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Embryo morphokinetics and PGT-A outcomes in recurrent pregnancy loss cases

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Research Article

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Summary

Recurrent pregnancy loss (RPL) is a clinically important condition in women undergoing assisted reproductive technologies (ART). This study evaluated embryo morphology and morphokinetic parameters using time-lapse monitoring (TLM) and assessed embryo ploidy by preimplantation genetic testing for aneuploidy (PGT-A) in women with RPL compared with unexplained infertility (UEI) controls. A total of 190 patients (100 RPL, 90 UEI) and 1169 embryos (634 RPL, 535 UEI) cultured under TLM were analyzed. Clinical characteristics, embryo morphology, morphokinetics and ploidy status were compared between groups, and logistic regression was used to identify predictors of aneuploidy. The euploidy rate was significantly lower in the RPL group than in controls (43.5% vs. 52.3%, $p = 0.018$). Group-wise analysis of all embryos revealed differences in selected morphokinetic parameters (t9 and tSC; $p < 0.05$). However, morphokinetic timing of euploid embryos did not differ between groups, suggesting that accelerated development alone was not associated with chromosomal abnormality. Embryo morphology was the strongest predictor of aneuploidy in both cohorts. Notably, direct uneven cleavage was associated with a 3.4-fold increased risk of aneuploidy specifically in the RPL group. Although embryos from RPL patients showed relatively faster developmental kinetics, morphokinetic speed alone did not predict chromosomal competence. Instead, embryo quality remained the key determinant of aneuploidy, while the association between direct cleavage and aneuploidy highlights the potential clinical value of TLM when combined with PGT-A. These findings support a complementary role for TLM in embryo assessment in RPL patients.

Introduction

Early and spontaneous pregnancy loss is one of the most common complications encountered during pregnancy. Recurrent pregnancy loss (RPL) is defined as two or three consecutive miscarriages occurring before the 20th week of gestation (Coulam *et al*., 1997). Approximately 20% of clinically recognized pregnancies result in spontaneous abortion, with nearly 75% occurring within the first 12 weeks of pregnancy (Kasak *et al*., 2023). RPL is considered a major cause of first-trimester pregnancy loss and has been associated with advanced maternal age (AMA) as well as genetic, endocrine and immunological factors (Ozer *et al*., 2023). Most couples undergoing assisted reproductive technologies (ART) have a history of RPL (ASRM Practice Committee, 2012). Many studies have investigated the relationship between genetic and clinical factors in RPL (Carbonnel *et al*., 2021; Klimczak *et al*., 2021; Pei *et al*., 2019; Shahine and Lathi, 2015; Yuksel *et al*., 2024). Genetic analyses have demonstrated that both female or male partners may harbour gene mutations or chromosomal abnormalities (Colley *et al*., 2019; Lanasa *et al*., 2001; Park *et al*., 2022). In a subset of RPL cases, pregnancy loss occurs despite the absence of detectable parental genetic abnormalities, suggesting a potential role for embryo aneuploidy (Blue *et al*., 2019). Aneuploidy represents a major embryonic cause of implantation failure, preimplantation genetic testing for aneuploidy (PGT-A) is frequently incorporated into ART treatments strategies for patients with RPL (Weissman *et al*., 2017).

Time-lapse monitoring (TLM) allows continuous and detailed assessment of embryo developmental dynamics. Although conflicting evidence exists regarding its clinical benefit, TLM provides valuable morphokinetic information that may assist in embryo selection or deselection by reflecting embryonic developmental competence (Herrero and Meseguer, 2013; Park *et al*., 2015). Nevertheless, TLM is generally considered an adjunctive tool that supports, rather than replaces, conventional embryo assessment methods (Campbell *et al*., 2013; Chavez *et al*., 2012; Pennetta et. al., 2018; Vera-Rodriguez *et al*., 2015).

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Despite the increasing use of both TLM and PGT-A in ART practice, the relationship between embryo morphokinetic patterns and chromosomal status in patients with RPL remains incompletely understood. In particular, it is unclear whether morphokinetic behaviour differs between RPL patients and infertile controls when embryo ploidy is taken into account, and which embryo-related parameters are most strongly associated with aneuploidy risk in this population. We hypothesized that embryos derived from patients with RPL exhibit distinct morphokinetic characteristics and lower euploidy rates compared with unexplained infertility (UEI) controls. Therefore, the present study aimed to comprehensively evaluate embryo morphology, morphokinetic characteristics and ploidy outcomes in RPL patients compared with UEI controls using a TLM system combined with PGT-A.

Materials and methods

Study design, ethical approval and study population

All procedures were performed in compliance with relevant laws and institutional guidelines and were approved by the Istanbul Yeni Yüzyıl University Clinical Research Ethics Committee (approval number: 07.04.2022/24). Written informed consent was obtained from all participants. This retrospective study was conducted in Istanbul Memorial Şişli Hospital ART&Reproductive Genetics Center between April 2021 and November 2022.

The study population comprised 100 patients with a history of RPL and 90 patients with UEI as controls. Although the American Society for Reproductive Medicine defines RPL as two first-trimester clinical pregnancy losses confirmed by ultrasonography or histopathological examination (Pennetta *et al.*, 2018), definitions of RPL vary across clinical guidelines and research settings (Chester *et al.*, 2022; Ozer *et al.*, 2023; Turesheva *et al.*, 2023). In particular, a threshold of three or more consecutive pregnancy losses (≥3) has traditionally been applied in epidemiological and research-based studies to ensure a more homogeneous study population and reduce inclusion of sporadic losses (Chester *et al.*, 2022; Ozer *et al.*, 2023; Shahine and Lathi, 2015; Turesheva *et al.*, 2023). Therefore, in the present study, RPL was defined as ≥3 pregnancy losses.

To minimize confounding factors, only UEI patients with an adequate number of high-quality blastocyst-stage embryos without additional poor prognostic risk factors were included. All UEI patients were normoresponders with normal baseline fertility test results. PGT-A was performed in all cases to optimize embryo selection and reduce the time for pregnancy.

Patients with non-PGT-A cycles, chromosomal translocations, Mendelian genetic diseases, maternal age >38 years, endometrial or uterine abnormalities (including Müllerian anomalies, severe endometriosis/adenomyosis, Asherman syndrome, or thin endometrium (<7mm)), or partners with abnormal sperm parameters according to the World Health Organization 2021 criteria were excluded from the study. A total of 1169 embryos (RPL: 634, UEI: 535) cultured in the TLM system were included in the analysis.

Controlled ovarian stimulation protocol

Controlled ovarian stimulation (COS) was initiated using individualized gonadotropin dosing based on patient characteristics including age, body mass index (BMI), antral follicle count and anti-Müllerian hormone (AMH) levels. Recombinant follicle-stimulating hormone (rFSH) (Gonal-f®, Merck, Switzerland), a combination of rFSH and recombinant luteinizing hormone (rLH) (Luveris®, Merck, Switzerland) or human menopausal gonadotropin (hMG) (hMG®, Ferring, Switzerland) were used for COS.

A gonadotropin-releasing hormone (GnRH) antagonist (Cetrotide®, Merck-Serono or Orgalutran®, MSD) was administered when at least one follicle was reached a diameter of ≥14 mm. Follicular development was monitored by transvaginal ultrasonography at regular intervals. Final oocyte maturation was triggered with 250 mcg recombinant human chorionic gonadotropin (rhCG) (Ovitrelle®, Merck, Switzerland) when at least two dominant follicles reached a diameter of 17 mm. Oocyte retrieval (OPU) was performed 36 hours later under transvaginal ultrasound guidance.

Embryo culture and morphological assessment

Embryos were cultured in single-step culture media (LifeGlobal®, Cooper Surgical, Belgium) supplemented with 10% human serum albumin (Life Global®, Cooper Surgical, Belgium) using 25-µl EmbryoSlide® (Vitrolife, Sweden) wells overlaid with 1.5 ml paraffin oil (Life Global®, Belgium). Culture dishes were equilibrated overnight in a TLM System (EmbryoScope™, Sweden) under controlled conditions of 6% CO_2, 5% O_2, 37°C and pH 7.26-7.30.

Embryos were cultured until day 5/6 with media refreshed on day 3. Blastocysts were graded according to Gardner's classification and categorized as follows: top-quality (TQ: Hatched AA, 6AA, 5AA, 4AA), good-quality (GQ: Hatched AB/BA/BB, 5AB/BA/BB, 4AB/BA/BB, 3AA), moderate-quality (MQ: 3AB/BA, 2AA), or poor-quality (PQ: remaining embryos).

Time-lapse monitoring and morphokinetic assessment

Embryo morphokinetic parameters were annotated using the EmbryoViewer (Vitrolife, Sweden) software by the same experienced embryologist, who was blinded to embryo ploidy status and clinical outcomes. Recorded time points (hours post-ICSI) included the appearance of two pronuclei (tPNa), pronuclear fading (tPNf), cleavage to two cells (t2), subsequent cleavages (t3, t4, t5, t6, t7, t8 and t9+), start of compaction (tSC) and morula stage (tM), start of blastulation (tSB), blastocyst formation (tB) and expanded blastocyst stage (tEB).

Developmental abnormalities, including direct uneven cleavage (DC; transition from one to three cells) and excluded/extruded blastomeres (EB) at the blastocyst stage, were also documented for each embryo.

Trophectoderm biopsy and PGT-A analysis

Artificial zona opening was performed on day 3 using a diode laser (RI Saturn 3, England). On day 5/6, five to eight trophectoderm cells were biopsied using a 30mm inner diameter biopsy pipette (Origio, Denmark) with the flicking method. The trophectoderm biopsy was not performed on PQ embryos.

Next-generation sequencing was used for PGT-A analysis. Genomic DNA amplification and sequencing were carried out using the ReproSeq™ kit (Thermo Fisher Scientific, USA) on the *Ion Torrent™ S5™* platform (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. Data analysis was performed using the Ion Reporter™ software (versions 5.2 and 5.6) (Thermo Fisher Scientific, USA).

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Table 1. Demographic and clinical characteristics of the study groups

Parameters	RPL ($n = 100$)	UEI ($n = 90$)	p-value
Maternal age (year)	33.12 ± 3.70	33.93 ± 3.21	0.153
Paternal age (year)*	35.51 ± 4.48	36.44 ± 5.24	0.189
BMI (kg/m^2)	24.48 ± 4.50	23.04 ± 3.80	**0.009**
AMH (ng/ml)	2.62 ± 1.92	2.80 ± 1.57	0.104
Basal FSH (mIU/mL)*	8.96 ± 3.27	8.79 ± 2.34	0.931
Duration of infertility (year)	4.37 ± 2.92	3.71 ± 3.49	**0.043**
Duration of stimulation (day)*	11.18 ± 1.56	11.01 ± 1.32	0.434
Previous IVF cycles (n)	2.86 ± 2.24	2.21 ± 1.87	**0.033**

Values were given as number (n) or mean ± standard deviation (SD). RPL: repeated pregnancy loss, UEI: unexplained infertility, BMI: body mass index, AMH: anti-mullerian hormone, FSH: follicle-stimulating hormone. Intergroup comparisons were made using Student's t-test* and Mann-Whitney U test according to the distribution of data. Bold values are statistically significant ($p < 0.05$).

Statistical analysis

Statistical analyses were conducted using IBM SPSS software (version 25). Data distribution was assessed using the Kolmogorov-Smirnov test. Normally distributed continuous variables (paternal age, duration of ART stimulation, two pronuclei (2PN)) were compared using Student's t-test, while non-normally distributed variables (remaining parameters) were analyzed using the Mann-Whitney U test. Categorical variables were compared using the chi-square test.

Comparisons presented in Table 1 were performed for descriptive purposes to assess baseline characteristics between groups; therefore, no formal adjustment for multiple comparisons was applied. Stepwise-backward logistic regression was performed to identify clinical and embryological risk factors associated with aneuploidy, and results were expressed as odds ratios (OR) with 95% confidence intervals (CIs). Variables or categories with no observations or no outcome events were excluded from the regression model to improve model stability and clarity. Data are presented as mean ± standard deviation (SD), mean ± standard error (SE) or frequencies (%). A p-value < 0.05 was considered statistically significant.

Results

Patient demographics and baseline characteristics

The demographic and clinical characteristics of the study groups are summarized in Table 1. No statistically significant differences were observed between the RPL and UEI groups with respect to maternal age, paternal age, AMH levels, basal FSH levels, or duration of COS ($p > 0.05$). However, BMI (RPL: 24.48 ± 4.50 vs. UEI: 23.04 ± 3.80; $p = 0.009$), duration of infertility (4.37 ± 2.92 vs. 3.71 ± 3.49; $p = 0.043$) and the number of previous ART cycles (2.86 ± 2.24 vs. 2.21 ± 1.87; $p = 0.033$) were significantly higher in the RPL group than in the UEI group.

Cycle outcomes and embryo morphology

Cycle characteristics and laboratory outcomes are presented in Table 2. No statistically significant differences were observed between the study groups in terms of the number of follicles ≥17mm on the trigger day, total oocytes retrieved, metaphase II (MII) oocytes, or normally fertilized oocytes (2PN) ($p > 0.05$). Oocyte maturation, fertilization and blastulation rates (%) were comparable between the RPL and UEI groups ($p > 0.05$).

The distribution of embryo morphological quality was similar in both groups, with comparable proportions of TQ embryos (RPL: 47.6% vs. UEI: 48.2%; $p > 0.05$). The frequency of developmental abnormalities, including DC or EB, did not differ significantly between the groups (RPL-DC: 14.7% vs. UEI-DC: 16.7%; RPL-EB: 11.5% vs. UEI-EB: 8.5%; $p > 0.05$). In contrast, the euploidy rate was significantly lower in the RPL group compared with the UEI group (43.5% vs. 52.3%; $p = 0.018$) (Table 2).

Embryo morphokinetics analysis

When embryo morphokinetic parameters were evaluated independently of PGT-A results, significant differences were observed between the RPL and UEI groups for t9 and tSC (Table 3). Embryos derived from RPL patients demonstrated faster developmental kinetics compared with those from UEI patients (t9 (mean ± SE): 67.30 ± 0.46 vs. 69.15 ± 0.58 h, $p = 0.012$; tSC (mean ± SE): 78.62 ± 0.46 vs. 80.61 ± 0.51 h, $p = 0.004$) (Table 3). However, when only euploid embryos were analyzed, no statistically significant differences were observed in any morphokinetic parameters between RPL and UEI groups (Table 4).

Comparisons of euploid and aneuploid embryos within each group revealed distinct patterns. In the RPL group, no statistically significant differences were observed between euploid and aneuploid embryos for any of the evaluated morphokinetic parameters ($p > 0.05$). In contrast, within the UEI group, euploid and aneuploid embryos demonstrated significantly different developmental timings. Aneuploid embryos in the UEI group demonstrated significantly delayed developmental timing compared with euploid embryos, including t4, t6, t7, t8, t9, tSC, tSB, tB and tEB (Table 5).

Within the RPL cohort, euploid embryos exhibited t4, t6, t7 and t8 values comparable to those observed in aneuploid embryos from the UEI group; however, these values were not statistically different from the aneuploid embryos within the RPL group. Notably, t9, tSC, tB and tEB values of euploid embryos in the RPL group were observed earlier than those of euploid embryos in the UEI group (Table 5).

Aneuploidy risk assessment and logistic regression analysis

Logistic regression analysis was performed to evaluate clinical and embryological factors associated with embryo aneuploidy (Table 6). Embryo morphological quality emerged as the strongest predictor of aneuploidy in both groups. Compared with TQ embryos, GQ and MQ embryos exhibited a significantly increased risk of aneuploidy in both the RPL group (GQ OR: 2.20 95% CI: 1.519–3.196, $p < 0.001$; MQ OR: 3.85 95% CI: 2.036-7.297, $p < 0.001$ and the UEI group (GQ OR: 2.24 95% CI: 1.293–3.899, $p = 0.004$; MQ OR: 3.00 95% CI: 1.175–7.704, $p = 0.022$).

Additionally, DC was identified as a 3.4-fold risk factor for aneuploidy exclusively in the RPL group, whereas no significant association was observed in the UEI group. Other clinical parameters including maternal age, paternal age, female BMI and the presence of EB at the blastocyst stage, were not significantly associated with an increased risk of aneuploidy (Table 6).

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Table 2. Cycle characteristics and embryo development outcomes of RPL and UEI groups

Parameters	RPL ($n = 100$)	UEI ($n = 90$)	p-value
≥17 mm follicle count on trigger day	6.49 ± 3.45	7.02 ± 2.89	0.154
Total oocytes retrieved in OPU	10.88 ± 5.70	11.44 ± 5.02	0.433
MII	9.47 ± 4.88	9.69 ± 4.08	0.579
2PN	7.81 ± 4.05	7.96 ± 3.40	0.785
Oocyte maturation rate (%)	88.50 ± 12.65	86.72 ± 13.87	0.438
Fertilization rate (%)	84.29 ± 15.18	83.24 ± 14.30	0.470
Blastulation rate (%)	71.57 ± 20.91	69.87 ± 21.31	0.585
Embryo quality (%)			
Top	47.6%	48.2%	
Good	42.0%	39.2%	0.558
Moderate	10.4%	12.6%	
Excluded/extruded blastomere (%)			
(+)	11.5%	8.5%	0.181
(−)	88.5%	91.5%	
Direct cleavage (%)			
(+)	14.7%	16.7%	0.353
(−)	85.3%	83.3%	
PGT-A results (%)			
Euploid	43.5%	52.3%	**0.018**
Mosaic	9.6%	11%	
Aneuploid	46.9%	36.7%	

Values were given as number (n) or mean ± standard deviation (SD) and percentage (%). RPL: repeated pregnancy loss, UEI: unexplained infertility, MII: metaphase II, OPU: oocyte pick up, PN: pronucleus, +: presence, −: absence. Mann-Whitney U test was used to compare the parameters. Those found to be at the statistical significance level ($p < 0.05$) are indicated in bold.

Discussion

RPL has been recognized as a major contributor to first-trimester pregnancy loss (Colley *et al.*, 2019; Blue *et al.*, 2019; Ozer *et al.*, 2023; Park *et al.*, 2022; Shahine and Lathi, 2015; Turesheva *et al.*, 2023; Yuksel *et al.*, 2024). In the present study, clinical and embryo-related parameters were comparatively analyzed in PGT-A cycles of patients with UEI and RPL to identify factors associated with embryo aneuploidy and to clarify the relationship between morphokinetic behaviour and chromosomal status.

Baseline patient characteristics, ovarian reserve markers and cycle-related parameters were largely comparable between the RPL and UEI groups, including AMH levels, basal FSH concentrations and rates of fertilization and blastulation. Although some studies have reported lower AMH levels in RPL cases (Bunnewell *et al.* 2020; Shahine *et al.* 2016), others have found no association between AMH and pregnancy loss (Bliddal *et al.*, 2023; Yuksel *et al.*, 2024), supporting the heterogeneity of RPL pathophysiology. Similarly, previous studies evaluating euploid embryo transfer cycles have reported inconsistent associations between female age and pregnancy loss (Del Carmen Nogales *et al.*, 2021; Nielsen *et al.*, 2024; Yuksel *et al.*, 2024), underscoring the multifactorial nature of RPL.

In the present cohort, the duration of infertility and the number of previous ART cycles were significantly higher in the RPL group. While some studies have reported no association between infertility duration and pregnancy loss (Cimadomo *et al.*, 2021; Del Carmen Nogales *et al.*, 2021), others have suggested that repeated ART attempts and prior biopsy exposure may reflect cumulative reproductive burden rather than direct causality (Ozer *et al.*, 2023; Yuksel *et al.*, 2024). Differences in study design, inclusion criteria, age thresholds and outcome-based grouping across studies likely contribute to these discrepancies. BMI was modestly but significantly higher in the RPL group (RPL: 24.48 ± 4.50 vs. UEI: 23.04 ± 3.80, $p = 0.009$). Increased BMI has been associated with adverse ART outcomes and pregnancy loss in previous reports (Del Carmen Nogales *et al.*, 2021; Ozer *et al.*, 2023; Yuksel *et al.*, 2024; Nielsen *et al.*, 2024), particularly in women with obesity (Metwally *et al.*, 2010). However, BMI was not identified as an independent predictor of embryo aneuploidy in the current analysis, suggesting that its adverse effects may be mediated through non-embryonic mechanisms.

When morphokinetic parameters were evaluated independently of ploidy status, embryos derived from RPL patients demonstrated accelerated development, particularly at t9 and tSC. However, these differences were no longer evident when only euploid embryos were analyzed, indicating that faster developmental timing alone was not associated with chromosomal competence. In the UEI group, aneuploid embryos demonstrated significantly delayed cleavage and blastulation timings compared with euploid embryos, consistent with prior time-lapse studies linking chromosomal imbalance to altered developmental kinetics (Campbell *et al.*, 2013; Chavez *et al.*, 2012; Bamford *et al.*, 2022).

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Table 3. Comparing the morphokinetic parameters in study groups regardless of PGT-A results

	RPL ($n = 634$)	UEI ($n = 535$)	
	Mean ± Std. error	**Mean ± Std. error**	***p*-Value**
PNa*	8.41 ± 0.09	8.40 ± 0.10	0.552
PNf*	23.88 ± 0.17	24.06 ± 0.17	0.432
t2*	26.38 ± 0.17	26.68 ± 0.19	0.347
t3	36.56 ± 0.22	36.76 ± 0.24	0.538
t4*	38.66 ± 0.24	38.94 ± 0.26	0.309
t5	48.53 ± 0.35	49.14 ± 0.38	0.238
t6	52.38 ± 0.36	52.35 ± 0.38	0.952
t7	55.18 ± 0.39	55.41 ± 0.43	0.680
t8*	58.39 ± 0.42	59.20 ± 0.50	0.233
t9	67.30 ± 0.46	69.15 ± 0.58	**0.012**
tSC	78.62 ± 0.46	80.61 ± 0.51	**0.004**
tM	87.57 ± 0.41	87.81 ± 0.45	0.695
tSB	97.77 ± 0.41	98.25 ± 0.44	0.430
tB	104.59 ± 0.41	105.06 ± 0.45	0.448
tEB	110.47 ± 0.40	110.77 ± 0.45	0.620

Times indicate hours post ICSI. RPL: repeated pregnancy loss, UEI: unexplained infertility, PNa: time that PN appears, PNf: time that PN faded, t2: time to reach two cells, t3: time to reach three cells, t4: time to reach four cells, t5: time to reach five cells, t6: time to reach six cells, t7: time to reach seven cells, t8: time to reach eight cells, t9: time to reach nine cells, tSC: time to start compaction, tM: time to reach morula, tSB: time to start blastulation, tB: time to reach blastocyst, tEB: time to reach expanded blastocyst. *Student's t-test was performed for normally distributed parameters. Other parameters were compared using non-parametric Mann Whitney U test. Statistically significant results ($p < 0.05$) are shown in bold.

Table 4. Comparison of euploid embryo morphokinetics in the RPL and UEI cases

Morphokinetic parameters	Study groups		
	RPL ($n = 168$)	**UEI ($n = 191$)**	***p*-Value**
PNa	8.01 ± 0.13	8.11 ± 0.15	0.612
PNf	23.50 ± 0.27	23.43 ± 0.29	0.844
t2	25.89 ± 0.27	25.90 ± 0.32	0.978
t3	36.55 ± 0.37	36.53 ± 0.39	0.963
t4	38.23 ± 0.34	37.97 ± 0.38	0.619
t5	49.29 ± 0.54	49.44 ± 0.56	0.844
t6	52.55 ± 0.52	51.68 ± 0.61	0.277
t7	54.48 ± 0.59	53.80 ± 0.70	0.456
t8	56.86 ± 0.68	56.35 ± 0.69	0.602
t9	66.98 ± 0.79	67.79 ± 0.85	0.482
tSC	76.78 ± 0.77	77.78 ± 0.69	0.342
tM	86.02 ± 0.69	86.22 ± 0.69	0.843
tSB	96.32 ± 0.75	96.40 ± 0.64	0.932
tB	103.46 ± 0.69	103.75 ± 0.65	0.758
tEB	109.83 ± 0.69	109.95 ± 0.67	0.906

Values were given as mean ± standard error. Times indicate hours post ICSI. UEI: unexplained infertility, PNa: time that PN appears, PNf: time that PN faded, t2: time to reach two cells, t3: time to reach three cells, t4: time to reach four cells, t5: time to reach five cells, t6: time to reach six cells, t7: time to reach seven cells, t8: time to reach eight cells, t9: time to reach nine cells, tSC: time to start compaction, tM: time to reach morula, tSB: time to start blastulation, tB: time to reach blastocyst, tEB: time to reach expanded blastocyst. Student's t-test was used for the comparisons between study groups.

Nevertheless, existing evidence regarding the predictive value of morphokinetics for embryo ploidy remains inconsistent. In a study by Campbell et al., morphokinetic parameters of 98 blastocysts were examined and blinded to ploidy, and it was found that there were delays in the tSC stage of aneuploid embryos compared to euploid embryos (Campbell *et al.*, 2013). In addition, a meta-analysis examining morphokinetic timing variables found that delayed t8, t9, tB, tEB were associated with aneuploidy which is consistent with our results in UEI group (Bamford *et al.*, 2022). However, Del Carmen Nogales *et al.* (2021) questioned the reliability of morphokinetics alone, stating that clinical and laboratory parameters are not always discriminative in cases of miscarriage after euploid transfer (Del Carmen Nogales *et al.*, 2021). A systematic review has reported that no single or combined morphokinetic parameter consistently predicts embryo ploidy status across different studies, underscoring significant methodological variability in design, outcome measures and patient populations (Reignier *et al.*, 2018). Moreover, individual cohort studies have reported that certain abnormal morphokinetic behaviours may correlate with higher aneuploidy rates but not necessarily with different clinical outcomes when euploid embryos are transferred, further illustrating the complexity and inconsistency of the current evidence base (Yen *et al.*, 2025). In the study of Yuksel et al., no significance was obtained in terms of embryo morphokinetics between pregnancy loss and live birth groups after single euploid transfers as well (Yuksel *et al.*, 2024). In the study of McQueen et al., it was suggested that the miscarriage of a euploid embryo is independent from its morphokinetic behaviour (McQueen *et al.*, 2021). These findings support the established association between chromosomal imbalance and delayed embryo development. In contrast to the UEI group, no significant morphokinetic differences were observed between euploid and aneuploid embryos in the RPL group, suggesting that embryo developmental kinetics in RPL patients may be influenced by factors beyond chromosomal status alone. In the present study, accelerated t9 and tSC observed between study groups, independent of PGT-A results, were not associated with higher aneuploidy rates, particularly in the RPL cohort. This pattern suggests that embryo developmental kinetics in RPL patients may follow a distinct trajectory, in which chromosomal imbalance does not translate into the expected morphokinetic delay. Interestingly, euploid embryos from the RPL group exhibited earlier t9, tSC, tSB, tB and tEB values compared with euploid embryos from the UEI group. Although embryos in the RPL group exhibited earlier tEB, the interval between t9 and tEB did not show a consistent discriminatory pattern between euploid and aneuploid embryos, particularly within the RPL cohort. Importantly, the present study was not powered to assess the t9-tEB interval as an independent predictor of embryo ploidy, and this limitation should be addressed in adequately powered prospective studies. Nonetheless, the accelerated blastulation observed in euploid embryos derived from RPL patients may reflect a compensatory developmental response or altered regulatory mechanisms during the later cleavage stages. Although laser-assisted hatching was uniformly applied on day 3 across all embryos, minimizing its impact on comparative analyses, its potential effect on tEB annotation cannot be excluded. Therefore, tEB-related findings may not be directly extrapolated to embryos

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Table 5. Comparison of euploid vs. aneuploid embryo morphokinetics in the RPL and UEI cases

	RPL			UEI		
Morphokinetic parameters	**Euploid ($n = 168$)**	**Aneuploid ($n = 181$)**	*p*-Value	**Euploid ($n = 191$)**	**Aneuploid ($n = 134$)**	*p*-Value
PNa	8.01 ± 0.13	8.35 ± 0.16	0.107	8.11 ± 0.14	8.37 ± 0.19	0.276
PNf	23.50 ± 0.26	23.22± 0.25	0.460	23.43 ± 0.28	23.83 ± 0.33	0.354
t2	25.89 ± 0.27	25.60 ± 0.25	0.452	25.90 ± 0.31	26.31 ± 0.32	0.374
t3	36.55 ± 0.37	36.73 ± 0.33	0.724	36.53 ± 0.38	37.65 ± 0.41	0.053
t4	38.23 ± 0.34	38.0 ± 0.35	0.661	37.97 ± 0.37	39.18 ± 0.43	**0.038**
t5	49.29 ± 0.54	49.59 ± 0.58	0.698	49.44 ± 0.56	50.36 ± 0.62	0.281
t6	52.55 ± 0.51	52.02 ± 0.53	0.480	51.68 ± 0.60	53.73 ± 0.68	**0.027**
t7	54.48 ± 0.58	54.74 ± 0.58	0.755	53.80 ± 0.69	56.46 ± 0.83	**0.015**
t8	56.86 ± 0.68	57.33 ± 0.68	0.621	56.35 ± 0.69	59.66 ± 0.96	**0.005**
t9	66.98 ± 0.79	67.72 ± 0.73	0.491	67.79 ± 0.84	71.05 ± 1.10	**0.019**
tSC	76.78 ± 0.77	78.07 ± 0.72	0.223	77.78 ± 0.68	80.56 ± 0.81	**0.011**
tM	86.02 ± 0.69	87.06 ± 0.71	0.297	86.22 ± 0.68	88.05 ± 0.80	0.087
tSB	96.32 ± 0.74	97.14 ± 0.72	0.344	96.40 ± 0.64	99.42 ± 0.83	**0.004**
tB	103.46 ± 0.68	105.03 ± 0.72	0.423	103.75 ± 0.65	107.17 ± 0.87	**0.002**
tEB	109.83 ± 0.68	111.57 ± 0.65	0.114	109.95 ± 0.67	113.16 ± 0.92	**0.005**
t9-tSC	10.18 ± 0.56	10.52 ± 0.57	0.670	10.00 ± 0.62	9.87 ± 0.77	0.896
t9-tEB	44.28 ± 0.89	44.28 ± 0.87	0.997	41.93 ± 0.78	42.22 ± 1.29	0.842

Values were given as mean ± standard error. Times indicate hours post ICSI. UEI: unexplained infertility, PNa: time that PN appears, PNf: time that PN faded, t2: time to reach two cells, t3: time to reach three cells, t4: time to reach four cells, t5: time to reach five cells, t6: time to reach six cells, t7: time to reach seven cells, t8: time to reach eight cells, t9: time to reach nine cells, tSC: time to start compaction, tM: time to reach morula, tSB: time to start blastulation, tB: time to reach blastocyst, tEB: time to reach expanded blastocyst. Student's *t*-test was used for the comparisons between study groups. Statistically significant results ($p < 0.05$) are shown in bold.

cultured without artificial hatching. Together, these findings indicate that morphokinetic behaviour of euploid embryos in RPL patients differs from that observed in infertile controls, and that apparently normal developmental speed does not necessarily reflect implantation competence in this population. Thus, our findings support that TLM alone may not be sufficient for embryo selection in RPL patients and that the use of PGT-A plays a more critical role in this group.

DC (from 1 to 3 cells) was associated with a 3.4-fold increased risk of aneuploidy in embryos from RPL cases, supporting the findings of Zhan et al., who reported that DC is linked to lower blastocyst formation, poorer embryo quality and indirectly reduced euploidy rates (Zhan *et al.*, 2016). Abnormal morphokinetic events such as DC have previously been associated with compromised chromosomal integrity in preimplantation embryos, providing a mechanistic basis for the association observed in our RPL cohort (Bamford *et al.*, 2022). DC may represent visible manifestations of underlying mitotic errors, which are known to increase aneuploidy risk during the early cleavage stages due to error-prone embryonic mitosis and limited cell cycle checkpoint control (McCoy *et al.*, 2023). Although the mechanisms underlying RPL are multifactorial, accumulating evidence indicates a high prevalence of chromosomal abnormalities and genomic alterations in RPL cases, suggesting impaired genomic integrity as a contributing factor to recurrent loss (Tise and Byers, 2021). The observation that DC was associated with aneuploidy exclusively in the RPL group may therefore reflect intrinsic differences in embryo vulnerability rather than uterine or endometrial factors, which were carefully excluded in both cohorts. Embryos derived from RPL patients may harbour subtle defects in oocyte cytoplasmic competence or chromosomal segregation machinery, predisposing them to mitotic errors manifested as DC, whereas UEI embryos, despite comparable euploid morphokinetic profiles, may exhibit greater tolerance to isolated abnormal cleavage events. From a clinical perspective, these findings indicate that DC represents a biologically meaningful marker of aneuploidy risk in RPL patients and support the use of TLM as a complementary tool alongside PGT-A, rather than as a standalone method for embryo selection in this population.

Regression analysis identified, embryo morphological quality as the strongest predictor of aneuploidy in both groups. Although embryo quality was not different between our study groups, the overall prevalence of aneuploidy in RPL cases was higher than in the UEI group. Our results were consistent with previous research, showing increased aneuploidy rates in RPL patients (Liu *et al.*, 2020; Kort *et al.*, 2018). Capalbo et al. demonstrated that blastocysts with suboptimal morphology had significantly increased aneuploidy rates, reinforcing the predictive value of morphological grading in embryo selection (Capalbo *et al.*, 2014). The risk of aneuploidy increased with decreasing embryo quality in our study, which is compatible with previous research (Capalbo *et al.*, 2014). Other clinical factors such as maternal and paternal ages, female BMI and EB were not found to be statistically significant for the aneuploidy increase in the current study. Our results are similar to the findings of Bamford et al., which included 8147 embryos for ploidy prediction by machine learning in their study and stated that blastocyst expansion and trophectoderm morphology are significantly associated with the euploidy, while male age did not

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Table 6. Analysis of risk factors for aneuploidy in study groups (logistic regression analysis)

Study groups	Model	Independent variables	OR	95% CI		*p*-Value
				Lower	Upper	
RPL	**Euploid vs Aneuploid**	Female age 31–35 (Ref. ≤30)	1.209	0.796	1.835	0.374
		Female age 36–38 (Ref. ≤30)	1.034	0.608	1.758	0.902
		Male age 40–49 (Ref. ≤39)	0.956	0.569	1.607	0.866
		Female BMI 25–29.9 (Ref. <25)	0.821	0.546	1.234	0.342
		Female BMI ≥30 (Ref. <25)	1.180	0.596	2.335	0.635
		Good qulity embryo (Ref. top quality)	**2.203**	**1.519**	**3.196**	**<0.001**
		Moderate quality embryo (Ref. top quality)	**3.854**	**2.036**	**7.297**	**<0.001**
		Presence of excluded/extruded blastomere (Ref. absence)	1.003	0.517	1.947	0.993
		Presence of Direct Cleavage (Ref.Absence)	**3.401**	**1.602**	**7.246**	**0.001**
UEI		Female age 31–35 (Ref. ≤30)	1.175	0.617	2.239	0.623
		Female age 36–38 (Ref. ≤30)	1.107	0.521	2.352	0.792
		Male age 40–49 (Ref. ≤39)	0.677	0.308	1.490	0.332
		Female BMI 25–29.9 (Ref. <25)	1.164	0.628	2.159	0.629
		Female BMI ≥30 (Ref. <25)	1.305	0.394	4.322	0.664
		Good quality embryo (Ref.top quality)	**2.245**	1.293	3.899	**0.004**
		Moderate quality embryo (Ref. top quality)	**3.008**	1.175	7.704	**0.022**
		Presence of excluded/extruded Blastomere (Ref.absence)	1.095	0.377	3.184	0.866
		Presence of direct cleavage (Ref.absence)	2.695	0.933	7.812	0.067

A stepwise-backward logistic regression analysis was performed. Categories with no observations or no outcome events were excluded from the regression model to ensure interpretability of odds ratios (ORs). ORs indicate the relative likelihood of aneuploidy for each variable in comparison with the reference group. OR values greater than 1 denote an increased relative risk of aneuploidy. RPL: repeated pregnancy loss, UEI: unexplained infertility, BMI: body mass index.
Dependent Variable: euploid/aneuploid.
Hosmer Lemeshow Test for RPL $p = 0.185$ and for UEI $p = 0.255$.
Model for RPL $p < 0.001$, for UEI $p = 0.030$.

show a correlation with euploidy when stratified for female age (Bamford *et al*, 2023). Regarding female BMI and aneuploidy risk, our findings support the previous research in which BMI was not found to be associated with blastocyst euploidy (Goldman *et al*., 2015; Hallisey *et al*., 2022).

In the current study, although the distribution of EB and DC in all embryos was similar between RPL and UEI groups, DC was found associated with aneuploidy in the RPL group. These findings contradict the results of Watanabe et al. suggesting that DC and EB could not be associated with blastocyst euploidy since blastomeres showing DC were mostly excluded from blastocyst (Watanabe *et al*., 2023). Our results are also consistent with Zhan's study, which found that the presence of DC in RPL patients increases the risk of aneuploidy (Herrero and Meseguer, 2013; Zhan *et al.*, 2016). On the other hand, in the meta-analysis study of Bamford et al., reverse cleavage was reported to be associated with euploidy while DC was not (Bamford *et al*., 2022). In this context, the present study adds nuance by specifically evaluating morphokinetic patterns in a well-defined RPL cohort using combined TLM and PGT-A, thereby providing a more integrated assessment of embryo developmental dynamics and chromosomal status.

The strength of our study lies in the fact that all analyses were conducted in cases with a confirmed diagnosis of RPL defined as ≥3 miscarriages and potential confounding factors such as male factor infertility, AMA, genetic causes and endometrial pathologies were excluded. In addition, all embryo culture procedures and morphokinetic annotations were performed in a single laboratory using standardized protocols, and all time-lapse annotations were conducted by the same experienced senior embryologist who was blinded to embryo ploidy and clinical outcomes, thereby minimizing inter-observer variability.

The major limitation of the study is ART outcomes of the cases were not evaluated. The use of patients with UEI as the control group was a deliberate methodological choice. In ART-based studies, direct comparison with fertile couples is often not feasible due to ethical and practical constraints, particularly the inability to obtain comprehensive embryo morphokinetic and ploidy data from fertile populations. UEI patients represent a clinically relevant comparison group because they lack identifiable female or male factor infertility, genetic abnormalities, or known uterine pathology that could independently affect embryo development or chromosomal status. Alternative control groups such as tubal factor or male factor infertility were considered; however, these groups may introduce additional biological confounders that could independently influence embryo morphokinetics and chromosomal outcomes. Tubal factor infertility often requires surgical or radiological confirmation and may overlap with underlying conditions such as endometriosis or adenomyosis, which have been shown to adversely affect ovarian reserve, oocyte competence and embryo development. Likewise, male factor infertility has been associated with altered embryo developmental dynamics and compromised chromosomal integrity, potentially mediated by increased sperm DNA fragmentation or abnormal sperm parameters (Kahraman *et al*., 2020; Pellegrini *et al*., 2024).

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Therefore, neither tubal factor nor male factor infertility can be assumed to represent a biologically neutral baseline for embryo morphokinetic or PGT-A analyses. Although the UEI group had previous ART attempts, this study was not designed to formally classify recurrent implantation failure (RIF). Contemporary ESHRE recommendations emphasize that RIF should not be defined solely by the number of IVF cycles, but rather by individualized estimates of implantation probability and repeated failure after transfers with an acceptable predicted chance of success (ESHRE Working Group on Recurrent Implantation Failure *et al*., 2023). Detailed implantation histories and cumulative implantation probability data-particularly from ART treatments performed outside our centre-were not uniformly available to allow standardized RIF classification. Therefore, the UEI cohort was not categorized according to RIF status but was treated as infertile controls without predefined implantation failure. Thus, the use of UEI controls allows for a more controlled evaluation of embryo morphokinetic behaviour and ploidy outcomes while minimizing confounding factors unrelated to RPL. Nevertheless, the use of UEI patients as controls may limit the generalizability of the findings. The results primarily apply to women undergoing ART treatment and may not be directly extrapolated to fertile populations or to patients with other infertility aetiologies. Therefore, the conclusions should be interpreted within the context of ART-treated RPL and UEI cohorts. In addition to these limitations, the retrospective design of the study introduces a potential risk of selection bias. Specifically, only embryos meeting predefined morphological and technical criteria were selected for PGT-A analysis, while PQ embryos or those not suitable for trophectoderm biopsy were excluded. This selection process may have resulted in an underestimation of the true prevalence of aneuploidy, particularly among lower-quality embryos that are more likely to harbour chromosomal abnormalities. Consequently, the observed associations between morphokinetic parameters and chromosomal status primarily reflect embryos eligible for biopsy and may not be fully generalizable to the entire embryo population in RPL patients. Therefore, the findings should be interpreted with caution when extrapolating to all embryos generated in RPL cycles, although this approach reflects routine clinical practice in ART settings.

In conclusion, although the morphokinetic characteristics of euploid embryos in RPL patients were not discriminative, our findings indicate that the use of TLM may be useful for the identification of cleavage anomalies such as DC. Importantly, accelerated morphokinetic parameters,including t9 and tSC, were not associated with an increased risk of aneuploidy in embryos from RPL patients, underscoring that faster developmental kinetics should not be interpreted as a surrogate marker of chromosomal competence in this population. In addition, despite the exclusion of factors such as AMA, male factor and known genetic causes that could independently increase aneuploidy risk in RPL patients, the strong association between embryo quality and aneuploidy represent important findings.Within this context, PGT-A may be considered a complementary tool for embryo selection in selected RPL cases, particularly to reduce the transfer of aneuploid embryos, although its impact on clinical outcomes requires further prospective evaluation.

In routine ART workflows, these findings underline the need for a cautious interpretation of morphokinetic data in patients with RPL. While TLM provides continuous and non-invasive developmental information, morphokinetic speed alone should not be used to infer embryo chromosomal status in this population. Instead, abnormal cleavage behaviours, particularly DC, may serve as an early warning sign during embryo assessment. In clinical decision-making, identifying such cleavage anomalies may assist embryologists in embryo ranking and in guiding discussions with patients regarding embryo selection strategies. When combined with PGT-A, time-lapse observations can contribute to a more structured and transparent embryo evaluation process, potentially improving counselling and expectation management in RPL cases.

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