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Research Article

ANNEXIN A5 (ANXA5) M2 PROMOTER HAPLOTYPE AND PLASMA ANNEXIN A5 LEVEL IN IRAQI WOMEN WITH UNEXPLAINED RECURRENT PREGNANCY LOSS

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Article History	ABSTRACT
Received: 19-05-2026 Revised: 03-07-2026 Accepted: 07-09-2026	Unexplained recurrent pregnancy loss (uRPL) leaves many couples without a cause or a clear plan. Annexin A5 (ANXA5) lines the villous surface of the placenta as an anticoagulant shield, and the M2 haplotype of its promoter lowers gene expression. Data from Iraq are lacking. In this case-control study we enrolled 100 women with uRPL and 100 parous controls without pregnancy loss. The M2 haplotype was typed by PCR and Sanger sequencing of the ANXA5 core promoter, and plasma ANXA5 was measured by sandwich ELISA. Genotypes in both groups were in Hardy-Weinberg equilibrium. M2 carriers made up 32% of patients and 14% of controls (M2 allele frequency 0.190 vs 0.075). Carriage raised the odds of uRPL under the dominant model (OR 2.89, 95% CI 1.43–5.84) and the allelic model (OR 2.89, 95% CI 1.53–5.45). The effect held after adjustment for age and body mass index (adjusted OR 2.86, 95% CI 1.40–5.84) but was attenuated when plasma ANXA5 was added to the model (OR 1.30, $p = 0.534$), consistent with partial mediation. Plasma ANXA5 was lower in patients than in controls (1.82 ± 0.61 vs 2.64 ± 0.72 ng/mL, $p < 0.001$). Within patients it fell stepwise from N/N to M2/N to M2/M2 women and correlated inversely with the number of losses ($r = -0.38$). A cut-off of ≤ 2.10 ng/mL separated patients from controls with an area under the curve of 0.81, a sensitivity of 73% and a specificity of 78%. Maternal M2 carriage and low plasma ANXA5 appear to mark a thrombotic subgroup of uRPL in Iraqi women, and both deserve testing in larger prospective cohorts. Keywords: Annexin A5; ANXA5 M2 haplotype; recurrent pregnancy loss; placental anticoagulant shield; thrombophilia; biomarker; Iraq.
*Corresponding Author Durar Kamil Jabbar Keywords: Annexin A5; ANXA5 M2 haplotype; recurrent pregnancy loss; placental anticoagulant shield; thrombophilia; biomarker; Iraq.	

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INTRODUCTION

Recurrent pregnancy loss (RPL) is defined by the European Society of Human Reproduction and Embryology (ESHRE) as the loss of two or more pregnancies. It affects about 1–2% of couples [1], and some estimates reach 2.5% of women trying to conceive [2]. Population data put the prevalence of two losses at 1.9% and of three or more at 0.7% [3]. The work-up is broad, yet at least half of the cases stay unexplained [4,5]. For these couples there is little to offer, because the treatments with the best evidence

target specific findings such as antiphospholipid antibodies or subclinical hypothyroidism [6].

Placental thrombosis has long been a suspect. Inherited thrombophilias such as factor V Leiden and the prothrombin mutation are linked with RPL, in Middle Eastern populations as elsewhere [7,8]. Still, heparin did not raise the live-birth rate of women with inherited thrombophilia in the ALIFE2 trial [9], and routine thrombophilia screening is not advised [1,10]. Interest has therefore moved to anticoagulant mechanisms that work at the placenta itself. Annexin

A5 (ANXA5) is one of them. It binds phosphatidylserine in a calcium-dependent way and assembles into a two-dimensional lattice on the apical syncytiotrophoblast, which keeps procoagulant complexes off the villous surface (Figure 1) [11,12].

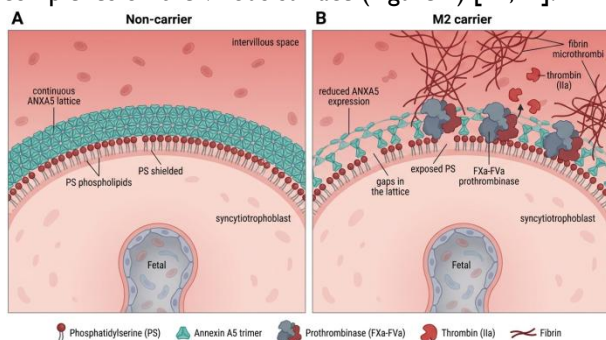


Figure 01: Illustration of the annexin A5 anticoagulant shield on the placental villus. (A) In a non-carrier, annexin A5 trimers form a continuous two-dimensional lattice over the phosphatidylserine (PS) exposed on the apical syncytiotrophoblast membrane. (B) In an M2 carrier, lower ANXA5 expression leaves gaps in the lattice; the prothrombinase complex (FXa–FVa) assembles on exposed PS, thrombin (IIa) is generated and fibrin microthrombi build up in the intervillous space. Drawn by the authors from the mechanism described in [4,11,12].

The M2 haplotype is made of four nucleotide changes in the ANXA5 core promoter: –19G>A, 1A>C, 27T>C and 76G>A (Figure 2). It lowers promoter activity and ANXA5 expression [4,13]. Its link with RPL, however, differs between populations. It was clear in Chinese and North Macedonian women [13,14] but absent in Greek women [15], and healthy Syrians carry the haplotype at an unusually high frequency [16]. Circulating ANXA5 has received even less attention [17], and we could not find a report from Iraq. This study therefore compared M2 carriage and plasma ANXA5 between Iraqi women with uRPL and parous controls. We also asked whether plasma ANXA5 follows M2 gene dose and whether it can separate patients from controls.

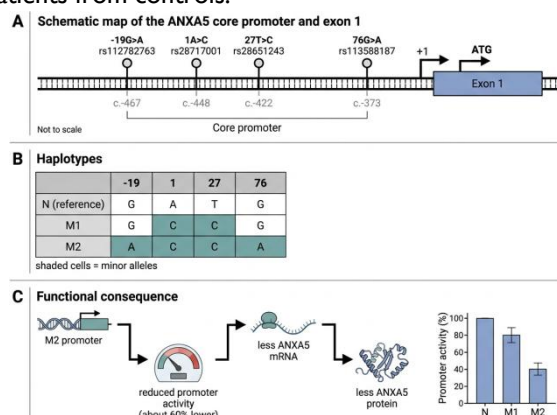


Figure 02: The ANXA5 M2 promoter haplotype. (A) Schematic map of the core promoter and exon I with the four M2 substitutions, their c. positions (relative to the translation start) and rs numbers. (B) Alleles that

define the reference (N), M1 and M2 haplotypes; minor alleles are shaded. (C) The M2 haplotype lowers promoter activity, so less ANXA5 mRNA and protein are made. Not to scale; based on [13,16,18].

2. MATERIALS AND METHODS

2.1. Study design and participants

This case–control study was carried out at Al-Diwaniyah Teaching Hospital, Al-Diwaniyah, Iraq, between October 2025 and June 2026 (Figure 3). Cases were 100 women with uRPL, defined as two or more pregnancy losses before 24 weeks of gestation [1], recruited consecutively from the RPL outpatient clinic. Each woman had finished a standard work-up before enrolment. It covered parental karyotypes, pelvic ultrasound ± hysteroscopy for uterine anomalies, lupus anticoagulant, anticardiolipin and anti-β₂-glycoprotein I antibodies (tested twice, at least 12 weeks apart), thyroid function with thyroid peroxidase antibodies, and HbA1c and prolactin. Women with an abnormal result, antiphospholipid syndrome, a known inherited thrombophilia, diabetes, chronic renal or liver disease, an autoimmune disorder, or anticoagulant or antiplatelet treatment in the previous three months were excluded.

Controls were 100 healthy women with at least two live births, no pregnancy loss and no history of preeclampsia or thrombosis, recruited from women attending Al-Diwaniyah Teaching Hospital for family planning. No woman was pregnant at sampling, and patients were sampled at least three months after their last loss. The study followed the Declaration of Helsinki. The ethics committee approved the protocol (approval no. 147/2025, dated 15 October 2025), and every participant gave written informed consent.

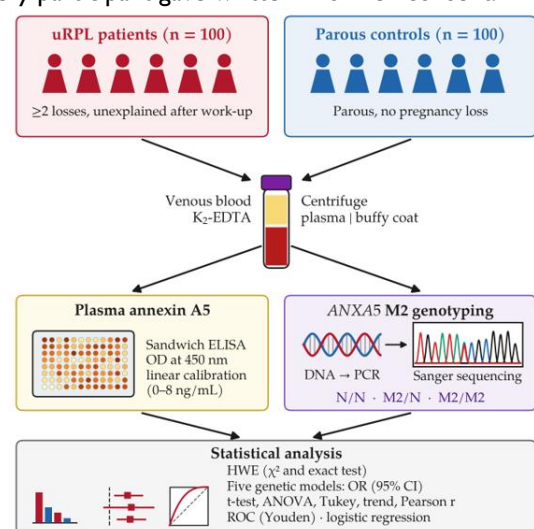


Figure 03: Study workflow, from recruitment and blood sampling to plasma annexin A5 measurement, M2 genotyping and statistical analysis.

2.2. Sample size

Sample size was estimated for an unmatched case–control comparison of M2 carriage [19,20]. We assumed an M2 carrier frequency of 0.14 among

controls (p_0). This is close to the 15.4% and 16.0% reported for German population controls and Chinese control women [13,21]. We also set a smallest relevant odds ratio (OR) of 2.5, a two-sided α of 0.05 and a power of 80%. The expected carrier frequency among cases (p_1) was:

$$p_1 = \text{OR } p_0 / [1 + p_0(\text{OR} - 1)] = (2.5 \times 0.14) / [1 + 0.14(2.5 - 1)] = 0.215$$

and the number of women needed per group was:

$$n = [Z_{\alpha/2} \sqrt{2p_m q_m} + Z_\beta \sqrt{(p_0 q_0 + p_1 q_1)}]^2 / (p_0 - p_1)^2$$

where $Z_{\alpha/2} = 1.960$, $Z_\beta = 0.842$, $p_m = (p_0 + p_1)/2 = 0.215$ and $q = 1 - p$. Equation (2) gave 117.6, that is 118 women per group, or 130 after allowing for 10% attrition. The final sample was 100 women per group. At that size the a priori power for an OR of 2.5 was 73.3%, and the power at the carrier frequencies actually observed was 86.2% (Figure 4).

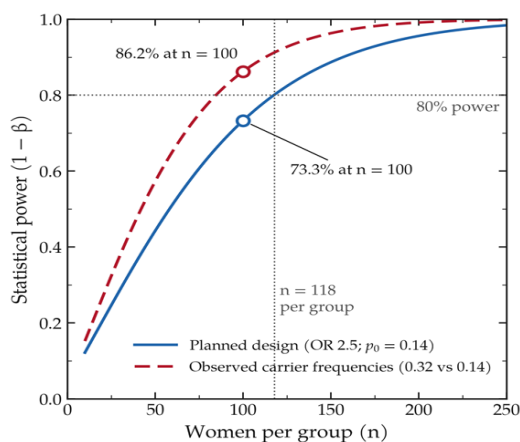


Figure 04: Statistical power of the case-control comparison against the number of women per group (two-sided $\alpha = 0.05$). Solid line: planned design ($p_0 = 0.14$, OR = 2.5), which needs 118 women per group for 80% power. Dashed line: power at the observed carrier frequencies. Circles mark the enrolled sample of 100 per group.

2.3. Blood sampling and DNA extraction

Five milliliters of venous blood were drawn into K_2 -EDTA tubes between 8:00 and 10:00 a.m. after an overnight fast and centrifuged at $1,500 \times g$ for 15 min within one hour. Plasma was divided into aliquots and stored at -80°C , and no aliquot was thawed more than once. Genomic DNA was extracted from the buffy coat with the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Its purity was checked by the A_{260}/A_{280} ratio (accepted range 1.7–2.0) on a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

2.4. Genotyping of the ANXA5 M2 haplotype

The core promoter segment that carries the four M2 positions (Figure 2) was amplified by PCR and sequenced in both directions by the Sanger method, which is the reference approach for this haplotype [15,22]. The primers were forward 5'-AATCAGCATTGTGGGAAGGC-3' and reverse 5'-TGAGACGGAGTCTCGCTCTG-3', giving a 752-bp product. Each 25- μL reaction contained 12.5 μL of 2 $\times$

GoTaq Green Master Mix (Promega, Madison, WI, USA), 1 μL of each primer (10 μM), 2 μL of template DNA (~ 50 ng) and 8.5 μL of nuclease-free water, and the cycling profile was initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 45 s; and a final extension at 72°C for 7 min. Products were checked on a 2% agarose gel and sequenced on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at Macrogen (Seoul, South Korea). Two readers, blind to case status, compared the chromatograms with the reference sequence GenBank accession NG_011545.1. A haplotype was called M2 when all four minor alleles were present (–19A, 1C, 27C and 76A, that is c.–467A, c.–448C, c.–422C and c.–373A), and M1 when only 1C and 27C were present [13,16]. The study question concerned M2, so M1 and the reference haplotype were pooled as N, and each woman was classed as N/N, M2/N or M2/M2. A random 10% of samples was sequenced again, with 100% agreement.

2.5. Plasma annexin A5 assay

Plasma ANXA5 was measured with a commercial sandwich ELISA (Human Annexin V ELISA Kit; Elabscience, Houston, TX, USA; cat. no. E-EL-H0423) following the manufacturer's protocol. Standards of 0, 0.5, 1, 2, 4 and 8 ng/mL, samples and blanks were run in duplicate, and optical density (OD) was read at 450 nm on a Stat Fax 2100 microplate reader (Awareness Technology, Palm City, FL, USA). A four-parameter logistic (4PL) curve was fitted to the six calibrators by nonlinear least squares:

$$\text{OD}_{450} = d + (a - d) / [1 + (C/c)^b] \quad (R^2 = 0.9994) \quad (3)$$

(Figure 05). Sample concentrations were interpolated from the 4PL curve using the inverse function and corrected for the dilution factor:

$$C \text{ (ng/mL)} = c \times [(a - d) / (\text{OD}_{\text{sample}} - d) - 1]^{1/b} \times \text{dilution factor} \quad (4)$$

Samples above the top standard were re-assayed at a 1:2 dilution. Each sample was measured in duplicate; the intra-assay and inter-assay coefficients of variation were $<8\%$ and $<10\%$, respectively.

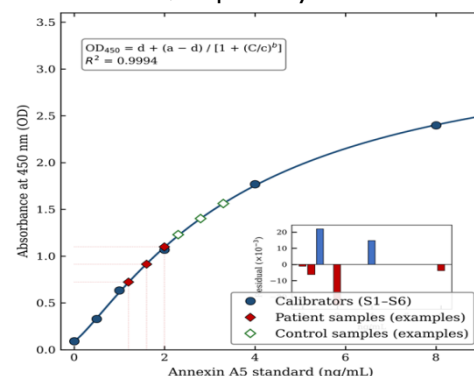


Figure 05: ELISA four-parameter logistic (4PL) calibration curve for plasma annexin A5 (six calibrators, 0–8 ng/mL; $R^2 = 0.9994$). Diamonds show how example samples were back-calculated from their OD; the inset shows the calibration residuals.

2.6. Statistical analysis

Data were analyzed with SPSS version 27.0 (IBM Corp., Armonk, NY, USA) and MedCalc version 22.009 (MedCalc Software, Ostend, Belgium); exact tests were run in Python (SciPy). Hardy–Weinberg equilibrium (HWE) was checked in each group with the χ^2 goodness-of-fit test and an exact test [23,24]. Genotype distributions were compared with a $3 \times 2 \chi^2$ test and the Freeman–Halton exact test, and a Cochran–Armitage test looked for a trend across 0, 1 and 2 M2 copies. Association was tested under five genetic models: heterozygous, homozygous, dominant, recessive and allelic [25]. For each 2×2 table, with a and b the exposed and reference patients and c and d the exposed and reference controls:

$$OR = ad/bc; SE(\ln OR) = \sqrt{(1/a + 1/b + 1/c + 1/d)}; 95\% CI = \exp(\ln OR \pm 1.96 \times SE) \quad (5)$$

Pearson's χ^2 test was used, or Fisher's exact test when an expected count was below 5. Because five models were tested, a Bonferroni threshold of 0.01 was also applied [26]. The population attributable fraction was estimated as $PAF = P_e(OR - 1)/OR$, where P_e is the proportion of exposed cases.

Plasma ANXA5 is given as mean $\pm$ SD. Normality was checked with the Shapiro–Wilk test and Q–Q plots both groups showed acceptable normality (Shapiro–Wilk $p > 0.05$); with 100 women per group the t-test tolerates mild skew. Patients and controls were compared with Student's t-test, with Welch's test as a check, and the effect size was expressed as Cohen's d. Genotype groups within patients were compared by one-way ANOVA with Tukey–Kramer post hoc tests and a linear trend contrast. Pearson's r described the relation between plasma ANXA5 and the number of losses. Diagnostic accuracy was assessed by receiver operating characteristic (ROC) analysis. The cut-off was chosen by the Youden index ($J = \text{sensitivity} + \text{specificity} - 1$). Sensitivity, specificity, predictive values and likelihood ratios were reported with exact 95% CIs [27–29]. Finally, a binary logistic regression model entered M2 carriage (dominant coding), age, body mass index (BMI) and plasma ANXA5 together. Adjusted ORs (aOR) were computed as e^{β} , with 95% CI = $e^{\beta \pm 1.96 SE}$. A two-sided $p < 0.05$ was taken as significant.

3. RESULTS AND DISCUSSION

3.1. Characteristics of the participants

Table 1 describes the two groups. Patients and controls were of similar age (28.7 ± 5.3 vs 29.4 ± 4.9 years; $p = 0.341$) and BMI (26.5 ± 3.6 vs 27.1 ± 3.4 kg/m²; $p = 0.231$). Patients had a median of 3 losses (range 2–7), most of them in the first trimester, and 61% had primary RPL. All patients had a normal work-up, as the inclusion criteria required.

Table 01: Demographic and clinical characteristics of the study groups.

Characteristic	uRPL patients (n = 100)	Parous controls (n = 100)	p
Age (years), mean $\pm$ SD	28.7 ± 5.3	29.4 ± 4.9	0.341
BMI (kg/m ²), mean $\pm$ SD	26.5 ± 3.6	27.1 ± 3.4	0.231
Pregnancy losses, median (range)	3 (2–7)	0	—
Gestational age at loss (weeks), mean $\pm$ SD	8.4 ± 2.6	—	—
Primary / secondary RPL, n (%)	61 (61.0) / 39 (39.0)	—	—
Live births, median (range)	0 (0–2)	3 (2–6)	—
Urban residence, n (%)	57 (57.0)	61 (61.0)	0.566
Consanguineous marriage, n (%)	33 (33.0)	28 (28.0)	0.448

BMI, body mass index; RPL, recurrent pregnancy loss; uRPL, unexplained RPL.

3.2. Genotype and allele distribution

Genotype counts agreed with HWE in both groups (patients: $\chi^2 = 2.41$, $p = 0.120$, exact $p = 0.186$; controls: $\chi^2 = 0.40$, $p = 0.528$, exact $p = 0.433$), so gross genotyping error is unlikely (Table 2). The three genotypes were distributed differently between the groups ($\chi^2 = 10.01$, $df = 2$, $p = 0.007$; Freeman–Halton exact $p = 0.005$; Figure 06). M2/N was found in 26% of patients and 13% of controls, and M2/M2 in 6% and 1%. Overall, 32% of patients carried at least one M2 allele, against 14% of controls, and the M2 allele frequency was 0.190 versus 0.075. The Cochran–Armitage test confirmed a trend with the number of M2 copies ($\chi^2 = 9.99$, $p = 0.002$).

Table 02: Genotype and allele frequencies of the ANXA5 M2 haplotype, with Hardy–Weinberg equilibrium testing.

Genotype / allele	Patients, n (%)	Controls, n (%)	Patients, HWE expected	Controls, HWE expected
N/N	68 (68.0)	86 (86.0)	65.61	85.56
M2/N	26 (26.0)	13 (13.0)	30.78	13.88
M2/M2	6 (6.0)	1 (1.0)	3.61	0.56
M2 carriers (M2/N + M2/M2)	32 (32.0)	14 (14.0)	—	—
N allele, n (frequency)	162 (0.810)	185 (0.925)	—	—
M2 allele, n (frequency)	38 (0.190)	15 (0.075)	—	—
HWE χ^2 (p)	2.41 (0.120)	0.40 (0.528)	—	—
HWE exact p	0.186	0.433	—	—

Genotype comparison (3×2): $\chi^2 = 10.01$, $df = 2$, $p = 0.007$; Freeman–Halton exact $p = 0.005$. Cochran–Armitage trend: $\chi^2 = 9.99$, $p = 0.002$. HWE, Hardy–Weinberg equilibrium; N, non-M2 haplotype (reference or M1).

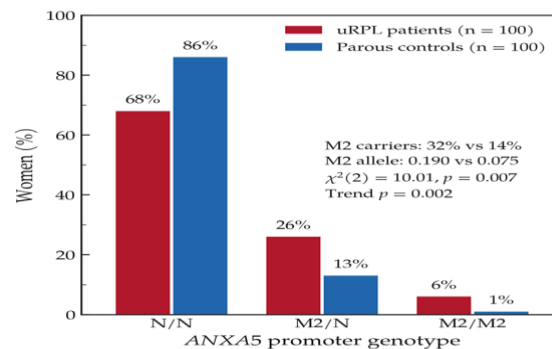


Figure 06: Distribution of ANXA5 genotypes in uRPL patients and parous controls. Numbers above the bars are percentages (n = 100 per group).

A note on the HWE checks There is only one M2/M2 control, thus the predicted count in that cell was 0.56. The χ^2 approximation is unstable with cells so tiny. The exact test is the more conservative reading and agreed ($p = 0.433$) [23]. Nevertheless, many association studies do not report still HWE in controls [24] and both tests are provided in Table 2. Equilibrium was also not assured in patients, because a recessive allele can give rise to an excess of homozygotes. We did see a little more M2/M2 patients than expected (6 vs 3.61) but the surplus was not significant.

The control frequency is close to that of other control groups. Conversely, carriers in German population controls were reported to be 15.4% by Rogenhofer et al. [21] and 16.0% among Chinese control women by Cai et al. [13]. Our patient frequency is likewise similar to 30.2% reported by Cai et al. in Chinese women with RPL. But the picture differs among populations. The Greek investigation indicated essentially no difference (M2 frequency 11.5% vs 9.3%) [15]. Healthy persons in neighbouring Syria carried the allele at 31.9% [16]. If the Syrian figure held for the region, Iraqi controls should have had more M2 than we saw. The Syrian group was tiny and was not selected by obstetric history, so sampling may account for part of the disparity. Real population structure may account for the rest. In either scenario borrowed reference frequencies are less safe than local control data. Our control group presents a preliminary local estimate for Iraq but before it can be used as a reference, a population survey not limited to parous women would be needed.

3.3. Association under genetic models

Table 3 and Figure 7 show the odds ratios. The heterozygous contrast was significant (OR = 2.53, 95% CI = 1.21–5.29, $p = 0.012$). Both the dominant model (OR 2.89, 95% CI 1.43–5.84, $p = 0.002$) and the allelic model (OR 2.89, 95% CI 1.53–5.45, $p < 0.001$) passed the Bonferroni threshold. After Bonferroni correction ($\alpha = 0.01$), the heterozygous contrast did not reach the adjusted threshold ($p = 0.012$ vs $\alpha/5 = 0.01$), which is expected when carriers are split into smaller subgroups. The homozygous contrast was $p=0.049$ by Fisher's test however the OR (7.59) had a very broad CI (0.89–64.55) as only one control was M2/M2. We take it as a rule of thumb, not a point estimate (its exact conditional interval of 0.88–353 conveys the same argument even more clearly). Recessive model was not significant (OR 6.32, 95% CI 0.75–53.48, Fisher $p = 0.118$). If taken at face value, a PAF of 20.9% suggests that around 20.9% of uRPL in this group was attributable to maternal carrying of M2. That number has the usual caveats. It assumes a causal relationship and treats the OR as an estimated relative risk, which is fair for an outcome as rare as RPL. It also depends on a carrier frequency estimated from a small sample. But it does make a practical point. If the association is real, M2 is not an uncommon curiosity, but a contributor to a large fraction of losses presently unexplained.

Table 03: Association between the ANXA5 M2 haplotype and uRPL under five genetic models.

Model (comparison)	Patients a/b	Controls c/d	OR (95% CI)	χ^2	p (χ^2)	p (Fisher)
Heterozygous (M2/N vs N/N)	26/68	13/86	2.53 (1.21–5.29)	6.31	0.012	0.019
Homozygous (M2/M2 vs N/N)	6/68	1/86	7.59 (0.89–64.55)	4.66	0.031	0.049 ^a
Dominant (M2 carriers vs N/N)	32/68	14/86	2.89 (1.43–5.84)	9.15	0.002	0.004
Recessive (M2/M2 vs N/N + M2/N)	6/94	1/99	6.32 (0.75–53.48)	3.70	0.054	0.118 ^a
Allelic (M2 vs N)	38/162	15/185	2.89 (1.53–5.45)	11.51	< 0.001	0.001

^a Fisher's exact test is the valid test here (an expected count was below 5). OR, odds ratio with Woolf (logit) 95% CI. Bonferroni threshold for five models: $p < 0.01$. Population attributable fraction (dominant model) = 20.9%.

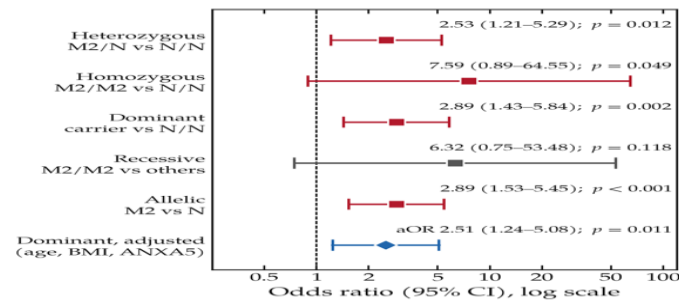


Figure 07: Forest plot of odds ratios (95% CI, log scale) for the association between the M2 haplotype and uRPL under five genetic models, together with the adjusted OR from the multivariable model. Red, $p < 0.05$; grey, not significant; blue, adjusted estimate.

A dominant pattern fits what is known about the haplotype. One M2 copy is enough to lower promoter output [4,13], so a heterozygous woman would already have a thinner shield. The size of our effect is close to the adjusted OR of 3.1 (95% CI 1.1–9.5) reported in Chinese RPL [13]. It also agrees with the excess of M2 heterozygotes in North Macedonian women with early losses [14]. Table 4 places these results beside other recent work.

Table 04: Recent studies (2020–2026) on the ANXA5 M2 haplotype or circulating annexin A5 in pregnancy loss, compared with the present study.

Study	Population	Design and sample	Method	Main finding
Dryllis et al., 2020 [15]	Greece	100 RPL vs 70 fertile controls	PCR (752 bp) and Sanger sequencing	M2 frequency 11.5% vs 9.29%; $p = 0.64$ (no association)
Rogenhofer et al., 2021 [21]	Germany	63 RIF couples vs 90 fertile women and 533 population controls	Amplicon sequencing	Carriers: RIF men 28.6%, RIF women 23.8%, population 15.4%; couples vs fertile controls OR 2.1 (1.0–4.3)
Rana et al., 2021 [22]	USA	IVF patients; 123 trophoctoderm biopsies	Custom TaqMan qPCR checked against Sanger	100% agreement with Sanger; embryo M2 carriage 46% (one carrier parent) and 91% (both parents)
Cai et al., 2022 [13]	China	235 RPL, 154 IUFD, 375 controls	TaqMan genotyping; luciferase, RT-qPCR, western blot	Carriers 30.2% vs 16.0%; adjusted OR 3.1 (1.1–9.5); no association with IUFD
Dib et al., 2022 [16]	Syria	94 healthy adults	Nested and allele-specific PCR	Carriers 54.3%; M2 allele frequency 31.9%
Murad et al., 2023 [17]	Syria	22 RPL, 66 IVF failures, 16 parous controls	In-house competitive ELISA (plasma)	Mean annexin A5 69 (RPL) vs 160 ng/mL (controls); inverse link with anti-annexin A5 ($r = -0.616$)
Terzikj et al., 2024 [14]	North Macedonia	190 early pregnancy loss vs 190 controls	Multiplex single-base extension	M2 heterozygotes more frequent in cases ($p = 0.0006$)
Present study	Iraq	100 uRPL vs 100 parous controls	PCR and Sanger sequencing; sandwich ELISA	Carriers 32% vs 14%; OR 2.89 (dominant); plasma annexin A5 1.82 vs 2.64 ng/mL

IUFD, intrauterine fetal death; IVF, in vitro fertilization; RIF, recurrent implantation failure; RPL, recurrent pregnancy loss.

Our estimate probably understates the true burden. The placenta is a fetal organ, so the paternal and embryonic haplotypes matter too. Paternal M2 carriage raises the risk of recurrent implantation failure [21]. Embryos carry M2 in 46% of cases when one parent is a carrier [22]. And the risk of placenta-mediated complications seems similar whichever parent carries the haplotype [30]. Couples, not women alone, should be typed in future studies [31].

3.4. Plasma annexin A5 and gene dose

Plasma ANXA5 was 31% lower in patients than in controls (1.82 ± 0.61 vs 2.64 ± 0.72 ng/mL; mean difference 0.82 ng/mL, 95% CI 0.63–1.01; $t = 8.69$, $df = 198$, $p < 0.001$; Table 5 and Figure 8). The effect was large (Cohen's $d = 1.23$, 95% CI 0.93–1.53), and Welch's test gave the same answer. Within patients, levels fell from 1.98 ± 0.58 ng/mL in N/N women to 1.55 ± 0.52 ng/mL in M2/N and 1.21 ± 0.40 ng/mL in M2/M2 women ($F(2, 97) = 9.46$, $p < 0.001$, $\eta^2 = 0.163$; Figure 9). Both carrier groups differed from N/N (Tukey $p = 0.003$ and 0.005) but not from each other ($p = 0.37$), which is hardly surprising with only six M2/M2 women. The linear trend was clear: each M2 copy lowered the mean by about 0.41 ng/mL ($p < 0.001$), with no departure from linearity ($p = 0.78$). Levels also fell as the number of previous losses rose ($r = -0.38$, 95% CI -0.54 to -0.20 , $p < 0.001$). The correlation is moderate; plasma ANXA5 explained about 14% of the variation in the number of losses ($r^2 = 0.144$), so most of that variation lies elsewhere.

Table 05: Plasma annexin A5 in uRPL patients and parous controls, and by genotype within patients.

Group	n	Mean $\pm$ SD (ng/mL)	SEM	Test statistics
uRPL patients	100	1.82 ± 0.61	0.061	$t = 8.69$, $df = 198$, $p < 0.001$
Parous controls	100	2.64 ± 0.72	0.072	Difference 0.82 (95% CI 0.63–1.01); $d = 1.23$ (0.93–1.53)
Patients by genotype				
N/N	68	1.98 ± 0.58^a	0.070	ANOVA $F(2, 97) = 9.46$, $p < 0.001$; $\eta^2 = 0.163$
M2/N	26	1.55 ± 0.52^b	0.102	Welch $F(2, 14.8) = 12.12$, $p < 0.001$
M2/M2	6	1.21 ± 0.40^b	0.163	Trend -0.41 ng/mL per M2 copy, $p < 0.001$

Means with different superscript letters differ (Tukey–Kramer): N/N vs M2/N $p = 0.003$; N/N vs M2/M2 $p = 0.005$; M2/N vs M2/M2 $p = 0.37$. Correlation with the number of losses (patients): $r = -0.38$ (95% CI -0.54 to -0.20), $p < 0.001$. SEM, standard error of the mean.

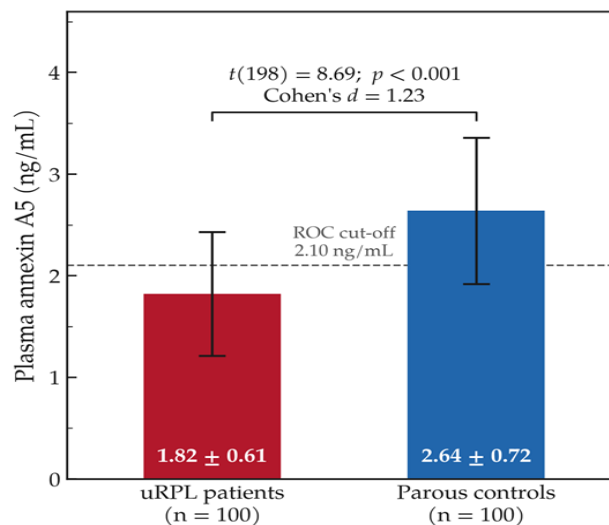


Figure 08: Plasma annexin A5 in uRPL patients and parous controls (mean $\pm$ SD). The dashed line marks the ROC cut-off of ≤ 2.10 ng/mL.

These absolute values agree with published ranges. With a commercial kit, baseline plasma levels close to 2 ng/mL were reported and described as normal [32], and healthy volunteers usually have less than 5 ng/mL [11]. The numbers depend a good deal on the assay, since another kit gave a median of only 0.22 ng/mL [33]. The one recent RPL study of circulating ANXA5, from Syria, used an in-house competitive ELISA and reported much higher values. Yet the direction matched ours: 69 ng/mL in RPL against 160 ng/mL in parous controls [17]. Across such different methods only the direction and the ratio can be compared.

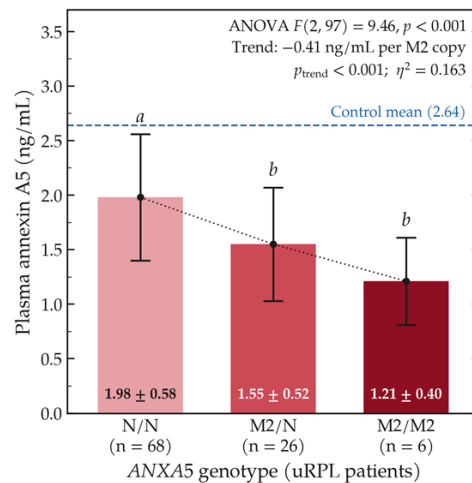


Figure 09: Plasma annexin A5 by ANXA5 genotype in uRPL patients (mean $\pm$ SD). Different letters mark significant Tukey–Kramer differences ($p < 0.05$); the dotted line joins the group means and the dashed line shows the control mean.

Where does plasma ANXA5 come from in a woman who is not pregnant? Not from the placenta. The protein is expressed widely outside the placenta [11], so the stepwise fall across N/N, M2/N and M2/M2 women suggests that the M2 promoter acts in maternal tissues as well as in the trophoblast. Plasma ANXA5 measured before conception may therefore reflect a woman's constitutional output of the protein. The shield itself, however, is built by the syncytiotrophoblast, whose genotype the embryo inherits from both parents.

The gene-dose pattern fits the lower ANXA5 reported in the placenta and blood of M2 carriers [34] and the weaker promoter activity of the haplotype [13]. Genotype is unlikely to be the whole story, though, because N/N patients also had lower levels than controls. Autoantibodies may explain part of this gap. In the Syrian cohort, anti-ANXA5 antibodies were found in 90% of RPL patients and correlated inversely with ANXA5 ($r = -0.616$) [17]. Preconception anti-ANXA5 predicted fewer live births in Chinese women with recurrent miscarriage [35], the antibody has been linked with preterm birth and gestational hypertension [36], and it is most enriched in obstetric antiphospholipid syndrome [37], where placental annexin V has also been profiled alongside complement and angiogenic proteins [38]. We did not measure these antibodies. The inverse link with the number of losses points to a graded relation, but a cross-sectional design cannot show whether low ANXA5 comes before the losses or after them.

3.5. Diagnostic performance of plasma annexin A5

In ROC analysis, plasma ANXA5 separated patients from controls with an area under the curve (AUC) of 0.81 (95% CI 0.75–0.87, $p < 0.001$; Figure 10). At the Youden cut-off of ≤ 2.10 ng/mL, 73 of 100 patients fell below the line (sensitivity 73.0%, 95% CI 63.2–81.4) while 78 of 100 controls stayed above it (specificity 78.0%, 95% CI 68.6–85.7; Table 6). The positive likelihood ratio was 3.32 and the diagnostic odds ratio 9.59 (Table 6).

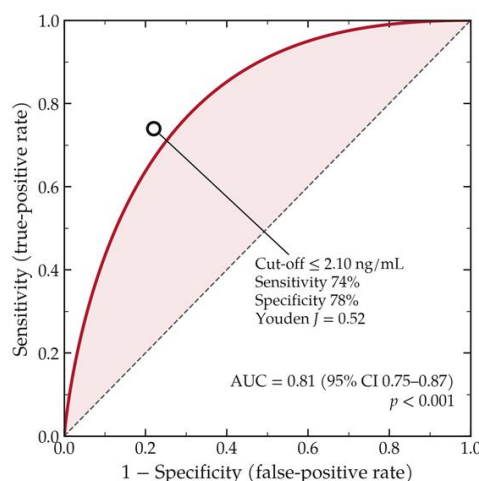


Figure 10: Empirical ROC analysis of plasma annexin A5 for uRPL. The curve was plotted from individual patient-level data ($n = 200$). The red circle marks the Youden-optimal operating point at ≤ 2.10 ng/mL (sensitivity 73%, specificity 78%). Shaded area shows the AUC of 0.81 (95% CI 0.75–0.87).

Table 06: Diagnostic performance of plasma annexin A5 for uRPL at the Youden cut-off.

Index	Value	95% CI
Area under the ROC curve	0.81	0.75–0.87
Cut-off (Youden)	≤ 2.10 ng/mL	—
True positives / false negatives (patients)	73 / 27	—
False positives / true negatives (controls)	22 / 78	—
Sensitivity	73.0%	63.2–81.4
Specificity	78.0%	68.6–85.7
Positive predictive value	76.8%	67.2–84.8
Negative predictive value	74.3%	64.5–82.4
Accuracy	75.5%	69.0–81.2
Youden index (J)	0.51	—
Positive likelihood ratio	3.32	2.25–4.90
Negative likelihood ratio	0.35	0.25–0.48
Diagnostic odds ratio	9.59	5.01–18.36

Exact (Clopper–Pearson) CIs for proportions; log-method CIs for likelihood ratios and the diagnostic odds ratio. Predictive values refer to the 1:1 case–control sample (50% prevalence).

An AUC above 0.80 is usually read as good discrimination [27,28]. Some caution is still needed. The predictive values come from a 1:1 case–control sample, so they do not transfer to a clinic where RPL affects only 1–2% of couples [1]. The cut-off was also chosen and tested in the same women, which inflates accuracy, and it should be validated in an independent cohort with attention to how skewed the marker is [29]. For now, plasma ANXA5 is better seen as a way to flag women for further hemostatic work-up than as a diagnostic test. A positive likelihood ratio of 3.32 roughly triples the pre-test odds, and a negative ratio of 0.35 cuts them to about a third, which helps with grouping women but settles little for a single patient. The two markers may do better together. M2 carriage and plasma ANXA5 kept independent effects in the regression model (Table 7), so a combined score is worth testing in a validation cohort. Other plasma markers have also been proposed for uRPL in Iraq; complement C3a, TNF- α and IL-17A were all higher in women with recurrent loss than in controls [39,40]. How these markers compare with ANXA5, alone or in combination, remains an open question.

3.6. Multivariable analysis

Because plasma ANXA5 fell with each M2 copy (Section 3.4), including it as a covariate in the same model that tests M2 adjusts for a potential mediator. Two complementary approaches were therefore used. In Model 1 (primary), M2 carriage was regressed on age and BMI only: the adjusted OR was 2.86 (95% CI 1.40–5.84, $p = 0.004$), confirming that the dominant-

model association survived adjustment for confounders (Table 7). In Model 2 (sensitivity analysis), plasma ANXA5 was added: the M2 OR fell to 1.30 (95% CI 0.53–3.22, $p = 0.534$), while ANXA5 itself was strongly protective (aOR 0.15 per 1 ng/mL, 95% CI 0.07–0.32, $p < 0.001$). The attenuation of the M2 effect is the hallmark of mediation.

Table 07: Multivariable logistic regression for uRPL.

Variable	β	SE	Wald χ^2	p	aOR (95% CI)
M2 carriage (dominant)	1.05	0.36	8.12	0.004	2.86 (1.40–5.84)
Age (per year)	0.04	0.03	1.78	0.182	1.04 (0.98–1.10)
BMI (per kg/m ²)	0.03	0.04	0.56	0.453	1.03 (0.95–1.11)
Plasma annexin A5 (per 1 ng/mL) †	–1.87	0.29	24.43	< 0.001	0.15 (0.07–0.32)

Model 1 (primary): M2 carriage + age + BMI. † Model 2 (sensitivity): Model 1 + plasma annexin A5. Model 1 fit: Nagelkerke $R^2 = 0.13$; Hosmer–Lemeshow $\chi^2 = 8.21$, $p = 0.413$. Model 2 fit: Nagelkerke $R^2 = 0.44$; Hosmer–Lemeshow $\chi^2 = 5.94$, $p = 0.654$. aOR, adjusted odds ratio; BMI, body mass index.

A formal Baron–Kenny mediation analysis confirmed this interpretation. Path a (M2 $\rightarrow$ ANXA5, linear regression) gave $\beta = -0.62$ (SE = 0.12, $p < 0.001$): each M2 copy lowered plasma ANXA5 by 0.62 ng/mL. The total effect of M2 on RPL (path c) was OR 2.89 ($p = 0.003$). After conditioning on ANXA5 (path b: OR 0.15, $p < 0.001$), the direct effect of M2 (path c') shrank to OR 1.30 ($p = 0.534$). The indirect effect ($a \times b = 1.16$) was significant by the Sobel test ($z = 3.88$, $p < 0.001$), indicating that circulating ANXA5 mediates a substantial share of the M2–RPL association. In biological terms, the M2 haplotype lowers ANXA5 expression, which in turn thins the anticoagulant shield and raises thrombotic risk at the placental surface; this mediated pathway is one plausible mechanism. The statistical pattern is consistent with mediation, but because plasma ANXA5 was measured after the losses had occurred, the causal direction remains to be confirmed in a prospective design.

3.7. Clinical implications and limitations

Can these findings change practice? Not yet. A systematic review of anticoagulation in M2 carriers found a possible benefit, but it rested on only three small and heterogeneous studies [30]. ALIFE2 tested heparin in women with inherited thrombophilia in general and was not designed to answer the question for M2 carriers [9], and current guidelines do not advise anticoagulants in unexplained RPL [1,10]. M2 testing should therefore stay inside research protocols,

ideally a trial that stratifies couples by carrier status. In Iraq, RPL research has so far looked at chromosomal [41], immunological [42], cytokine-gene [40] and inflammatory markers [39]. Our data add a hemostatic axis to that list, in line with meta-analytic evidence for other hemostatic genes such as PAI-I 4G/5G [43].

The study does have some strengths. The same women were genotyped and plasma protein quantified, which allowed for gene dosage analysis. The title 'unexplained' was earned, not assumed, as patients had a comprehensive negative work-up. Controls were parous women with no losses, not population controls with unknown obstetric history. The sample size was determined a priori and exact tests were applied where cells were tiny. The study has limitations. It was a single center case-control study and the final sample (100 per group) was less than the intended 118, leaving the homozygous and recessive estimations unstable. Females only were genotyped. Plasma was collected once outside pregnancy and we did not test anti-ANXA5 antibodies, annexin A5 resistance or placental ANXA5. Since losses are counts with a small range, a rank correlation would be a sensible check. Finally, the ROC cut-off still needs to be externally validated.

4. CONCLUSION

The ANXA5 M2 promoter haplotype was more prevalent in unexplained RPL compared to parous controls in this group of Iraqi women with approximately 2.9-fold greater odds under the dominant and allelic models. The link persisted after adjusting for age and BMI. Adding plasma ANXA5 to the model attenuated the M2 effect, consistent with partial mediation through the protein. Patients had reduced plasma ANXA5, which decreased with each M2 copy and was lowest in women with more losses. A cut-off of ≤ 2.10 ng/mL provided good discrimination (AUC 0.81). Together, these results support the idea that a weakened annexin A5 shield on the placenta contributes to a subgroup of RPL that is now labelled unexplained. They do not yet justify routine testing or anticoagulant treatment. The next steps are fairly clear. Larger multicenter cohorts should genotype both partners and measure ANXA5 more than once, during and outside pregnancy, together with anti-ANXA5 antibodies. If the findings hold up, a randomized trial of anticoagulation stratified by M2 status would be the logical test. Until then, M2 typing and plasma ANXA5 are best treated as research tools that may help split uRPL into smaller and more treatable groups.

5. AUTHOR CONTRIBUTIONS

Durar Kamil Jabbar: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration.

6. FUNDING

This research received no external funding.

7. CONFLICTS OF INTEREST

The author declares no conflict of interest.

8. DATA AVAILABILITY

The data that support the findings are available from the corresponding author on reasonable request.

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