Precision Journal of Disease Biology
Volume 1, **2026**, **pp.** 1-24, **Article ID:** PD2601205006

Systematic Review Article

Mitochondrial DNA Copy Number and Genomic Variants in Recurrent Pregnancy Loss and Recurrent Implantation Failure: A Systematic Review

Inshal Mehmood[1, *]

[1]*Patni Charitable Hospital, Karachi, Pakistan*

Article History

Received: 03 August, 2026 Revised: 05 October, 2026 Accepted: 08 October, 2026 Published: 19 November, 2026

Abstract:

Introduction: Recurrent pregnancy loss and recurrent implantation failure remain unexplained in a substantial proportion of patients after evaluation for established causes. Mitochondrial DNA number and genomic alterations have been proposed as markers of oocyte competence, embryo development, endometrial receptivity and placental function.

Methods: To synthesize evidence on associations between mtDNA copy number, deletion burden, sequence variants, haplogroups, heteroplasmy, recurrent pregnancy loss, recurrent implantation failure and related reproductive outcomes. Moreover, to distinguish direct evidence from contextual evidence derived from in vitro fertilisation and embryo-transfer populations, PubMed/MEDLINE, Scopus, and Web of Science Core Collection were searched on June 30, 2026.

Results: A total of 29 studies involving primary human studies evaluating mtDNA markers in recurrent pregnancy loss, recurrent miscarriage, early pregnancy loss, recurrent implantation failure or relevant implantation, miscarriage and live-birth outcomes were included. Due to substantial clinical and methodological heterogeneity, we synthesized findings narratively by mtDNA marker type, biological source, and clinical directness. Sequence variants showed the most consistent direct signal among the available direct recurrent pregnancy loss studies.

Conclusion: The available evidence suggests tissue- and context-specific biological and epidemiological associations between alterations in mitochondrial DNA and reproductive outcomes but does not validate mtDNA alterations as diagnostic biomarkers for recurrent pregnancy loss, recurrent implantation failure, or independent embryo-selection tests. Before clinical implementation, prospective multicentre studies using standardized assays, harmonized definitions, proper confounder control, and independent validation are required.

Keywords: Mitochondrial DNA, copy number, recurrent pregnancy loss, recurrent implantation failure, heteroplasmy, embryo implantation, miscarriage.

1. INTRODUCTION

Recurrent Pregnancy Loss (RPL) and Recurrent Implantation Failure (RIF) are different conditions of reproductive failure, which sometimes overlap, but cannot be used interchangeably. Infertility occurs in about one in every six adults around the world [1, 2], whereas recurrent pregnancy loss has severe physical, psychological, and economic implications [3]. These burdens support the clinical interest in exploring other mechanisms in patients whose recurrent reproductive failures are not explained by standard evaluation.

Definitions vary across professional guidelines. ESHRE defines RPL as the loss of two or more pregnancies. In contrast, the RCOG guideline distinguishes between RPL and early miscarriage by retaining the traditional definition of recurrent miscarriage: three or more first-trimester pregnancy losses, but moving the line of investigation of these early miscarriages to earlier in the pregnancy when clinically indicated [4, 5]. No single universally accepted numerical threshold defines Recurrent Implantation Failure (RIF), and its interpretation is influenced by maternal age, embryo competence, the number

*Address correspondence to this author at Patni Charitable Hospital, Karachi, Pakistan; E-mail: inshals1102@gmail.com

© 2026 Copyright by the Author.
Licensed as an open access article using a CC BY 4.0 license.

Cite as: Mehmood I. Mitochondrial DNA copy number and genomic variants in recurrent pregnancy loss and recurrent implantation failure: A systematic review. Prec. J. Dis. Biol. 2026; 1: 1-24, Article ID: PD2601205006.

and quality of embryo transfers, and, where available, embryo ploidy [6].

Established contributors to RPL include chromosomal, uterine, endocrine, immunological, thrombophilic and lifestyle factors, yet a clinically important proportion of cases remains unexplained after standard evaluation [4, 5, 7]. Mitochondrial biology is relevant to this residual group because oocytes, early embryos, endometrium, and placental tissues have stage-specific energy demands and depend on tightly regulated mitochondrial function.

Mitochondria support oocyte competence, fertilisation, early embryonic development, endometrial function and placentation [8-10]. Mitochondrial DNA (mtDNA) copy number reflects the number of mitochondrial genomes in a sample and may vary with mitochondrial biogenesis, cell composition, stress-related compensatory replication, and the normalization strategy used. In contrast, mtDNA sequence variants reflect genomic integrity and may have tissue- and heteroplasmy-dependent effects on oxidative phosphorylation [11-14].

Mitochondrial dysfunction can lead to reproductive failure through a biologically plausible pathway-namely oxidative stress. Mitochondria both generate and respond to Reactive Oxygen Species (ROS), and dysregulated oxidative stress has been associated with miscarriage and other pregnancy complications [15-17]. None of the above is specific to oxidative stress, but mitochondrial dysfunction can also be evaluated using mitochondrial DNA copy number and genetic changes.

Variations in study populations, biological tissues, reproductive outcomes, and assay methods make interpretation of the literature challenging. Few direct RPL/RIF studies exist. However, several larger in vitro fertilisation (IVF) studies compare embryo or trophectoderm mtDNA with implantation or live birth across populations that may not have RPL or RIF. Previous reviews focused on mitochondrial dysfunction or reproductive ageing. Accordingly, this review distinguishes direct RPL/RIF evidence from contextual embryo-transfer evidence and compares quantitative and qualitative changes in mtDNA across biological tissue compartments.

Population-Exposure-Outcome (PEO) framework was used because the review evaluated biomarker associations rather than therapeutic interventions [18]. The population comprised women or couples with RPL, RM, EPL, RIF, or a history of repeated failed embryo transfer. The exposures included quantitative and qualitative mtDNA alterations, including copy number, deletion burden, D-loop variants, coding-region variants, mt-tRNA variants, haplogroups, and heteroplasmy.

Accordingly, the review addressed the following question: Among women or couples with recurrent pregnancy loss or recurrent implantation failure, how are mtDNA copy number and mitochondrial genomic alterations associated with embryo competence, implantation, miscarriage recurrence, and live birth?

The synthesis aimed to investigate mtDNA quantity and genomic integrity; assess the effect of tissue source; and evaluate the clinical relevance of direct evidence over contextual evidence (rather than treating every reproductive study as equally applicable to RPL/RIF).

This review adds value by integrating these strands of evidence within a single tissue- and context-specific framework. Rather than treating mtDNA copy number, sequence variation and embryo-transfer findings as interchangeable evidence, the review distinguishes direct RPL/RIF associations from contextual IVF evidence. It evaluates biological association, prediction, and clinical utility separately.

2. METHODS

2.1. Design and Reporting Framework

This systematic review followed the PRISMA 2020 [19] reporting items. The review was not prospectively registered in PROSPERO; therefore, no registration number is available, and we acknowledge the absence of prospective protocol registration as a methodological limitation. Since the review evaluated associations between mitochondrial genomic markers and reproductive outcomes rather than therapeutic interventions, we planned a structured narrative synthesis before the final synthesis. Meta-analysis was not performed because studies differed substantially in population definitions, tissue source, mtDNA assays, normalization approaches, embryo ploidy control and outcome definitions. Additional limitations include single-reviewer screening and data extraction, heterogeneity in mtDNA assays and biological sources, limited replication of direct RPL/RIF studies, and inability to establish clinical predictive validity.

2.2. PEO Framework and Research Question

The PEO framework comprised of the following elements:

- Population- women or couples with RPL, RM, EPL, RIF, or repeated failed embryo transfer.
- Exposure- mtDNA copy number, deletion burden, sequence variants, haplogroups, or heteroplasmy.
- Outcomes- implantation, embryo competence, miscarriage or recurrent pregnancy loss, clinical pregnancy and live birth.

Studies providing direct RPL or RIF evidence constituted as primary evidence base. Those studies that retained general IVF or embryo-transfer as contextual evidence when they evaluated clinically relevant implantation, miscarriage, or live-birth outcomes. These contextual studies were not used to infer RPL/RIF-specific associations or causality, but to assess whether mtDNA-related biological or predictive patterns observed in embryo-transfer settings were consistent with the direct evidence.

2.3. Eligibility Criteria

Primary human studies in English between 2000 and 2026 that measured an of the mtDNA marker such as copy number or content, deletion burden, sequence variant or haplogroup, or heteroplasmy and correlated it with RPL, RM, EPL, RIF, or a predefined reproductive outcome were qualified in the study.

Eligible designs included case-control, cohort, analytical cross-sectional, molecular, and non-randomized intervention or follow-up studies. Reviews, editorials, commentaries, conference-only reports, animal studies, mechanistic studies without a relevant reproductive outcome, and duplicate or overlapping reports that did not provide additional information were excluded. General IVF studies were not counted as specific evidence for RPL/RIF if the study population did not expressly refer to RPL/RIF.

2.4. Information Sources and Search Strategy

PubMed/MEDLINE, Scopus and Web of Science Core Collection were searched using a combination of terms related to mtDNA, copy number/content, variants/mutations, deletion, heteroplasmy, haplogroup, D-loop, mt-tRNA and reproductive failure (*e.g.* RPL, recurrent miscarriage, RIF, implantation failure, embryo transfer, miscarriage and live birth). The exact strings, fields, and limits for each database are provided in Appendix **A** and Appendix Fig. (**A1**-**A3**).

Searches were limited to human studies published in English between January 2000 and June 2026. Database filters were applied for publication language and document type where available. Only original research articles with extractable primary human data were considered eligible. In addition to database searching, backward citation searching of included studies and relevant reviews was performed by screening reference lists. Forward citation searching was conducted using Scopus and Web of Science Core Collection to identify potentially eligible studies published after the original reports. No additional eligible studies were identified through backward or forward citation searching. All the records retrieved from the citation search were evaluated by the same screening questions. Before duplicate removal, 628 records were identified from the three databases: PubMed/MEDLINE (n = 164), Scopus (n = 333) and Web of Science Core Collection (n = 131). The search screenshots and exact database-specific syntax is specified in Appendix **A**.

2.5. Study Selection

Title and abstract screening along with full text screening were conducted based on the eligibility criteria. The screening process did not involve duplicate and independent screening, but rather a re-audition of the final set that were included for "publication type" and "relevant" to the eligibility criteria. It is admitted that there is a methodological limitation in the fact that independent duplicate screening is not done. To facilitate the selection process for the final exclusion categories and the number of exclusions, these are detailed in Appendix **B** and reconfirmed with the PRISMA flow. Appendix Table **B1** details the reasons and numbers for exclusion at the title/abstract and full-text stages. The databases-wise identification and duplicate-removal accounting are presented in Appendix Table **B2** and the stepwise PRISMA transitions in Appendix Table **B3**. A citation-level log linking each excluded full-text report to its specific exclusion reason was not retained.

2.6. Data Extraction

Data extraction was performed using the following predefined fields: author/year, country, design, population and sample size, control/comparator, biological material, mtDNA marker, assay method, reproductive outcome, quantitative effect data (where reported) and major limitations/relevance to the review question. Extraction was not independently duplicated. During revision, study-level sample, control, tissue, assay and quantitative outcome information was cross-checked against the available primary full text or authoritative primary article record. Unavailable effect estimates or confidence intervals were recorded as not reported rather than inferred. Supplementary Table **S1** provides the study-level verification matrix. The verification process was used to reconcile study-level sample sizes, comparator groups, tissue sources, assays and reported effect estimates before finalising. The PRISMA 2020 study-selection flow is presented in Fig. (**1**). The database-specific identification is reported in Appendix **A**, and the merged screening, eligibility and final-study allocation are reported in Appendix **B**, Table **B1**-**B4**. Since the screening and extraction were undertaken by a single reviewer, verification during revision was used to reduce transcription and eligibility errors but does not substitute for independent duplicate reviews.

2.7. Risk of Bias Assessment

Observational case-control, cohort, analytical cross-sectional, and case-series studies were appraised using the relevant Joanna Briggs Institute (JBI) Critical Appraisal Checklists [20] (Supplementary Table **S2A**). The two non-randomized ooplasmic-transplantation studies were assessed using ROBINS-I [21] because they evaluated an intervention and follow-up rather than a natural exposure. Domain-level ROBINS-I judgments were presented in Supplementary Table **S2B**, including confounding, participant selection, intervention classification, deviations from intervention, missing data, outcome measurement, and selective reporting domains.

Risk of bias was evaluated using design-specific tools rather than combining multiple instruments into a composite score (Supplementary Table **S2** and **S2B**). The risk-of-bias appraisal was undertaken and was not independently duplicated. It is acknowledged as a limitation in this study.

The domains under consideration included participant selection, comparability/confounding, validity of exposure or mtDNA measurement, outcome ascertainment, completeness of follow-up where applicable, and appropriateness of statistical analysis. Item-level assessments were completed study by study using the full design-specific JBI checklists (10-item case-control, 10-item case-series, and 11-item cohort checklists) and are reported in Supplementary Table **S2A**. Only Yes, No, Unclear, and Not applicable response categories were used; no “Partial” category or abbreviated checklist was used. The two non-randomized ooplasmic-transplantation reports were evaluated separately with ROBINS-I and are reported by domain in Supplementary Table **S2B**. Overall narrative judgements were derived from the pattern and severity of item/domain-level concerns rather than from a numerical score.

Risk-of-bias judgments weight the narrative synthesis, but risk judgments were not used to exclude studies. There was an independent consideration of methodological quality and clinical directness: a study that was more methodologically

robust on embryo transfer could give valid evidence on embryo mtDNA but may have been indirect on RPL/RIF as its population was not selected on those grounds. Table **1** presents the design-specific appraisal framework that is employed regarding the included studies. Completed study-specific item-level JBI assessments are presented in Supplementary Table S2A, while domain-level ROBINS-I assessments for the ooplasmic-transplantation reports are presented in Supplementary Table **S2B**. The JBI appraisal reported confounding factors for some case-control studies and strategies to address confounding, but did not identify the factors. Caution needs to be taken in interpreting these entries, as the latter means that a strategy was reported or judged to be present but does not mean that relevant confounders are fully identified and/or controlled. This apparent discrepancy should not be interpreted as effective confounder control, as it reflects the underlying study reports.

Among the 29 primary studies, six were judged at low overall risk of bias (Table **2**), 20 at moderate risk and three at high risk. Observational design alone was not treated as an automatic high-risk designation. Recurrent concerns included small or non-disease-specific cohorts, population-specific variant findings without functional replication, retrospective embryo-selection analyses, assay and normalization heterogeneity, incomplete confounder control, and limited external validation.

Larger embryo-transfer studies generally had stronger outcome ascertainment and, in several cases, controlled embryo ploidy through euploid-transfer designs. Their principal limitation for this review was clinical indirectness because most were not conducted specifically in RPL/RIF populations. Conversely, direct RPL/RIF studies were more clinically relevant but typically smaller and more vulnerable to population stratification, residual confounding, and limited replication.

The risk-of-bias assessment therefore supports cautious, hypothesis-generating interpretation. Neither mtDNA copy-number nor sequence-variant evidence is sufficiently validated for diagnostic or embryo-selection use. Future studies should use prospective multicentre designs, standardized assays, matched controls, prespecified confounder adjustment and independent validation.

2.8. Synthesis Strategy

A meta-analysis was not performed because effect measures and biological substrates were not sufficiently comparable. Narrative synthesis was organized into five prespecified domains:

(1) mtDNA sequence variants in RPL/RM.

(2) mtDNA copy number and deletion burden in pregnancy loss.

(3) Endometrial mtDNA copy number in RIF.

(4) Embryo/trophectoderm mtDNA content and implantation, miscarriage or live birth.

(5) Heteroplasmy and ooplasmic-transfer evidence.

Within each domain, direct RPL/RIF evidence was interpreted separately from contextual IVF evidence. Within each domain, findings were compared for direction and consistency of association, methodological strength, clinical directness and evidence of replication, rather than summarized solely by study-level statistical significance. The PEO framework used to structure the review question is presented in Table **3**, and the core concepts used to develop the database searches are summarised in Table **4**.

Table 1. Design-specific appraisal framework.

Study Design	Appraisal Tool	Rationale	Key Domains Assessed
Case-control RPL/RM genetic studies	JBI Case-Control Checklist	Direct comparison of affected women with fertile/healthy controls.	Case definition; control selection; comparability; exposure measurement; confounding; analysis.
Pregnancy-tissue / early-loss studies	JBI Case-Control Checklist	Cases and control pregnancy tissues were compared for mtDNA markers.	Selection; tissue comparability; assay validity; confounding; outcome classification; analysis.
Case series / mutation-screening without controls	JBI Case Series Checklist	Some mutation-screening studies did not include a parallel control group.	Clear inclusion criteria; consecutive/complete inclusion; valid measurement; demographics; reporting.
RIF endometrial study	JBI Case-Control Checklist	Endometrial mtDNA was compared between RIF cases and fertile controls.	RIF definition; control appropriateness; cycle timing; assay reliability; confounding.
IVF embryo-transfer outcome studies	JBI Cohort Checklist	Embryos/participants were followed from mtDNA measurement to implantation or pregnancy outcome.	Cohort selection; ploidy/embryo selection; exposure measurement; outcome ascertainment; follow-up; confounding.
Ooplasmic-transplantation studies	ROBINS-I	Non-randomized intervention/follow-up studies.	Confounding; participant selection; intervention classification; deviations; missing data; outcome measurement; selective reporting.

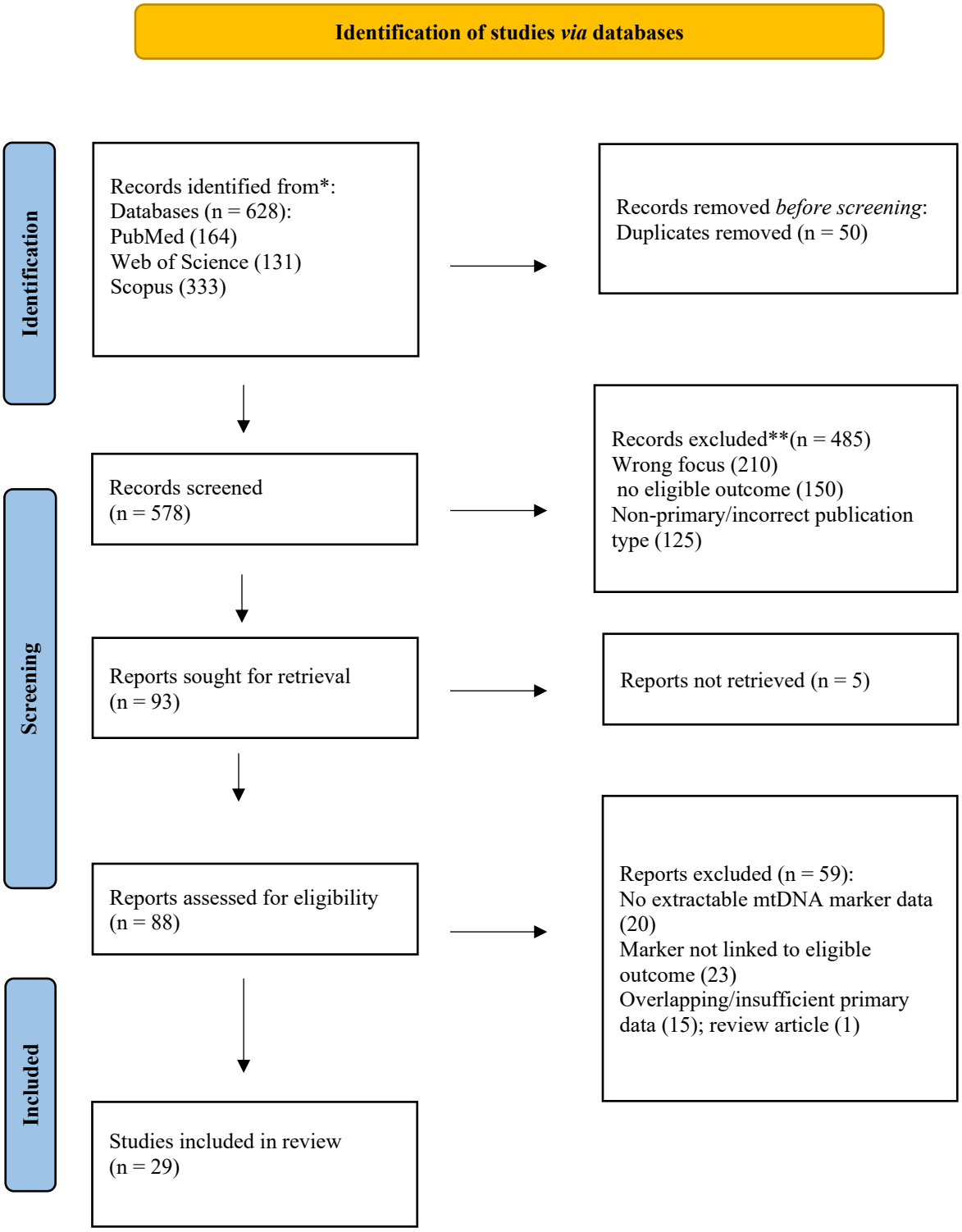


Fig. (1). PRISMA 2020 flow diagram.

Table 2. Risk-of-bias summary.

Overall Risk	Number of Studies	Studies
Low	6	Fragouli *et al.* (2017) [22], Ravichandran *et al.* (2017) [23], Treff *et al.* (2017) [24], Victor *et al.* (2017) [25], Lee *et al.* (2019) [26], Zhou *et al.* (2021) [27].
Moderate	20	Kaare *et al.* (2009) [28], Seyedhassani *et al.* (2010a, 2010b) [29, 30], Vanniarajan *et al.* (2011) [31], Colagar *et al.* (2013) [32], Azadi *et al.* (2018) [33], Ye *et al.* (2020) [34], Ji *et al.* (2020) [35], Eker *et al.* (2021) [36], Mojodi *et al.* (2023) [37], Lledó *et al.* (2018) [38], Diez-Juan *et al.* (2015) [39], Fragouli *et al.* (2015) [40], Stigliani *et al.* (2014) [41], Klimczak *et al.* (2018) [42], Łukaszuk *et al.* (2022) [43], Podolak *et al.* (2022) [44], El-Damen *et al.* (2021) [45], Wang *et al.* (2021) [46], Chuang *et al.* (2023) [47].
High	3	Brenner *et al.* (2000) [48], Barritt *et al.* (2001) [49], Victor *et al.* (2019) [50].

Table 3. Population-exposure-outcome framework used to structure the review question.

PEO Component	Definition in This Review	Examples
Population	Women/couples with RPL, RM, EPL, RIF, or repeated failed embryo transfer	Recurrent miscarriage, recurrent implantation failure, early pregnancy loss
Exposure	Quantitative or qualitative mtDNA alteration	Copy number, deletion rate, D-loop variants, tRNA variants, haplogroups, heteroplasmy
Outcome	Clinical or embryological reproductive outcome	Embryo quality, implantation failure, miscarriage, live birth

Table 4. Core search concepts.

Concept	Example search terms
Mitochondrial DNA	"mitochondrial DNA" OR mtDNA OR "mitochondrial genome" OR heteroplasmy OR haplogroup OR "D-loop" OR "copy number" OR deletion
Recurrent pregnancy loss	"recurrent pregnancy loss" OR "recurrent miscarriage" OR "repeated pregnancy loss" OR "early pregnancy loss" OR miscarriage
Implantation failure	"recurrent implantation failure" OR "implantation failure" OR "failed embryo transfer" OR "embryo transfer outcome"
Assisted reproduction outcomes	IVF OR ICSI OR blastocyst OR trophectoderm OR embryo OR "live birth" OR implantation OR miscarriage

Note: *Appendix A provides exact database-specific search strategies for each database.*

3. RESULTS

3.1. Study Profile and Evidence Structure

Twenty-nine primary human studies met the criteria for final synthesis. Each included study was assigned to one mutually exclusive evidence category based on its primary population and clinical relevance. The complete study-level allocation is provided in Appendix Table **B4**. Studies were assigned to mutually exclusive evidence categories based on their primary population and clinical relevance: 10 direct reproductive-failure studies (nine RPL/RM/EPL studies and one direct RIF study), four direct/mixed RIF/RPL intervention or embryo studies, and 15 contextual embryo-transfer, implantation, miscarriage or live-birth studies. A study-to-domain mapping is provided in Appendix Table **B4** to ensure transparent allocation without double counting.

A study-level mapping of all 29 included studies is provided in Appendix Table **B4**. Each study was assigned once according to its primary population and clinical context to avoid double counting. Direct RPL/RM/EPL evidence was prioritised when studies directly investigated recurrent reproductive loss, whereas IVF embryo-transfer studies without a primary RPL/RIF population were classified as contextual evidence.

Table 5. Characteristics and principal findings of the 29 included primary studies.

Study / Setting	Population / Sample	mtDNA Marker / Method	Principal Finding	Appraisal and Relevance
1. Kaare *et al.,* 2009 [28] Finland/Europe	Recurrent miscarriage women *vs* controls; 48 RM + 48 controls	Maternal blood; heteroplasmic mtDNA variants; DHPLC + sequencing	Heteroplasmic mtDNA variants were found in both RM and controls; overall, the study did not support a strong causal role of maternal heteroplasmic mtDNA mutations in recurrent miscarriage.	Strength: Direct RPL topic; good control comparison Limitation: Small sample; mainly maternal blood, not all reproductive tissues Relevance: Use as negative/limited association evidence
2. Seyedhassani *et al.,* 2010 [29] Iran	Repeated pregnancy loss *vs* controls; 96 RPL + 96 controls	Peripheral blood; mtDNA D-loop mutations; mtDNA deletions; multiplex PCR + sequencing	No mtDNA deletions detected; D-loop point mutations were more frequent in the RPL group.	Strength: Direct RPL + mtDNA control-region analysis Limitation: D-loop only; functional validation absent Relevance: Use in mtDNA D-loop/RPL section
3. Seyedhassani *et al.,* 2010 [30] Iran	96 women with idiopathic repeated pregnancy loss; no parallel control group in this mutation-screening study	Peripheral blood; mt-tRNA Thr/Pro variants; sequencing	Four mt-tRNA Thr variants were reported: A15907G 2/96 (2.08%), A15924G 3/96 (3.12%), G15928A 10/96 (10.42%), and G15930A 2/96 (2.08%) in the full-text results table; T15972C in mt-tRNA Pro occurred in 1/96 (1.04%). The abstract reports G15930A as 3/96, so we used the full-text table.	Strength: Direct RPL mutation-screening evidence Limitation: No concurrent control group; variants require functional and external validation Relevance: Supportive hypothesis-generating mt-tRNA evidence only
Vanniarajan *et al.,* 2011 [31] India	100 women with RPL + 12 aborted fetal tissues; primary article also reported RPL patients for confirmation and age- and ethnicity-matched fertile controls.	The original report clearly described complete mitochondrial genome sequencing in the discovery cohort comprising 100 RPL women and 12 aborted fetal tissues. The sequencing status of the independent confirmation cohort (55 RPL patients)	Found 681 mtDNA variants, many in ND/Complex-I genes; the study linked these variants with RPL among Indian women.	Limitation: Cohort details and control groups require careful interpretation; the reported control cohort and confirmation groups were considered in the revised appraisal.
Colagar *et al.,* 2013 [32] Iran	33 RPL women + 100 controls	ND1 T4216C mutation; PCR + direct sequencing	T4216C found in 10/33 RPL women = 30% *vs* 11/100 controls = 11%, $p < 0.05$.	Strength: Clear case-control comparison with specific mutation Limitation: Small case sample; single variant focus Relevance: Use in ND1/Complex-I mutation section
Azadi *et al.,* 2018 [33] Iran	73 RPL women + 100 controls	Complex-I mtDNA variants; direct sequencing	Reported seven mtDNA complex-I variants including T4216C, A5153G, C10142T, C12062T, A12662G, G14179A, T14263C.	Strength: Direct RPL; variant-focused Limitation: Journal category weaker; functional proof absent Relevance: Use as coding-region mtDNA variant evidence
Ye *et al.,* 2020 [34] China	75 EPL cases + 75 healthy controls	Chorionic villi; mtDNA copy number + 4977-bp deletion rate; qPCR/CNV sequencing	EPL cases had higher mtDNA copy number ($p < 0.001$) and deletion rate ($p = 0.005$). Both were independently associated with EPL: CN OR 1.71 (95% CI 1.17-2.49; $p = 0.005$); DR OR 1.07 (95% CI 1.02-1.12; $p = 0.006$).	Strength: Direct pregnancy-tissue study with adjusted analysis Limitation: EPL rather than recurrent loss; single-centre cohort Relevance: Core copy-number/deletion evidence for pregnancy loss

Ji *et al.,* 2020 [35] China	150 villous samples: 75 spontaneous abortions + 75 induced abortions	Chorionic villi; mtDNA copy number + heteroplasmy; qPCR + NGS	mtDNA copy number: SA 566 (397–791) *vs* IA 614 (457–739), $p = 0.768$; logistic regression: $p = 0.196$; pathogenic mtDNA mutations higher in SA: 70.7% *vs* 54.7%, $p < 0.05$; ND2 mutation: 26.67% *vs* 13.33%, $p = 0.041$.	Strength: Strong stats; tissue-specific miscarriage evidence Limitation: Spontaneous abortion, not strictly recurrent loss Relevance: Use as strong miscarriage-tissue mutation evidence
Eker *et al.,* 2021 [36] Turkey	25 women with RIF + 25 fertile controls	Endometrial biopsy; MT-ND1 and MT-CO2 copy number by ddPCR; GAPDH normalization	Normalized MT-ND1 and MT-CO2 copy numbers were higher in RIF endometrium than in fertile controls; Mann-Whitney and ROC analyses were performed.	Strength: Most direct endometrial RIF mtDNA study Limitation: Small single-centre case-control study; no independent threshold validation Relevance: Core but exploratory RIF evidence
Mojodi *et al.,* 2023 [37] Iran	100 women with idiopathic recurrent miscarriage + 100 controls	Peripheral blood; mt-tRNA Cys/Tyr; PCR-SSCP + sequencing	Four variants were identified in cases: 5824C>T (MT-TC), 5836T>C, 5849C>T, and novel 5868T>A (MT-TY); bioinformatic analyses suggested possible pathogenicity.	Strength: Direct recurrent-miscarriage case-control evidence Limitation: Rare variants; population-specific; functional validation and replication absent Relevance: Recent supportive mt-tRNA evidence; not a diagnostic marker
Brenner *et al.,* 2000 [48] USA	23 recurrent implantation failure cases after ooplasmic transfer	Donor-recipient mtDNA heteroplasmy; mtDNA analysis	Demonstrated mtDNA heteroplasmy after human ooplasmic transplantation in RIF context.	Strength: Direct RIF context; clinically important Limitation: Intervention-based; not a natural RIF biomarker Relevance: Use only in RIF heteroplasmy/ooplasmic transfer subsection
Barritt *et al.,* 2001 [49] USA	Offspring after ooplasmic transplantation in RIF context; case/follow-up study	Donor/recipient mtDNA; mtDNA fingerprinting	Reported mitochondria/mtDNA inheritance issues in offspring from ooplasmic transplantation.	Strength: Human follow-up; RIF-related heteroplasmy Limitation: Not a conventional RPL/RIF cohort Relevance: Use carefully as heteroplasmy safety evidence
Lledó *et al.,* 2018 [38] Spain	159 non-mosaic euploid embryos from 142 couples undergoing single embryo transfer	Blastocyst/trophectoderm mtDNA analysis; comprehensive mtDNA testing	mtDNA >0.003 was associated with lower ongoing pregnancy (42.96% *vs* 17.65%, $p < 0.05$) and higher miscarriage (6.15% *vs* 40.00%, $p < 0.05$). Heteroplasmy count was not significantly associated with ongoing pregnancy.	Strength: Euploid single-transfer design with adjusted analysis Limitation: IVF cohort not restricted to RPL/RIF; threshold requires external validation Relevance: Contextual bridge evidence for IVF outcomes
Diez-Juan *et al.,* 2015 [39] Spain	270 single euploid embryo transfers (205 day-3; 65 day-5) plus 10 double-embryo transfers	Embryo mtDNA content; MitoScore/quantification	Higher MitoScore categories showed lower implantation in this cohort. Day-3 embryos with Ms >160 (n = 22) and day-5 embryos with Ms >60 (n = 7) did not implant.	Strength: Euploid-transfer study that generated a testable threshold hypothesis Limitation: Retrospective single-laboratory threshold; later studies did not consistently replicate it Relevance: Historical positive evidence; interpret with later contradictory studies
Fragouli *et al.,* 2015 [40] UK/USA	379 embryos (39 cleavage-stage and 340 blastocysts)	Embryo/trophectoderm mtDNA level; NGS/PGT-related analysis	Altered mtDNA levels associated with female age, aneuploidy, and implantation potential.	Strength: High-quality genetics journal; mechanistic relevance Limitation: Embryo-focused, not RPL/RIF-specific Relevance: Use in implantation potential section

Stigliani *et al.,* 2014 [41] Italy	699 day-3 embryo spent-culture-medium samples	Spent culture medium mtDNA/gDNA ratio; non-invasive quantification	Higher spent-medium mtDNA/gDNA ratio was associated with blastocyst development ($p = 0.0251$) and implantation of good-quality embryos ($p = 0.0452$).	Strength: Large non-invasive culture-medium dataset Limitation: Extracellular mtDNA is not equivalent to intracellular embryo mtDNA; contamination/standardization concerns Relevance: Contextual evidence for a distinct non-invasive compartment
Fragouli *et al.,* 2017 [22] UK/USA	199 transferred euploid blastocysts from 174 couples	Trophectoderm mtDNA quantification	190/199 had mtDNA below the prespecified threshold; 9/57 non-implanting embryos had elevated mtDNA, and none of the elevated group implanted. Ongoing pregnancy was 64% with normal mtDNA *vs* 0% with elevated mtDNA ($p < 0.0011$).	Strength: Blinded prospective non-selection design Limitation: Single-clinic transfer context and threshold portability remain concerns Relevance: Strong positive prospective evidence, balanced by later negative studies
Ravichandran *et al.,* 2017 [23] USA	1505 euploid blastocysts from 490 couples, 35 IVF clinics	Trophectoderm mtDNA level; qPCR after PGT-A/WGA	Among 282 transferred euploid blastocysts, 249 had normal mtDNA and 185 implanted (74.3%); none of the 33 blastocysts with elevated mtDNA implanted ($p < 0.0011$).	Strength: Large multicentre euploid embryo dataset Limitation: Embryo-focused, not direct RIF-only Relevance: Use as strong implantation-failure biomarker evidence
Treff *et al.,* 2017 [24] USA	187 patients; 374 sibling euploid blastocyst biopsies, with paired implantation comparison in the informative sibling-outcome subset	Trophectoderm mtDNA level	Found mtDNA levels did not predict reproductive potential of sibling embryos.	Strength: Strong negative comparator evidence Limitation: Embryo-only, not RPL/RIF cohort Relevance: Use as contradictory evidence
Victor *et al.,* 2017 [25] USA/UK	1,396 blastocysts from 259 patients; qPCR validation in 150 samples	Adjusted mtDNA quantification	After correction for chromosomal/genetic factors, mtDNA levels were broadly uniform and did not meaningfully differ by ploidy, maternal age, or implantation potential.	Strength: Large dataset with NGS and qPCR comparison Limitation: Retrospective single-centre analysis; method differences limit cross-study comparability Relevance: Key negative evidence against a universal mtDNA threshold
Klimczak *et al.,* 2018 [42] USA	259 patients; 1,510 blastocyst biopsies (717 euploid embryos in restricted analysis)	Embryonal mtDNA content	Higher mtDNA was associated with poorer embryo grade in the full dataset ($p = 0.003$), but the association disappeared in euploid embryos ($p = 0.834$); maternal age showed negligible correlation.	Strength: Large embryo dataset with euploid subgroup analysis Limitation: Retrospective; embryo-focused rather than RPL/RIF-specific Relevance: Shows confounding by ploidy/embryo characteristics
Łukaszuk *et al.,* 2022 [43] Poland	716 embryos from 275 PGT-A patients; includes RIF ≥2 and recurrent miscarriage ≥2 indications	Trophectoderm mtDNA/nDNA ratio from NGS data	Aneuploid embryos had higher mtDNA than euploid embryos, $p = 0.02$; day-5 blastocysts higher than day-6, $p = 0.001$; clinical outcome difference not significant, $p = 0.39$.	Strength: Includes RIF/RM indications; detailed embryo stats Limitation: Outcome association weak/non-significant Relevance: Use as supportive RIF/RM indication + implantation evidence

Podolak *et al.,* 2022 [44] Poland	69 couples; 314 cleavage-stage embryos	Cleavage-stage embryo mtDNA copy number	mtDNA content differed by chromosomal status (reported $p < 0.004$) but did not show a robust association with embryo morphology; final reproductive associations were not sufficiently consistent for clinical prediction.	Strength: Cleavage-stage NGS dataset Limitation: Retrospective single-centre study; stage-specific and not RPL/RIF-specific Relevance: Contextual developmental-stage evidence
Lee *et al.,* 2019 [26] Taiwan	1,617 embryos from 380 infertile couples; 267 transferred blastocysts analysed for implantation	Adjusted embryo mtDNA quantity	Implanted (n = 103) and non-implanted (n = 164) blastocysts had similar adjusted mtDNA (median 0.00097 *vs* 0.00088; $p = 0.21$).	Strength: Large double-masked prospective observational dataset Limitation: Single Asian centre; mtDNA associated with ploidy but not implantation Relevance: Strong negative evidence for implantation prediction
Victor *et al.,* 2019 [50] USA/UK	109 transferred blastocysts in 100 women	Trophectoderm mtDNA content	mtDNA did not differ significantly between implanted (n = 55) and non-implanted (n = 54) blastocysts; very high mtDNA did not preclude healthy pregnancy and birth.	Strength: Direct transfer-outcome counterexample to exclusion thresholds Limitation: Selected transfer cohort; retrospective Relevance: Important evidence against deselecting embryos solely for high mtDNA
El-Damen *et al.,* 2021 [45] UAE/IVF setting	355 single euploid frozen embryo transfers in 314 patients	Blastocyst/trophectoderm mtDNA content	Pregnancy rate 66.2% (235/355); clinical pregnancy 52.4% (186/355); miscarriage 5.6% (20/355). mtDNA did not differ between pregnant/non-pregnant transfers (p = 0.095) or clinical pregnancy/miscarriage ($p = 0.136$).	Strength: Single-euploid transfer with miscarriage endpoint Limitation: Retrospective; not RPL-specific Relevance: Strong negative evidence for miscarriage prediction
Zhou *et al.,* 2021 [27] China	316 euploid blastocysts from 233 couples; includes RIF/RM indications	Trophectoderm mtDNA content; NGS PGT-A data	No mtDNA difference across live-birth, miscarriage, and non-implantation groups ($p = 0.999$); no association with implantation ($p = 0.965$) or adjusted live birth ($p = 0.567$). Elevated *vs* normal mtDNA live-birth rates were 26.7% *vs* 33.6% ($p = 0.780$).	Strength: Live-birth endpoint; euploid embryo control Limitation: Not purely RPL/RIF-only Relevance: Use as negative live-birth biomarker evidence
Wang *et al.,* 2021 [46] China	769 euploid blastocysts in primary analysis; day-5 n = 355, day-6 n = 388, day-7 n = 26	Trophectoderm mtDNA content	Aneuploid blastocysts had higher mtDNA ($p < 0.001$). Day-5 euploid blastocysts had higher mtDNA than day-6/day-7 ($p < 0.001$); better trophectoderm quality was associated with lower mtDNA ($p = 0.026$). Lower mtDNA in day-5 euploid blastocysts was associated with better implantation/live birth, but mtDNA was not supported as an independent predictor.	Strength: Large euploid embryo dataset Limitation: Retrospective; embryo-development effects complicate clinical interpretation Relevance: Contextual positive association with explicit limits on prediction
Chuang *et al.,* 2023 [47] Taiwan	1,675 embryos from 1,305 individuals; 1,340 euploid single-transfer outcomes analysed	Embryo/trophectoderm mtDNA content	Day-6 eSET (n = 139) showed lower mtDNA in adverse outcome groups (fetal heartbeat $p = 0.004$; ongoing pregnancy $p = 0.007$; live birth $p = 0.01$); no corresponding association was observed in day-5 eSET (n = 1,201).	Strength: Large stratified euploid-transfer dataset Limitation: Retrospective; association confined to day-6 subgroup Relevance: Strong evidence that developmental day modifies mtDNA-outcome associations

3.2. Thematic Analysis

3.2.1. mtDNA Sequence Variants in Recurrent Pregnancy Loss

Copy-number alterations may reflect compensatory mitochondrial biogenesis, cellular. composition, developmental stage, or tissue-specific responses rather than a single disease-associated mechanism. Kaare *et al.* [28] also identified heteroplasmic variants in both cases and controls and found no evidence supporting a strong causal role for maternal heteroplasmic mtDNA mutations. Vanniarajan *et al.* [31], Colagar *et al.* [32] and Azadi *et al.* [33], however, found enrichment of specific mtDNA variants (including Complex-I related variants) in affected populations.

The sequencing approach should be interpreted according to the cohort structure reported by Vanniarajan *et al.* [31]. Complete mtDNA sequencing was described for the primary investigated RPL cohort and fetal tissues, whereas the extent of sequencing within the confirmation and control groups was not clearly specified in the original publication.

The D-loop and mt-tRNA studies were similarly hypothesis-generating and were limited by their population-specific contexts. Seyedhassani *et al.* [29] reported a higher frequency of D-loop point mutations in women with repeated pregnancy loss, whereas the separate Seyedhassani *et al.* mt-tRNA Thr/Pro mutation-screening study [30] included 96 women with idiopathic RPL and no concurrent control group. Mojodi *et al.* [37] determined four mt-tRNA-Cys/Tyr variants in 100 Iranian women with Idiopathic Recurrent Miscarriage (IRM) and 100 controls. These findings do not necessarily imply universally causal mutations; rather, they identify candidate susceptibility loci that require further functional validation and external replication.

3.2.2. mtDNA Copy Number and Deletion Burden in Pregnancy Loss

Level of pregnancy-related copy number and deletions were not even. Ye *et al.* [34] analysed 75 EPL cases and 75 controls and reported significant increased copy number of mtDNA and increased chorionic-villus deletion rate in the EPL cases; in the logistic regression analysis, the copy number of mtDNA was independently associated with EPL (OR 1.71, 95% CI 1.17-2.49), as was the increased degree of chorionic-villus deletion (OR 1.07, 95% CI 1.02-1.12). In contrast, Ji *et al.* [35] found no significant difference in copy numbers between villus from spontaneous abortions and villus of induced abortions and potentially pathogenic mutations in the mtDNA were more common in the spontaneous abortion. These two studies pinpoint two different biological processes with the amount of mtDNA and the integrity of mtDNA genome.

The clinical significance of this difference is of interest. The number of copies may be affected by mitochondrial biogenesis, cell composition, trophoblast stress and normalization method; while sequence level changes may offer a more specific reflection of the integrity of the mitochondrial genome. Both should only be used in conjunction with the source of the tissue, embryonic chromosome status, and/or analytic method.

3.2.3. Endometrial mtDNA Copy Number in Recurrent Implantation Failure

Direct evidence of RIF was limited. Eker *et al.* [36] performed an analysis of 25 women with RIF and 25 fertile women by ddPCR normalised to the GAPDH gene for both MT-ND1 and MT-CO2. The copy-numbers of both mitochondrial targets were elevated in the RIF group. This is a clinically relevant study as the endometrium is directly investigated; however, the small sample size and single-centre case-control study design renders the results preliminary and further validation in larger independent cohorts is necessary.

The level of Endometrial mtDNA copy number may be affected by cycle timing, cell composition, the presence of inflammation and normalization strategy. Eker *et al.* [36] therefore report some preliminary evidence of an association between mitochondrial state in ECL and the diagnosis of RIF, though the study is small and cross-section at one site, does not so far reproduce a diagnostic threshold, predicts the diagnosis in an independent cohort, or shows clinical usefulness.

3.2.4. Heteroplasmy and Mitochondrial Replacement-Related Evidence in Implantation Failure

Brenner *et al.* [48] and Barritt *et al.* [49] provide intervention-based evidence from cytoplasmic transplantation in patients with prior implantation failure. These studies demonstrated donor-recipient mtDNA heteroplasmy after cytoplasmic transfer and in resulting offspring. Their relevance to this review is biological and safety-related; they do not estimate the prevalence or diagnostic significance of endogenous heteroplasmy in natural RIF.

Lledó *et al.* [38] evaluated 159 non-mosaic euploid embryos from 142 couples undergoing single embryo transfer. Elevated mtDNA above the study threshold was associated with a lower ongoing-pregnancy rate (42.96% *vs* 17.65%, $p < 0.05$) and a higher miscarriage rate (6.15% *vs* 40.00%, $p < 0.05$), while heteroplasmy burden was not significantly associated with ongoing pregnancy. Because the cohort was not restricted to RPL/RIF, this study is best considered bridge evidence rather than direct RIF evidence.

3.2.5. Embryonic mtDNA Copy Number and Implantation Potential: Positive Evidence

Several early studies reported an inverse association between embryonic mtDNA content and implantation. For example, no embryos with the highest mtDNA levels implanted, and Diez-Juan *et al.* [39] similarly reported declining implantation rates with increasing MitoScore. Ravichandran *et al.* [23] similarly reported that none of the 33 transferred euploid blastocysts with mtDNA levels above the study threshold implanted ($p < 0.0011$). Fragouli *et al.* [40] also reported associations between mtDNA levels and aneuploidy, implantation potential, and maternal age.

A distinct biological compartment was examined through analysis of spent culture medium. Stigliani *et al.* [41] analysed 699 day-3 spent-culture-medium samples and found that the mtDNA/gDNA ratio was positively associated with blastocyst formation ($p = 0.0251$) and implantation of good-quality

embryos (p = 0.0452). Findings derived from spent culture medium should not be interpreted as equivalent to measurements of intracellular embryonic mtDNA. Wang *et al.* [46] reported associations between mtDNA content and embryo characteristics and outcomes among day-5 euploid blastocysts; however, the findings did not support the use of mtDNA as a stand-alone predictor.

3.2.6. Embryonic mtDNA Copy Number and Implantation Potential: Negative and Conflicting Evidence

Subsequent studies challenged the reproducibility of a universal mtDNA threshold. Treff *et al.* [24] found that trophectoderm mtDNA levels did not predict the reproductive potential of sibling embryos. Victor *et al.* [25], analysing 1,396 blastocysts from 259 patients, reported broadly uniform mtDNA levels irrespective of ploidy, maternal age or implantation potential. Lee *et al.* [26] analysed 1,617 embryos from 380 couples and found no significant mtDNA difference between implanted and non-implanted transferred blastocysts (median 0.00097 *vs* 0.00088; p = 0.21). These negative studies directly weaken the case for using a single mtDNA threshold to discard embryos.

The conflicting embryo literature is plausibly explained by both methodological and biological heterogeneity: qPCR *versus* NGS-derived ratios; effects of whole-genome amplification; different normalization algorithms; day-3 *versus* day-5/day-6 sampling; untested, aneuploid or euploid embryos; fresh *versus* frozen transfer; and inconsistent outcome definitions. Consequently, a threshold derived in one laboratory or embryo-stage context cannot be assumed to transfer to another.

3.2.7. mtDNA Copy Number in Euploid Embryo Transfer, Miscarriage, and Live Birth

Euploid-transfer studies are especially informative because they reduce confounding by embryo aneuploidy. Fragouli *et al.* [22] prospectively evaluated 199 transferred euploid blastocysts and found that all nine embryos with mtDNA above the prespecified threshold failed to implant; ongoing pregnancy was 64% in the normal-mtDNA group and 0% in the elevated group ($p < 0.0011$). However, later euploid-transfer studies produced non-significant or directionally different results, demonstrating that ploidy control alone does not resolve assay and cohort heterogeneity.

El-Damen *et al.* [45] analysed 355 single euploid frozen embryo-transfer cycles and found no significant difference in mtDNA content between pregnant and non-pregnant transfers (p = 0.095) or between clinical pregnancies and miscarriages (p = 0.136); mtDNA therefore did not predict miscarriage. Zhou *et al.* [27] likewise concluded that mtDNA content was not a reliable live-birth screening biomarker after single euploid blastocyst transfer. Victor *et al.* [50] reported 109 transferred blastocysts and found no significant mtDNA difference between implanted (n = 55) and non-implanted (n = 54) embryos, including healthy births from embryos with very high mtDNA. These data argue against using mtDNA content as an exclusion criterion.

3.2.8. Embryo Developmental Stage and Sample Source as Modifiers of mtDNA Association

Developmental stage is an important modifier. Podolak *et al.* [44] studied 314 cleavage-stage embryos from 69 couples and found significant differences in mtDNA content by chromosomal status but no robust relationship with morphology or final clinical outcome. In 716 embryos from 275 PGT-A (Preimplantation Genetic Testing for Aneuploidy) patients, Łukaszuk *et al.* [43] found higher mtDNA in aneuploid than euploid embryos (p = 0.02) and higher values in day-5 than day-6 blastocysts (p = 0.001), while the clinical-outcome difference was non-significant (p = 0.39). Klimczak *et al.* [42] similarly found an association between mtDNA and poorer embryo grade across 1,510 biopsies (p = 0.003), but the association disappeared when analysis was restricted to 717 euploid embryos (p = 0.834).

Chuang *et al.* [47] further demonstrated stage dependence: among single-euploid transfers, lower mtDNA ratios were associated with adverse outcomes in day-6 embryos (fetal heartbeat p = 0.004; ongoing pregnancy p = 0.007; live birth p = 0.01), whereas no corresponding association was observed in the much larger day-5 transfer group. Collectively, these findings suggest that mtDNA content is more closely tied to embryo developmental context than to a universally reproducible clinical endpoint.

3.2.9. Integrated Comparative Synthesis

Across the direct evidence, sequence-variant studies report the most consistent RPL/RM signal, but positive findings are generally small, population-specific, and incompletely replicated. Direct RIF evidence is much more limited and rests mainly on one small endometrial case-control study. Embryo-transfer studies are methodologically larger and often better controlled for ploidy, but they are usually indirect for RPL/RIF.

Accordingly, internal validity and clinical directness were interpreted separately. General IVF findings were used to evaluate biological plausibility and prediction in embryo-transfer settings, not as evidence of RPL/RIF causality.

Across embryo studies, early threshold-based associations were not consistently replicated in sibling-embryo designs, adjusted quantification studies, or later euploid-transfer cohorts. Consequently, the evidence does not support mtDNA copy number as a universal threshold, a stand-alone embryo-selection test, or a diagnostic test for RPL/RIF.

Clinical meaning also differs by level of inference. Accordingly, the synthesis prioritised evidence of reproducibility, discrimination of clinically relevant outcomes and demonstrated improvement in patient management over statistical significance alone. A between-group mtDNA difference represents a biological association; an adjusted statistical relationship with a reproductive outcome represents an epidemiological association; reproducible discrimination of future implantation, miscarriage or live birth represents a predictive association; and evidence that using the marker improves selection, management or patient outcomes would establish clinical utility. The included literature contains

examples of biological and epidemiological associations, inconsistent evidence for prediction, and no convincing evidence that mtDNA-guided management improves clinical outcomes. Based on this, studies with small, population-specific findings were interpreted as hypothesis-generating unless there was support either through replication or more powerful comparative designs and were not given greater evidential weight because they were found statistically significant. In order not to mix up the concepts of statistical significance and clinical usefulness, the discussion separates these four levels of evidence in a very reflective manner (Table **6**).

These data support a tissue-specific model rather than a single mitochondrial biomarker. Maternal blood can indicate inherited background differences, pregnancy tissue can indicate loss-related mutation load, the endometrium may indicate receptivity-related mitochondrial strain, and embryo or culture-media measurements can indicate development. Future researchers ought to have a prospective test of these compartments instead of assuming that the compartments are interchangeable.

4. DISCUSSION

The data currently available suggests that association of mtDNA changes with reproductive outcomes is condition and method specific and depends on the type of biological tissue analyzed. Though direct RIF evidence is limited to rather small endometrial datasets, evidence of sequence level variation according to direct RPL was reported more often. Embryo-transfer studies were most convincing, but not consistent and in many studies populations did not meet the definition of RPL/RIF.

Table 6. Clinical interpretation of the synthesis by evidence domain.

Evidence Domain	Biological Association	Epidemiological Association	Predictive Association	Clinical Utility
Sequence variants in RPL/RM	Variant enrichment and recurrent locus-specific findings reported in several direct studies.	Some case-control differences were statistically significant, but replication across populations is limited.	No externally validated model predicts future miscarriage/RPL from these variants.	No evidence that variant-guided testing improves management or live-birth outcome.
Pregnancy-tissue mtDNA copy number/deletion	EPL tissue studies show altered copy number/deletion or mutation burden in some cohorts, but findings are not uniform.	Ye *et al.* [34] reported adjusted associations for copy number and deletion rate; Ji *et al.* did not find a copy-number association.	No prospective, externally validated prediction of future loss.	No validated diagnostic threshold or management benefit.
Endometrial mtDNA copy number in RIF	One small case-control study reported higher MT-ND1/MT-CO2 copy number in RIF endometrium.	The same small sample reported a group-level association, with ROC analysis.	No independent threshold validation or prospective prediction.	Clinical utility is unproven; not ready for diagnostic use.
Embryo/trophectoderm mtDNA content	Early studies reported threshold-related differences; later studies showed strong dependence on ploidy, developmental day, and quantification method.	Associations with implantation/live birth are inconsistent after controlling for embryo characteristics.	Threshold performance was not reproducible across sibling-embryo and euploid-transfer cohorts.	No evidence that mtDNA-guided selection improves outcomes over established embryo-selection approaches.
Heteroplasmy / ooplasmic-transfer evidence	Human ooplasmic-transfer studies demonstrate donor-recipient heteroplasmy and inheritance of donor mtDNA.	These intervention studies do not establish an endogenous association between RPL/RIF and disease.	No validated prediction of implantation or recurrent loss.	Relevant mainly to biological/safety interpretation, not RPL/RIF diagnostic utility.

Note: *Biological association = a difference in mtDNA biology between groups; epidemiological association = a statistical relationship with an outcome; predictive association = reproducible discrimination of a future outcome; clinical utility = evidence that using the marker improves selection, management, or patient outcomes.*

Results of direct reproductive-loss studies point to higher uniformity of the alterations of the sequence of mtDNA than of their copy number. The increased copy-number and deletion levels observed in Early Pregnancy Loss (EPL) tissue in Ye *et al.* [34] were not seen by Ji *et al.* [35] who found an increased frequency of potentially pathogenic variants but did not observe a significant difference in copy-number of mtDNA between EPL and induced abortion tissues. The conflicting results suggest that the amount of mtDNA and the integrity of the mtDNA genome are two distinct biological signatures and hence should not be considered as synonymous.

To differentiate between what is clinical "useful" and what is statistically significant, these 4 grades of evidence are clearly differentiated (Table **6**). In some studies, there were indications of biological or epidemiological differences; however, the predictive value of mtDNA- based evaluation of non-invasive measurements for reproductive outcomes remains to be evaluated and no study has borne out that a diagnosis based on mitochondrial DNA or embryo selection results in higher live birth rates or lower rates of miscarriage or alters care compared to standard care. It must be evident that recorded p-values, odds ratio analysis or ROC analysis cannot prove clinical utility.

There is a tissue-specific pattern since failure of the reproduction process may occur at various points, such as embryo competence before implantation, interaction between the embryo and the endometrium during implantation, and maintenance of pregnancy after implantation. Numerous differences in the requirements of the mitochondria exist among these stages, so that it is not likely that the same measurement of mtDNA would have the same meaning in maternal blood, villous tissue, endometrium, trophectoderm, and spent culture medium.

Of the direct RPL studies, mtDNA sequence variation was more consistent than copy number changes, and more variation existed among copy-number findings based on the biological context, tissue source and analytical techniques. In view of these modest successes by Kaare *et al.* [28] and the population specific nature of each of these studies, as well as the fact that most of the identified variants have not yet been functionally validated, it seems an inherently weak claim to describe any of these as simple causes of RPL.

A more conservative view is that the variants of mtDNA could affect susceptibility among specific populations. Association is necessary but not sufficient evidence for biological plausibility of complex I and mitochondrial translation variants as functional pathogenicity. Results need to be reproduced in other ancestry groups and have to be confirmed on a functional level.

Additionally, genome integrity and copy number have been shown to be uncoupled in studies of pregnancy tissue. EPL has a higher number of mtDNA copy number and mtDNA deletion burden than control in Ye *et al.* [34] but not found by Ji *et al.* [35] and they reported a higher frequency of potentially pathogenic mtDNA variants in spontaneous abortions compared to control. The results of these studies do not necessarily contradict each other since the quantity and mutational load of mtDNA would embody different aspects of mitochondrial biology.

There is limited direct evidence for RIF, which has not yet been translated to the clinic. With fewer individuals being studied (25 cases and 25 controls), Eker *et al.* [36] reported higher copy counts of endometrial-MT-ND1 and MT-CO2 in RIF. A diagnostic biomarker would need to be independently validated, have prespecified thresholds, and have a consistency of performance between laboratories, in addition to being reproducible and cycle timed, which would also require that endometrium be pathological.

Ooplasmic-transfer follow-up studies show that donor and recipient mitochondrial populations may persist in the oocytes, embryos, or offspring. These studies support the biological plausibility of heteroplasmy and the safety concerns, however they are not indicative of a common or diagnostic characteristics of RIF.

The risk of prematurely translating biomarkers containing risks has been reported in the literature related to embryo transfer. Preliminary studies using threshold criteria identified a high negative predictive value of high mtDNA. Later prospective studies and sibling-embryo studies, adjusted-quantification and euploid-transfer studies, however, have failed to demonstrate such a general relationship. Any changes in assay platform, day of the assay, embryo selection, embryo normalisation or transfer conditions will affect the apparent direction and strength of association.

Embryo transfer studies have not provided consistent evidence of correlation between increased mtDNA content and/or reproductive outcome. An important counter example does exist; according to Victor *et al.* [50] embryos containing high levels of "mtDNA" may lead to healthy pregnancies and healthy births. Unlike these findings, Lee and colleagues [26] reported no significant correlation between adjusted embryo mtDNA content with implantation, while Zhou and colleagues [27] found no association between embryo mtDNA content with euploid embryo outcomes of implantation or miscarriage or live birth. Overall, these results indicate that embryo mtDNA quantity is not likely to be an independent predictor of reproductive success.

The differences among these various studies might be explained by variations in embryo development, the level of quantification of mtDNA, the methods for normalizing the results of the prenatal results, patient selection criteria, and embryo ploidy control.

Consequently, heterogeneity should be viewed in a positive sense, and not as a problem. Three measurements involving information on varying mtDNA copy number within with the intracellular trophectoderm compartment, the extracellular compartment (in the culture medium) as well as information from the maternal blood, and information of the mutation burden within pregnancy tissues are biologically distinct. Putting them together would have made their meaning "soft" not hard or easier to interpret.

Currently these results should be considered as investigational use of the mtDNA test in RPL/RIF only and should not be substituted for a derived diagnosis, a proven embryo selection parameter. In any research use the exact

tissue, the assay, normalisation procedure, the ploidy state of the embryos, maternal age, environment of transfer and clinical endpoints should be reported.

However, future prediction models will require the incorporation of other well-established embryo and maternal features, such as ploidy and maternal age, and embryo morphology and endometrial features.

However, the sequencing of maternal blood and the sequencing of the pregnancy tissue may prove useful in RPL to distinguish between variants present in the background and those secondary to tissue lost during pregnancy. Standardisation of cycle timing and a predetermined RIF requirement, and multicentre replication, should all form part of endometrial research for RIF. The best design to determine if there is predictive information in the contribution of mtDNA besides other variables that would give greater value to embryo research would be a single euploid transfer to which the endpoint result was live birth.

Important, however, is that the available evidence that suggests that the use of mtDNA measurements as a standalone clinical biomarker is not supported at this time. The associations noted in individual studies should be explained by larger prospective cohorts of women using standardized assays for mtDNA, ploidy of the embryo, maternal factors, and appropriate clinical confounders.

4.1. Conceptual and Theoretical Framework

The conceptual framework derived from the synthesis distinguishes exposure of mtDNA on two bases: marker type as well as tissue compartment. The quantitative markers are CN and Deletion burden, while qualitative markers include D-loop, mt-tRNA variants, Coding Region variants, haplogroups and hysteroplasty. The observed clinical implications of these exposures will relate to mitochondrial energy production, oxidative stress and cellular competence and will vary depending on whether measured in maternal blood, in pregnancy tissue, in endometrium, in embryo/trophectoderm, or in culture medium.

The following schema is designed to explicate the different implications of the same nominal marker in different studies. The use of blood-based mtDNA will give a maternal background; pregnancy tissue will result in the capture of local mutation burden; mtDNA from the endometrium will reflect cell-stress related to receptivity; and embryo or spent-medium mtDNA could reflect developmental stage, cell number, stress or DNA release. Tissue source is therefore a biological variant not simply something of technical detail. This results in a tissue specific conceptual framework (Fig. **2**).

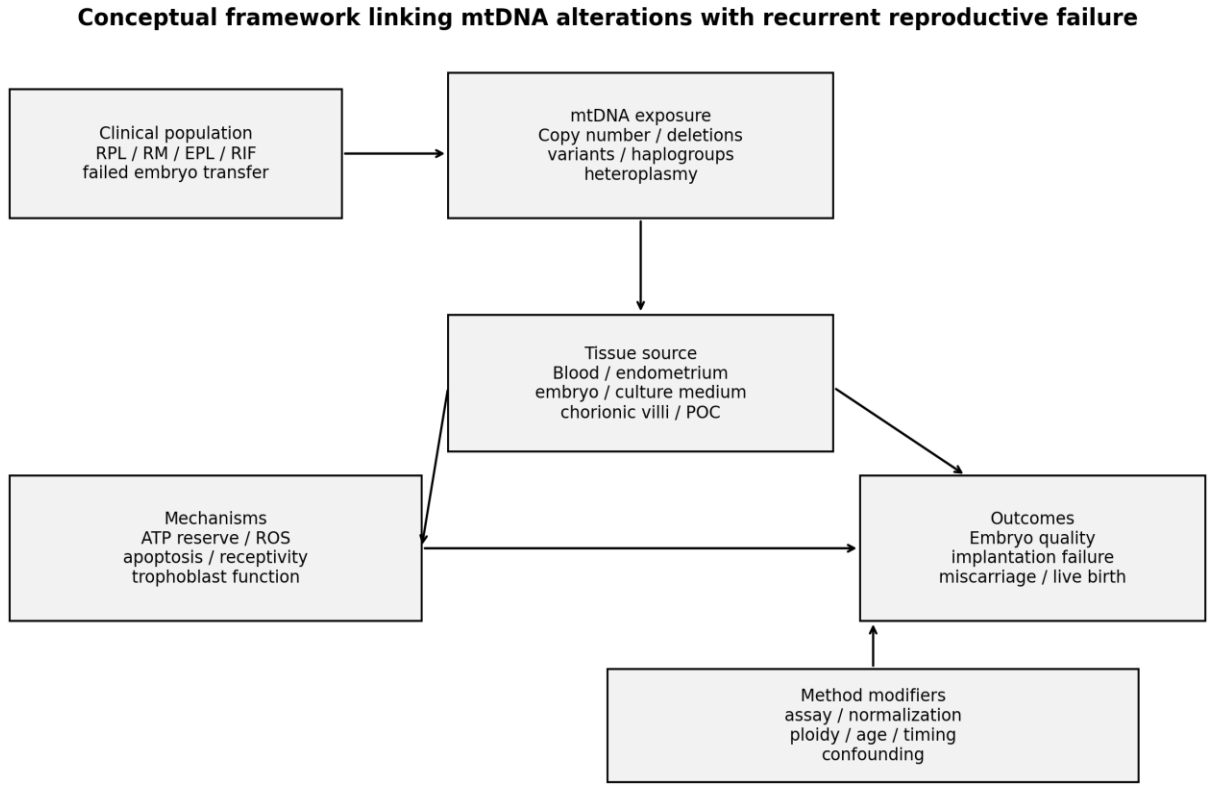


Fig. (2). Tissue-specific conceptual framework linking mtDNA copy-number and genomic-variant pathways with recurrent pregnancy loss, recurrent implantation failure and related reproductive outcomes.

STRENGTHS AND LIMITATIONS

The strengths of this review are that the evidence supporting IVF and direct RPL/RIF evidence was separated, considerations of the amount and number of mtDNA molecules and the level of integrity of the mtDNA sequence were considered separately, the evidence was originally synthesized across the maternal, pregnancy, endometrial, and embryonic compartments of the tissue, and design appropriate JBI or ROBINS-I appraisal was provided while separately considering clinical directness with respect to internal validity Supplementary Table **S2A** and **S2B**.

Important limitations remain. Direct RPL/RIF evidence is often few, and are population- and centre-specific, with many positive results being non-replicated and unfunctionalized. The review was not proprioceptively registered, and screening, data extraction and risk-of-bias appraisal were not duplicated separately, so that there is a risk of selection, extraction and appraisal error. Since there is no registration of all prospective protocols, deviations from the original review plan cannot be assessed independently. In the evidence, assay/normalization heterogeneity and inadequate controlling for maternal age and embryo ploidy, and limited external validation, constrict comparability and clinical translation. Other restrictions are the screening and data extraction by a single person, lack of uniformity in mtDNA assays and biological matrices, inadequate replication in direct RPL/RIF studies, and the lack of predictive validity in clinical use.

IMPLICATIONS AND RECOMMENDATIONS

Pregnancy-tissue paired sequencing, ancestry-aware replication in combination with functional annotations of origin from maternal and fatal tissues should be prioritized for future RPL studies. RIF studies need to be reproduced in larger multicentre studies of well-defined endometrial covariates with cycle timing. There should be no universal thresholds for mtDNA in pre-implantation embryos to use until assays are harmonized and incremental predictive value is proven in the cohort of single-euploid embryos they aim to transfer, which is prospectively determined.

Future studies on all the different domains should be explicit on RPL/RIF definition, use validated mtDNA assays, report on the extraction/amplification and normalization procedures, and also control maternal age and embryo ploidy (where applicable) and must include independent validation cohorts with clinically meaningful endpoints preferably live birth.

CONCLUSION

Biologically plausible, but varying associations between mitochondrial genomic changes and reproductive outcomes have been observed. The only consistently reported direct RPL signals are sequence variants, which lack adequate replication or functional validation in populations other than that in which they are found. One small case-control study indicates an association between increased copy numbers of mtDNA and RIF per unit endometrial area, but there is no established limit for diagnosis. Embryo mtDNA content is inconsistent in association on each platform, among each developmental stage and among each euploid-transfer cohort and therefore should not be used as sole criterion when selecting embryos. Thus, biological and epidemiological associations continue to be independent of predictive performance and clinical utility, which are lacking in RPL/RIF. There are a few direct RPL studies that show the best association signal at the sequence level with mtDNA alterations, but the available evidence is still not enough to confirm a diagnostic biomarker. Future prospective large-scale multi-center studies using standardised assays, carefully prespecified confounder control and external validation are needed before clinical implementation.

LIST OF ABBREVIATIONS

ATP	=	Adenosine triphosphate
CI	=	Confidence Interval
CN	=	Copy Number
CNV	=	Copy Number Variant/Variation
ddPCR	=	Droplet Digital Polymerase Chain Reaction
DHPLC	=	Denaturing high-performance liquid chromatography
D-loop	=	Displacement loop (mitochondrial DNA control region)
DR	=	Deletion Rate
EPL	=	Early Pregnancy Loss
ESHRE	=	European Society of Human Reproduction and Embryology
eSET	=	Elective Single Embryo Transfer
gDNA	=	Genomic DNA
IA	=	Induced Abortion
ICSI	=	Intracytoplasmic Sperm Injection
IRM	=	Idiopathic Recurrent Miscarriage
IVF	=	In vitro Fertilisation
JBI	=	Joanna Briggs Institute
MeSH	=	Medical Subject Headings
mtDNA	=	Mitochondrial DNA
MT-CO2	=	Mitochondrially encoded cytochrome c oxidase II
MT-ND1	=	Mitochondrially encoded NADH dehydrogenase 1
mt-tRNA	=	Mitochondrial transfer RNA
nDNA	=	Nuclear DNA
ND1 / ND2	=	NADH Dehydrogenase Subunit 1 / 2 (mitochondrially encoded)

NGS	=	Next-Generation Sequencing
OR	=	Odds Ratio
PCR	=	Polymerase Chain Reaction
PCR-SSCP	=	Polymerase Chain Reaction–Single-Strand Conformation Polymorphism
PEO	=	Population–Exposure–Outcome
PGT-A	=	Preimplantation Genetic Testing for Aneuploidy
PRISMA	=	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PROSPERO	=	International Prospective Register of Systematic Reviews
qPCR	=	Quantitative (real-time) Polymerase Chain Reaction
RCOG	=	Royal College of Obstetricians and Gynaecologists
RIF	=	Recurrent Implantation Failure
RM	=	Recurrent miscarriage
ROBINS-I	=	Risk Of Bias in Non-randomised Studies of Interventions
ROC	=	Receiver Operating Characteristic
ROS	=	Reactive Oxygen Species
RPL	=	Recurrent Pregnancy Loss
SA	=	Spontaneous Abortion
SEFET	=	Single Euploid Frozen Embryo Transfer
TE	=	Trophectoderm
WGA	=	Whole-Genome Amplification
WHO	=	World Health Organization

AUTHOR'S CONTRIBUTION

I.M. has contributed to the study conceptualization, methodology, data analysis, interpretation of results, and manuscript writing.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data will be made available on reasonable request by contacting the corresponding author [I.M.].

FUNDING

None.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest regarding the publication of this article.

ACKNOWLEDGEMENTS

Declared none.

DECLARATION OF AI

The author utilized ChatGPT for linguistic refinement and improvement of manuscript readability. All suggested changes were reviewed and verified by the author, who remains fully accountable for the final published content.

SUPPLEMENTARY MATERIAL

PRISMA checklist is available as supplementary material on the publisher's website along with the published article.

APPENDIX

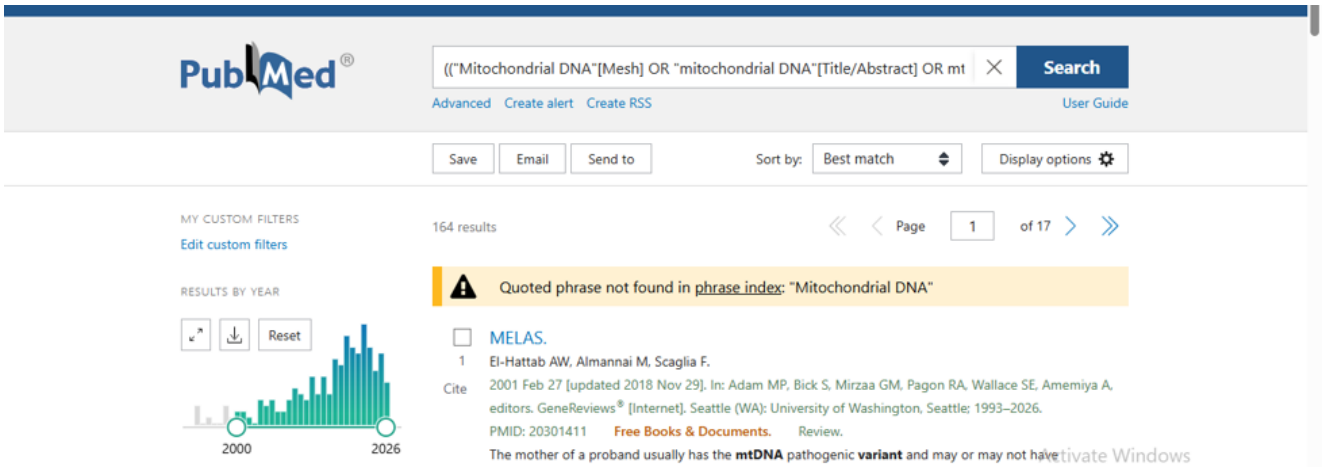
Appendix A. Database Search Strategy, Outcomes and Screenshots

The exact database-specific search strings and retrieval counts used for the PRISMA flow diagram are presented below. Screenshots of the database search outcomes are provided in Appendix Figs. **A1-A3**.

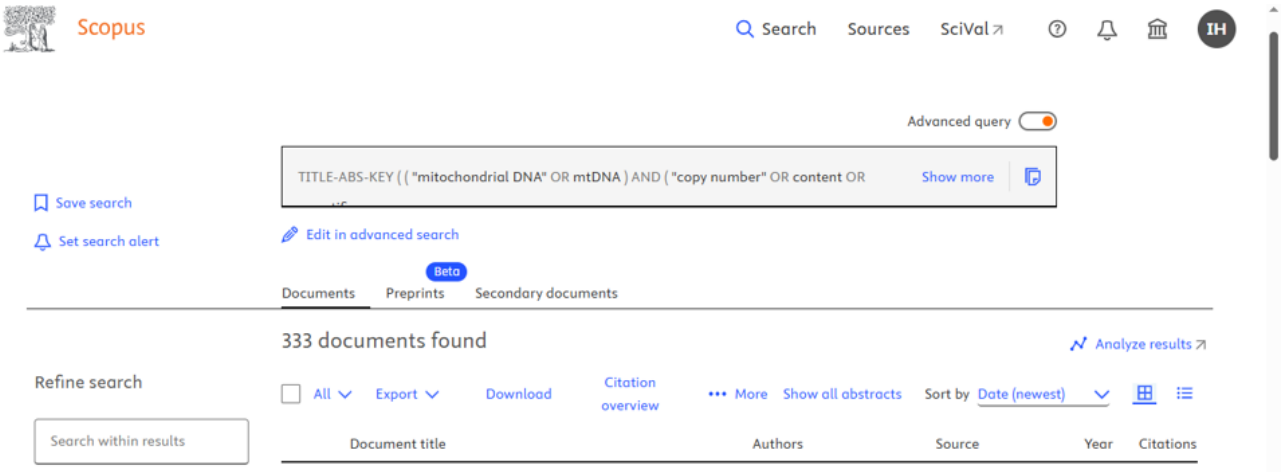
Appendix Table A1. Database search strings and retrieval outcomes by source

Database	Exact search string used	Limits/field	Outcome
PubMed/MEDLINE	(("Mitochondrial DNA"[Mesh] OR "mitochondrial DNA"[Title/Abstract] OR mtDNA[Title/Abstract]) AND ("copy number"[Title/Abstract] OR content[Title/Abstract] OR quantification[Title/Abstract] OR variant*[Title/Abstract] OR mutation*[Title/Abstract] OR deletion*[Title/Abstract] OR heteroplasmy[Title/Abstract] OR haplogroup*[Title/Abstract] OR "D-loop"[Title/Abstract] OR "tRNA"[Title/Abstract]) AND ("recurrent pregnancy	MeSH + Title/Abstract; 2000-2026; English; humans/journal articles during screening.	164 records

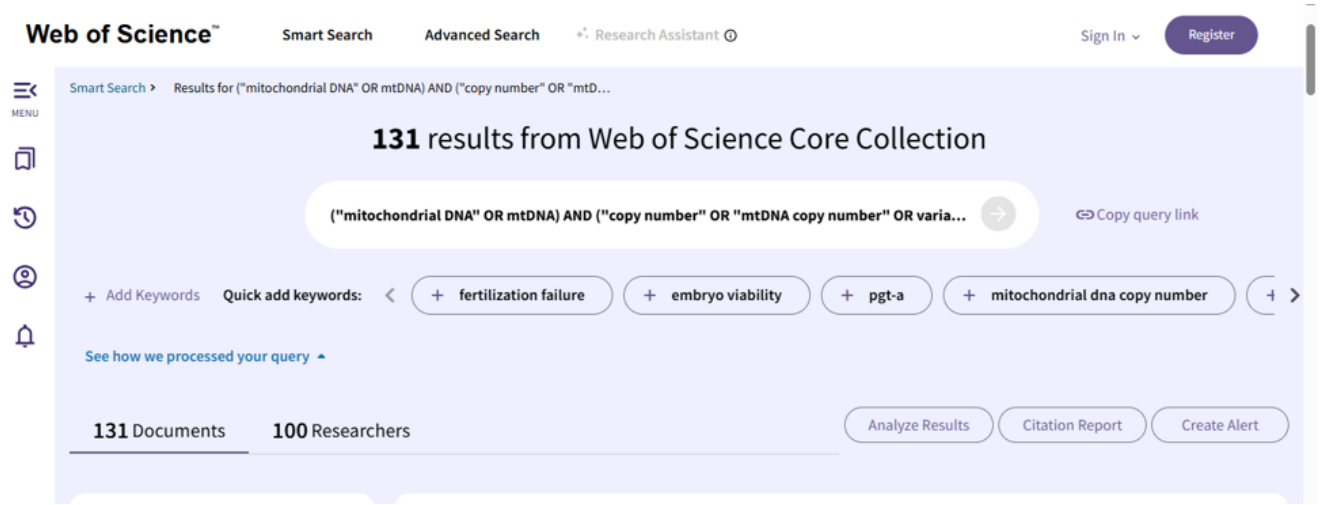
	loss"[Title/Abstract] OR "recurrent miscarriage"[Title/Abstract] OR "repeated pregnancy loss"[Title/Abstract] OR "early pregnancy loss"[Title/Abstract] OR miscarriage[Title/Abstract] OR "spontaneous abortion"[Title/Abstract] OR "recurrent implantation failure"[Title/Abstract] OR "implantation failure"[Title/Abstract] OR "implantation potential"[Title/Abstract] OR implantation[Title/Abstract] OR "embryo transfer"[Title/Abstract] OR "blastocyst transfer"[Title/Abstract] OR "pregnancy outcome"[Title/Abstract] OR "live birth"[Title/Abstract]))		
Scopus	TITLE-ABS-KEY (("mitochondrial DNA" OR mtDNA) AND ("copy number" OR content OR quantification OR variant* OR mutation* OR deletion* OR heteroplasmy OR haplogroup* OR "D-loop" OR "tRNA") AND ("recurrent pregnancy loss" OR "recurrent miscarriage" OR "repeated pregnancy loss" OR "early pregnancy loss" OR miscarriage OR "spontaneous abortion" OR "recurrent implantation failure" OR "implantation failure" OR "implantation potential" OR "embryo transfer" OR "blastocyst transfer" OR "pregnancy outcome" OR "live birth")) AND PUBYEAR > 1999 AND PUBYEAR < 2027	TITLE-ABS-KEY; PUBYEAR > 1999 and < 2027; English articles.	333 records
Web of Science Core Collection	TS=(("mitochondrial DNA" OR mtDNA) AND ("copy number" OR "mtDNA copy number" OR variant* OR mutation* OR heteroplasmy OR haplogroup* OR deletion*) AND ("recurrent pregnancy loss" OR "recurrent miscarriage" OR "pregnancy loss" OR miscarriage OR "early pregnancy loss" OR "recurrent implantation failure" OR "implantation failure" OR "failed implantation") AND (human* OR women OR embryo* OR endometrium OR endometrial OR blastocyst* OR trophectoderm)) NOT TS=(review OR "systematic review" OR "meta-analysis")	Topic/Smart Search; review/meta-analysis terms excluded; article records screened.	131 records



Appendix Fig. (A1). PubMed/MEDLINE search outcome showing 164 records.



Appendix Fig. (A2). Scopus search outcome showing 333 records.



Appendix Fig. (A3). Web of Science Core Collection search outcome showing 131 records.

Appendix B. PRISMA Stepwise Justification and Exclusion Allocation

Appendix B reports the database-wise and merged PRISMA accounting used in the main body. The search identified 628 database records in total (PubMed 164; Scopus 333; Web of Science 131). After 50 duplicates were removed, 578 unique records entered screening. No separate register source contributed records to the final accounting. Screening and full-text exclusions are reported as distinct stages below.

Appendix Table B1. Exclusion reasons at screening and eligibility stages.

Stage	Exclusion Reason	Number Excluded	Explanation
Title/abstract screening	Wrong mitochondrial/reproductive focus	210	The record mentioned mitochondria/mtDNA or reproduction but did not address mtDNA copy number, variants or related mitochondrial genomic markers in relevant reproductive failure.
Title/abstract screening	Non-primary, non-human or wrong publication type	125	Reviews, editorials, letters, conference-only items, animal-only studies or non-human laboratory studies were excluded.
Title/abstract screening	No eligible RPL/RIF, pregnancy-loss, implantation, miscarriage or live-birth outcome	150	Records did not include a relevant outcome such as RPL, RM, EPL, RIF, implantation failure, embryo-transfer outcome, miscarriage or live birth.

Full-text eligibility	No extractable mtDNA marker data	20	Full text did not provide extractable mtDNA copy-number, deletion, variant, haplogroup or heteroplasmy data.
Full-text eligibility	mtDNA measure not linked to eligible outcome	23	The mtDNA measure was not analysed against an eligible clinical or embryological reproductive outcome after full-text assessment.
Full-text eligibility	Overlapping cohort, duplicate dataset or insufficient primary outcome data	15	Reports used duplicate/overlapping cohorts or lacked sufficient primary outcome data for synthesis.
Full-text eligibility	Non-primary publication identified during verification	1	Viotti *et al.* (2017) was a review article and was removed from the primary evidence set.

Appendix Table B2. Database-wise identification and merged duplicate-removal accounting.

Source	Records Identified	Duplicate Removal After Merge	Records Entering Screening
PubMed/MEDLINE	164	-	-
Scopus	333	-	-
Web of Science Core Collection	131	-	-
Merged total	628	50	578

Appendix Table B3. Stepwise PRISMA flow after database merging.

PRISMA Step	Count	Arithmetic / Transition
Records identified from databases	628	164 + 333 + 131 = 628
Duplicates removed before screening	50	628 − 50 = 578
Records screened	578	—
Records excluded at title/abstract	485	578 − 485 = 93
Reports sought for retrieval	93	—
Reports not retrieved	5	93 − 5 = 88
Reports assessed for eligibility	88	—
Reports excluded at full text	59	88 − 59 = 29
Studies included in review	29	Final included set

Appendix Table B4. Allocation of the 29 Included Studies by Synthesis Domain

Study-level mapping of included studies according to primary evidence category.

No.	Study	Year	Country	Primary Population / Source	mtDNA Marker	Assigned Evidence Category
1	Kaare *et al.* [28]	2009	Finland	Women with recurrent miscarriage + controls	Heteroplasmic mtDNA variants	Direct RPL/RM evidence
2	Seyedhassani *et al.* (2010a) [29]	2010	Iran	Repeated pregnancy loss + controls	mtDNA deletions and D-loop variants	Direct RPL/RM evidence
3	Seyedhassani *et al.* (2010b) [30]	2010	Iran	Idiopathic recurrent pregnancy loss mutation screening without controls	mt-tRNA variants	Direct RPL/RM evidence
4	Vanniarajan *et al.* [31]	2011	India	Women with RPL, fetal tissues, confirmation cohort	Complete mtDNA sequencing	Direct RPL/RM evidence

5	Colagar *et al.* [32]	2013	Iran	RPL women + controls	ND1 T4216C mutation	Direct RPL/RM evidence
6	Azadi *et al.* [33]	2018	Iran	RPL women + controls	Complex-I mtDNA variants	Direct RPL/RM evidence
7	Ye *et al.* [34]	2020	China	Early pregnancy loss chorionic villi + controls	mtDNA copy number and deletion burden	Direct pregnancy-loss evidence
8	Ji *et al.* [35]	2020	China	Spontaneous abortion *vs* induced abortion tissues	Copy number and pathogenic variants	Pregnancy-loss tissue evidence
9	Eker *et al.* [36]	2021	Turkey	RIF women + fertile controls	Endometrial mtDNA copy number	Direct RIF evidence
10	Mojodi *et al.* [37]	2023	Iran	Idiopathic recurrent miscarriage + controls	mt-tRNA variants	Direct RPL/RM evidence

Direct / Mixed RIF-RPL Evidence.

No.	Study	Year	Country	Primary Population / Source	mtDNA Marker	Assigned Evidence Category
11	Zhou *et al.* [27]	2021	China	Euploid blastocysts including RIF/RM indications	Trophectoderm mtDNA content	Mixed RIF/RM embryo evidence
12	Łukaszuk *et al.* [43]	2022	Poland	PGT-A embryos including RIF/RM indications	Trophectoderm mtDNA/nDNA ratio	Mixed RIF/RM embryo evidence
13	Brenner *et al.* [48]	2000	USA	RIF patients after ooplasmic transfer	Donor-recipient mtDNA heteroplasmy	RIF intervention evidence
14	Barritt *et al.* [49]	2001	USA	Offspring after ooplasmic transplantation	Donor/recipient mtDNA inheritance	RIF intervention evidence

Contextual IVF / Embryo Transfer / Implantation / Live Birth Evidence.

No.	Study	Year	Country	Primary Population / Source	mtDNA Marker	Assigned Evidence Category
15	Fragouli *et al.* [22]	2017	UK/USA	Transferred euploid blastocysts	Trophectoderm mtDNA	Contextual IVF evidence
16	Ravichandran *et al.* [23]	2017	USA	Euploid blastocysts from IVF clinics	Trophectoderm mtDNA	Contextual IVF evidence
17	Treff *et al.* [24]	2017	USA	Sibling euploid blastocyst comparison	Trophectoderm mtDNA	Contextual IVF evidence
18	Victor *et al.* [25]	2017	USA/UK	Blastocysts after PGT-A	Adjusted mtDNA quantification	Contextual IVF evidence
19	Lee *et al.* [26]	2019	Taiwan	Infertile couples and transferred blastocysts	Adjusted embryo mtDNA quantity	Contextual IVF evidence
20	Lledó *et al.* [38]	2018	Spain	Euploid single embryo transfers	Blastocyst mtDNA analysis	Contextual IVF evidence
21	Diez-Juan *et al.* [39]	2015	Spain	Euploid embryo transfers	Embryo mtDNA content	Contextual IVF evidence
22	Fragouli *et al.* [40]	2015	UK/USA	Embryos undergoing genetic testing	Embryo/trophectoderm mtDNA	Contextual IVF evidence

23	Stigliani *et al.* [41]	2014	Italy	Spent culture medium samples	Extracellular mtDNA/gDNA ratio	Contextual IVF evidence
24	Klimczak *et al.* [42]	2018	USA	Blastocyst biopsy dataset	Embryonic mtDNA content	Contextual IVF evidence
25	Podolak *et al.* [44]	2022	Poland	Cleavage-stage embryos	Embryo mtDNA copy number	Contextual embryo evidence
26	El-Damen *et al.* [45]	2021	UAE	Single euploid frozen embryo transfers	Blastocyst mtDNA content	Contextual IVF evidence
27	Wang *et al.* [46]	2021	China	Euploid blastocysts	Trophectoderm mtDNA content	Contextual IVF evidence
28	Chuang *et al.* [47]	2023	Taiwan	Euploid single embryo transfers	Embryo/trophectoderm mtDNA content	Contextual IVF evidence
29	Victor *et al.* [50]	2019	USA/UK	Transferred blastocysts with live birth outcomes	Trophectoderm mtDNA content	Contextual IVF evidence

REFERENCES

[1] World Health Organization. *Infertility prevalence estimates, 1990–2021*. Geneva: World Health Organization; 2023. Available from: https://www.who.int/publications/i/item/9789240068315

[2] Cox CM, Thoma ME, Tchangalova N, Mburu G, Bornstein MJ, Johnson CL, Kiarie J. Infertility prevalence and the methods of estimation from 1990 to 2021: a systematic review and meta-analysis. Hum Reprod Open. 2022;2022(4): hoac051. https://doi.org/10.1093/hropen/hoac051

[3] Quenby S, Gallos ID, Dhillon-Smith RK, Podesek M, Stephenson MD, Fisher J, *et al.* Miscarriage matters: the epidemiological, physical, psychological, and economic costs of early pregnancy loss. *Lancet*. 2021;397(10285):1658–1667. https://doi.org/10.1016/S0140-6736(21)00682-6

[4] ESHRE Guideline Group on RPL, Bender Atik R, Christiansen OB, Elson J, Kolte AM, Lewis S, *et al.* ESHRE guideline: recurrent pregnancy loss: an update in 2022. *Hum Reprod Open*. 2023;2023(1). https://doi.org/10.1093/hropen/hoad002

[5] Regan L, Rai R, Saravelos S, Li TC. Recurrent miscarriage: Green-top Guideline No. 17. *BJOG*. 2023;130(12)–e39. https://doi.org/10.1111/1471-0528.17515

[6] ESHRE Working Group on Recurrent Implantation Failure, Cimadomo D, de Los Santos MJ, Griesinger G, Lainas G, Le Clef N, *et al.* ESHRE good practice recommendations on recurrent implantation failure. *Hum Reprod Open*. 2023;2023(3). https://doi.org/10.1093/hropen/hoad023

[7] Practice Committee of the American Society for Reproductive Medicine. Evaluation and treatment of recurrent pregnancy loss: a committee opinion. *Fertil Steril*. 2012;98(5):1103–1111. https://doi.org/10.1016/j.fertnstert.2012.06.048

[8] Babayev E, Seli E. Oocyte mitochondrial function and reproduction. *Curr Opin Obstet Gynecol*. 2015;27(3):175–181. https://doi.org/10.1097/GCO.0000000000000164

[9] May-Panloup P, Boucret L, Chao de la Barca JM, Desquiret-Dumas V, Ferré-L'Hotellier V, Morinière C, *et al.* Ovarian ageing: the role of mitochondria in oocytes and follicles. *Hum Reprod Update*. 2016;22(6):725–743. https://doi.org/10.1093/humupd/dmw028

[10] St John JC, Okada T, Andreas E, Penn A. The role of mtDNA in oocyte quality and embryo development. *Mol Reprod Dev*. 2023;90(7):621–633. https://doi.org/10.1002/mrd.23640

[11] Taylor RW, Turnbull DM. Mitochondrial DNA mutations in human disease. *Nat Rev Genet*. 2005;6(5):389–402. https://doi.org/10.1038/nrg1606

[12] Wai T, Ao A, Zhang X, Cyr D, Dufort D, Shoubridge EA. The role of mitochondrial DNA copy number in mammalian fertility. *Biol Reprod*. 2010;83(1):52–62. https://doi.org/10.1095/biolreprod.109.080887

[13] Stewart JB, Chinnery PF. The dynamics of mitochondrial DNA heteroplasmy: implications for human health and disease. *Nat Rev Genet*. 2015;16(9):530–542. https://doi.org/10.1038/nrg3966

[14] Stewart JB, Chinnery PF. Extreme heterogeneity of human mitochondrial DNA from organelles to populations. *Nat Rev Genet*. 2021;22(2):106–118. https://doi.org/10.1038/s41576-020-00284-x

[15] Burton GJ, Jauniaux E. Placental oxidative stress: from miscarriage to preeclampsia. *J Soc Gynecol Investig*. 2004;11(6):342–352. https://doi.org/10.1016/j.jsgi.2004.03.003

[16] Duhig K, Chappell LC, Shennan AH. Oxidative stress in pregnancy and reproduction. *Obstet Med*. 2016;9(3):113–116. https://doi.org/10.1177/1753495X16648495

[17] Zejnullahu VA, Zejnullahu VA, Kosumi E. The role of oxidative stress in patients with recurrent pregnancy loss: a review. *Reprod Health*. 2021;18:207. https://doi.org/10.1186/s12978-021-01257-x

[18] Munn Z, Stern C, Aromataris E, Lockwood C, Jordan Z. What kind of systematic review should I conduct? A proposed typology and guidance for systematic reviewers in the medical and health sciences. *BMC Med Res Methodol*. 2018;18(1):5. https://doi.org/10.1186/s12874-017-0468-4

[19] Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, *et al.* The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. *BMJ*. 2021;372. https://doi.org/10.1136/bmj.n71

[20] Aromataris E, Munn Z, editors. *JBI Manual for Evidence Synthesis*. JBI; 2020. https://doi.org/10.46658/JBIMES-20-01

[21] Sterne JAC, Hernán MA, Reeves BC, Savović J, Berkman ND, Viswanathan M, *et al.* ROBINS-I: a tool for assessing risk of bias in non-randomised studies of interventions. *BMJ*. 2016;355. https://doi.org/10.1136/bmj.i4919

[22] Fragouli E, McCaffrey C, Ravichandran K, Spath K, Grifo JA, Munné S, *et al.* Clinical implications of mitochondrial DNA quantification on pregnancy outcomes: a blinded prospective non-selection study. *Hum Reprod*. 2017;32(11):2340–2347. https://doi.org/10.1093/humrep/dex292

[23] Ravichandran K, McCaffrey C, Grifo J, Morales A, Perloe M, Munné S, Wells D, Fragouli E. Mitochondrial DNA quantification as a tool for embryo viability assessment: retrospective analysis of data from single euploid blastocyst transfers. *Hum Reprod*. 2017;32(6):1282–1292. https://doi.org/10.1093/humrep/dex070

[24] Treff NR, Zhan Y, Tao X, Olcha M, Han M, Rajchel J, *et al.* Levels of trophectoderm mitochondrial DNA do not predict the reproductive potential of sibling embryos. *Hum Reprod*. 2017;32(4):954–962. https://doi.org/10.1093/humrep/dex034

[25] Victor AR, Brake AJ, Tyndall JC, Griffin DK, Zouves CG, Barnes FL, Viotti M. Accurate quantitation of mitochondrial DNA reveals uniform levels in human blastocysts irrespective of ploidy, age, or implantation potential. *Fertil Steril*. 2017;107(1):34–42.e3. https://doi.org/10.1016/j.fertnstert.2016.09.028

[26] Lee YX, Chen CH, Lin SY, Lin YH, Tzeng CR. Adjusted mitochondrial DNA quantification in human embryos may not be applicable as a biomarker of implantation potential. *J Assist Reprod Genet*. 2019;36(9):1855–1865. https://doi.org/10.1007/s10815-019-01542-6

[27] Zhou X, Liu X, Shi W, Ye M, Chen S, Xu C. Mitochondrial DNA content may not be a reliable screening biomarker for live birth after single euploid blastocyst transfer. *Front Endocrinol (Lausanne)*. 2021;12:762976. https://doi.org/10.3389/fendo.2021.762976

[28] Kaare M, Götz A, Ulander VM, Ariansen S, Kaaja R, Suomalainen A, Aittomäki K. Do mitochondrial mutations cause recurrent miscarriage? *Mol Hum Reprod*. 2009;15(5):295–300. https://doi.org/10.1093/molehr/gap021

[29] Seyedhassani SM, Houshmand M, Kalantar SM, Modabber G, Aflatoonian A. No mitochondrial DNA deletions but more D-loop point mutations in repeated pregnancy loss. *J Assist Reprod Genet*. 2010a;27(11):641–648. https://doi.org/10.1007/s10815-010-9435-2

[30] Seyedhassani SM, Houshmand M, Kalantar SM, Aflatoonian A, Modabber G, Hadipour F, Fallahzadeh MH. The point mutations of mitochondrial tRNA threonine and proline in idiopathic repeated pregnancy loss. *Iran J Reprod Med*. 2010b;8(1):45–50. Available from: https://utoronto.scholaris.ca/items/04570e11-00a5-4b37-a093-fb6080176bfa (Accessed on: 13-10-2026).

[31] Vanniarajan A, Govindaraj P, Carlus SJ, Aruna M, Aruna P, Kumar A, *et al.* Mitochondrial DNA variations associated with recurrent pregnancy loss among Indian women. *Mitochondrion*. 2011;11(3):450–456. https://doi.org/10.1016/j.mito.2011.01.002

[32] Colagar AH, Mosaieby E, Seyedhassani SM, Mohajerani M, Arasteh A, Kamalidehghan B, Houshmand M. T4216C mutation in NADH dehydrogenase I gene is associated with recurrent pregnancy loss. *Mitochondrial DNA*. 2013;24(5):610–612. https://doi.org/10.3109/19401736.2013.772150

[33] Azadi A, Seo DJ, Jafari Sasansara H, Van Haute M. Mitochondrial DNA variations are associated with recurrent pregnancy loss. *Mitochondrial DNA A DNA Mapp Seq Anal*. 2018;29(5):674–678. https://doi.org/10.1080/24701394.2017.1350854

[34] Ye M, Shi W, Hao Y, Zhang L, Chen S, Wang L, He X, Li S, Xu C. Associations of mitochondrial DNA copy number and deletion rate with early pregnancy loss. *Mitochondrion*. 2020;55:48–53. https://doi.org/10.1016/j.mito.2020.07.006

[35] Ji L, Liao T, Yang J, Su H, Song J, Qian K. Deep sequencing shows that accumulation of potentially pathogenic mtDNA mutations rather than mtDNA copy numbers may be associated with early embryonic loss. *J Assist Reprod Genet*. 2020;37(9):2181–2188. https://doi.org/10.1007/s10815-020-01893-5

[36] Eker C, Basdas R, Karamustafaoglu Balci B, Bastu E, Gunel T. The genomic analysis of endometrial mitochondrial DNA copy number variation on recurrent implantation failure. *J Gynecol Obstet Hum Reprod*. 2021;50(2):101945. https://doi.org/10.1016/j.jogoh.2020.101945

[37] Mojodi E, Mehrjardi AM, Naeimzadeh Y, Ghasemi N, Falahati A, Moshtaghioun SM. The sequence variation of mitochondrial tRNA tyrosine and cysteine among Iranian women with idiopathic recurrent miscarriage: a case-control study. *Int J Reprod Biomed*. 2023;21(7):567–576. https://doi.org/10.18502/ijrm.v21i7.13894

[38] Lledó B, Ortiz JA, Morales R, García-Hernández E, Ten J, Bernabeu A, Llácer J, Bernabeu R. Comprehensive mitochondrial DNA analysis and IVF outcome. *Hum Reprod Open*. 2018;2018(4). https://doi.org/10.1093/hropen/hoy023

[39] Diez-Juan A, Rubio C, Marin C, Martinez S, Al-Asmar N, Riboldi M, Díaz-Gimeno P, Valbuena D, Simón C. Mitochondrial DNA content as a viability score in human euploid embryos: less is better. *Fertil Steril*. 2015;104(3):534–541.e1. https://doi.org/10.1016/j.fertnstert.2015.05.022

[40] Fragouli E, Spath K, Alfarawati S, Kaper F, Craig A, Michel CE, Kokocinski F, Cohen J, Munne S, Wells D. Altered levels of mitochondrial DNA are associated with female age, aneuploidy, and provide an independent measure of embryonic implantation potential. *PLoS Genet*. 2015;11(6). https://doi.org/10.1371/journal.pgen.1005241

[41] Stigliani S, Persico L, Lagazio C, Anserini P, Venturini PL, Scaruffi P. Mitochondrial DNA in Day 3 embryo culture medium is a novel, non-invasive biomarker of blastocyst potential and implantation outcome. *Mol Hum Reprod*. 2014;20(12):1238–1246. https://doi.org/10.1093/molehr/gau086

[42] Klimczak AM, Pacheco LE, Lewis KE, Massahi N, Richards JP, Kearns WG, Saad AF, Crochet JR. Embryonal mitochondrial DNA: relationship to embryo quality and transfer outcomes. *J Assist Reprod Genet*. 2018;35(5):871–877. https://doi.org/10.1007/s10815-018-1147-z

[43] Lukaszuk K, Podolak A. Does trophectoderm mitochondrial DNA content affect embryo developmental and implantation potential? *Int J Mol Sci*. 2022;23(11):5976. https://doi.org/10.3390/ijms23115976

[44] Podolak A, Liss J, Kiewisz J, Pukszta S, Cybulska C, Rychlowski M, *et al.* Mitochondrial DNA copy number in cleavage stage human embryos—impact on infertility outcome. *Curr Issues Mol Biol*. 2022;44(1):273–287. https://doi.org/10.3390/cimb44010020

[45] El-Damen A, Elkhatib I, Bayram A, Arnanz A, Abdala A, Samir S, Lawrenz B, De Munck N, Fatemi HM. Does blastocyst mitochondrial DNA content affect miscarriage rate in patients undergoing single euploid frozen embryo transfer? *J Assist Reprod Genet*. 2021;38(3):595–604. https://doi.org/10.1007/s10815-020-02050-8

[46] Wang J, Diao Z, Zhu L, Zhu J, Lin F, Jiang W, *et al.* Trophectoderm mitochondrial DNA content associated with embryo quality and Day-5 euploid blastocyst transfer outcomes. *DNA Cell Biol*. 2021;40(5):643–651. https://doi.org/10.1089/dna.2020.6271

[47] Chuang TH, Chen CY, Kuan CS, Lai HH, Hsieh CL, Lee MJ, *et al.* Reduced mitochondrial DNA content correlate with poor clinical outcomes in cryotransfers with day 6 single euploid embryos. *Front Endocrinol (Lausanne)*. 2022;13:1066530. https://doi.org/10.3389/fendo.2022.1066530

[48] Brenner CA, Barritt JA, Willadsen S, Cohen J. Mitochondrial DNA heteroplasmy after human ooplasmic transplantation. *Fertil Steril*. 2000;74(3):573–578. https://doi.org/10.1016/S0015-0282(00)00681-6

[49] Barritt JA, Brenner CA, Malter HE, Cohen J. Mitochondria in human offspring derived from ooplasmic transplantation: brief communication. *Hum Reprod*. 2001;16(3):513–516. https://doi.org/10.1093/humrep/16.3.513

[50] Victor AR, Griffin DK, Gardner DK, Brake AJ, Zouves CG, Barnes FL, Viotti M. Births from embryos with highly elevated levels of mitochondrial DNA. *Reprod Biomed Online*. 2019;39(3):403–412. https://doi.org/10.1016/j.rbmo.2019.03.214