETAL INTERFACE

Serum galectin-3 increased across the groups and correlated with infection and inflammation markers. Galectin-3 is important for healthy placentation: in experimental models its deficiency causes placental insufficiency, malperfusion and fetal growth restriction [20]. Interestingly, a placental tissue study found that galectin-3 was not consistently altered in abortive placentas even though several prototype galectins were reduced [19], implying that the circulating elevation we observed may reflect a systemic inflammatory and tissue-remodelling response rather than a simple change in placental expression. We therefore interpret the rise in serum galectin-3 as part of the broader inflammatory milieu of infection-associated loss, and caution against over-interpreting it as a direct placental signal.

HMGB1 AS AN ALARMIN LINKING INFECTION AND LOSS

Of the four markers, HMGB1 showed the greatest relative increase and the strongest discrimination from controls. HMGB1 is a damage-associated molecular pattern that, when released, propagates sterile inflammation. Zhu et al. and Wang et al. showed that HMGB1 is over-expressed at the maternal–fetal interface in unexplained recurrent spontaneous abortion, where it activates pyroptosis and contributes to interface destruction [21,22]. In parallel, Ayarci et al. reported significantly elevated serum HMGB1 in brucellosis [23]. Our results sit precisely at the intersection of these findings: HMGB1 was highest in infected women, correlated positively with Brucella IgG and ESR and inversely with IL-35, and tracked with the number of miscarriages. HMGB1 thus represents a plausible mechanistic bridge between Brucella infection and pregnancy loss, although the present design cannot establish causation.

THE DIVERGENT BEHAVIOUR OF IL-10 AND VEGF

Two findings merit specific comment because they differ from part of the existing literature. First, IL-10 decreased rather than increased across our groups. Some studies of idiopathic RPL, including Ozkan et al., have reported higher IL-10 as a compensatory regulatory response [15]. The opposite direction in our cohort may reflect the infection-specific context: Brucella drives a strongly Th1-polarised response and can suppress regulatory cytokines, so that the parallel decline of IL-35 and IL-10 plausibly represents a genuine failure of immunoregulation in infected women rather than a compensatory surge. Second, VEGF decreased across the groups. Although angiogenic factors are often elevated in inflammatory states, impaired VEGF-dependent angiogenesis and defective spiral-artery remodelling are well-recognised contributors to pregnancy loss, and the reduction we observed is consistent with compromised placental vascular adaptation in women with recurrent loss, most severe in those with infection. We present both observations transparently and without overstating their mechanistic certainty.

OTHER MEDIATORS AND ROUTINE MARKERS

The concurrent elevation of TNF-α, IL-6, TGF-β1 and MMP-9 reinforces the picture of a pro-inflammatory, tissue-remodelling phenotype, in keeping with the Th1/Th17-weighted cytokine imbalance described in RPL [12,13]. The sharp, graded increases in CRP and ESR provide an independent, routinely available confirmation of systemic inflammation, whereas the absence of leucocytosis is consistent with the characteristically normal or even low white-cell counts of brucellosis. The mild reductions in haemoglobin and albumin and the modest rises in transaminases in infected women align with the subclinical hepatic involvement and anaemia of chronic infection commonly reported in brucellosis, while preserved renal function argues against significant systemic organ damage.

COMPARISON WITH IRAQI AND REGIONAL STUDIES

Our work complements a small but growing Iraqi literature on recurrent miscarriage. Khamees and Al-Ouqaili, studying Iraqi couples with RPL, emphasised chromosomal and immunological contributions, including antiphospholipid antibodies and IL-6 [24], and Hamadi and Lafta, in nearby Thi-Qar province, reported elevated antiphospholipid and anticardiolipin antibodies and complement activation among women with recurrent miscarriage [25]. Neither study examined IL-35, PTX3, galectin-3 or HMGB1, nor stratified women by infection status; to our knowledge the present study is the first in Iraq to profile this biomarker panel specifically in Brucella-associated pregnancy loss. The epidemiological context is provided by national data showing high and female-predominant brucellosis seroprevalence in Iraq and clustering linked to fresh dairy consumption [2,3], which is consistent with the strong gradients in animal contact and raw-milk consumption we observed. Beyond Iraq, our findings parallel those of Ejaz et al., who reported markedly higher Brucella seropositivity among Pakistani women with spontaneous abortion than among healthy pregnant women, with raw-milk consumption a major risk factor [10]. They also accord broadly with systematic and clinical evidence that maternal brucellosis increases the risk of spontaneous abortion [4–7], while we acknowledge that some seroprevalence surveys, such as the Iranian study by Ahmadi et al., found no significant association [11]; this heterogeneity underscores the value of biomarker-based, infection-stratified designs such as ours.

MECHANISTIC SYNTHESIS

Taken together, the data are compatible with a model in which Brucella, a facultative intracellular pathogen with tropism for placental trophoblasts, provokes local and systemic innate-immune activation (rising PTX3 and HMGB1, CRP and ESR) and a Th1-polarised inflammatory response (rising TNF-α and IL-6) while suppressing immunoregulatory cytokines (falling IL-35 and IL-10); the accompanying changes in galectin-3, TGF-β1, MMP-9 and VEGF point to disturbed placental remodelling and angiogenesis [9,20]. This combination would be expected to undermine the immune tolerance and vascular adaptation required to sustain pregnancy. We emphasise that this synthesis is hypothesis-generating; the cross-sectional design precludes inference about temporal sequence or causality.

CLINICAL IMPLICATIONS

From a practical standpoint, the findings suggest that women with recurrent pregnancy loss in brucellosis-endemic areas may warrant assessment for Brucella infection, particularly where animal contact or raw-milk consumption is reported, since infection is potentially treatable. The biomarker panel — especially HMGB1 and PTX3 — may, after validation, help characterise the inflammatory burden of infection-associated loss. We deliberately refrain from proposing any of these markers as a stand-alone diagnostic test on the basis of a single-centre study.

STRENGTHS AND LIMITATIONS

The strengths of the study include a balanced three-group design that separates the effect of Brucella infection from that of RPL, well-matched groups for most baseline variables, serological confirmation of infection by two independent methods, and analysis of a comprehensive biomarker and laboratory panel using rigorous, appropriately chosen statistics. Several limitations temper the conclusions. First, the cross-sectional design cannot establish causality or the temporal relationship between infection, biomarker changes and pregnancy loss. Second, the study was conducted in a single governorate, and the findings require confirmation in other populations. Third, Brucella infection was confirmed serologically rather than by blood culture or molecular methods, so the infecting species and bacterial load were not determined. Fourth, the near-perfect ROC discrimination of HMGB1 and PTX3 against healthy controls almost certainly reflects the large biological gap between uninfected and infected women in this sample and is liable to optimism; these cut-offs must be regarded as provisional and validated externally before any clinical use. Finally, although recognised non-infectious causes of RPL were excluded clinically, residual confounding cannot be entirely ruled out, and some endocrine and anatomical evaluations relied on available records.

FUTURE DIRECTIONS

Prospective cohort studies that measure these biomarkers before and during pregnancy, ideally with culture- or PCR-confirmed Brucella infection and longitudinal follow-up of pregnancy outcome, are needed to test the temporal and potentially causal relationships suggested here. Multicentre studies across Iraqi provinces would improve generalisability and allow robust, externally validated diagnostic thresholds to be established. Finally, interventional studies examining whether timely antibrucellar treatment modifies the biomarker profile and improves pregnancy outcome would help clarify the clinical relevance of these findings.

CONCLUSION

In women from Maysan, southern Iraq, Brucella-associated recurrent pregnancy loss was characterised by a distinct serum profile combining reduced immunoregulatory cytokines (IL-35 and IL-10) with elevated innate-immune and alarmin mediators (PTX3, galectin-3 and HMGB1) and a broader pro-inflammatory, pro-remodelling response. These changes were graded across healthy controls, Brucella-negative RPL and Brucella-positive RPL, and were accompanied by strong, biologically coherent correlations with Brucella antibody titres and systemic inflammatory markers. The results support the view that Brucella infection adds a measurable immunological burden to recurrent pregnancy loss in endemic settings. Given the single-centre, cross-sectional design, these biomarkers should be regarded as promising candidates that require prospective, multicentre validation before any diagnostic or clinical application.

DECLARATIONS

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Research Ethics Committee of the College of Science, University of Misan, Maysan, Iraq. The study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants prior to enrolment.

CONSENT FOR PUBLICATION

Not applicable; the manuscript contains no individually identifiable data.

AVAILABILITY OF DATA AND MATERIALS

The datasets generated and analysed during the current study are 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. Material preparation, data collection and laboratory analysis were performed by the named authors; statistical analysis and the first draft of the manuscript were prepared collaboratively. All authors read, critically revised and approved the final manuscript.

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