

Classification of recurrent implantation failure patients using peripheral blood immunological and metabolic markers

Sina Baharaghdam^{1,2}, Özkan Yildirim³, Mohammadbagher Pirouzpanah⁴, Danial Abbas Kabiri³, Shima Karimi^{1,2}, Mohammad Esfani Farahani⁵, Amirhossein Aghapour^{1,2}, Ali Hassanzadeh⁶, Amin Kamrani^{1,2}, Reza Mousavi Ardehaie⁴, Amir Mehdizadeh⁷, Parvin Hakimi⁸, Javad Ahmadian Heris⁹, Leili Aghebati-Maleki^{1,2}, Mehdi Yousefi^{4,2*}

¹Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

²Department of Immunology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

³Department of Histology and Embryology, Institute of Health Sciences, Atatürk University, Erzurum, Türkiye

⁴Molecular Medicine Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

⁵Department of Toxicology and Cancer Biology, University of Kentucky, Lexington, USA

⁶Department of Applied Cell Sciences, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran

⁷Hematology and Oncology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

⁸Women's Reproductive Health Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

⁹Department of Allergy and Clinical Immunology, Pediatric Hospital, Tabriz University of Medical Sciences, Tabriz, Iran

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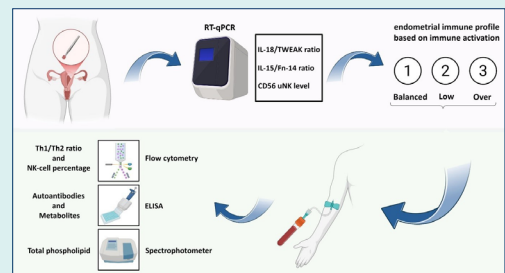
Abstract

Introduction: Recurrent implantation failure (RIF) is a complex condition that makes it one of the most challenging cases in the field of infertility. The diagnosis of this condition with the immunological etiology may be aided by determining the endometrial immune profile for the classification of these patients. Current diagnostic approaches are based on invasive endometrial biopsies to classify patients into balanced, low, or over-immune activation profiles, which have limitations for routine use and serial monitoring. This study aimed to develop and validate a minimally invasive peripheral blood-based classification system using immunological and metabolic markers to mirror endometrial immune profiles in RIF patients.

Methods: Endometrial tissue and peripheral blood samples were collected during the mid-luteal phase from 163 RIF patients and 28 fertile controls. Endometrial immune profiles were determined via RT-qPCR for IL-18, IL-15, TWEAK, Fn-14, and CD56, classifying RIF into balanced, low, and over-immune activation subgroups, with sample sizes of 32, 47, and 84 women, respectively. Peripheral blood was analyzed by flow cytometry to determine the Th1/Th2 ratio and NK cell percentage; by ELISA to measure nuclear antibodies (ANA, anti-dsDNA), phospholipid-related antibodies (anticardiolipin, anti- β 2-glycoprotein I, antiphospholipid antibodies), thyroid-related antibodies (anti-TPO, anti-TG), anti-tissue transglutaminase (anti-TTG), and metabolites (S1P, adiponectin, leptin, PGE2, phosphatidylserine, IGF-1); and by spectrophotometry to quantify total phospholipids.

Results: The over-immune activation group showed significantly elevated Th1/Th2 ratios, NK-cell percentages, and autoantibodies (ANA, anti-phospholipid, anti- β 2-glycoprotein I, anti-TG, anti-TPO) compared to balanced and low-immune activation groups. Metabolic profiles revealed higher leptin, and total phospholipids but lower adiponectin, S1P, and PGE2 in over-immune activation group. The low-activation group exhibited lower Th1/Th2 ratios, reduced leptin, but elevated adiponectin, S1P, and PGE2 versus balanced and over-immune activation groups. No significant differences were found in phosphatidylserine or IGF-1 across groups.

Conclusion: Our results demonstrate that peripheral blood immunological and metabolic markers can effectively distinguish RIF immune endotypes, offering a non-invasive alternative to endometrial biopsy for personalized assisted reproductive technology (ART) management and potentially improving implantation success through targeted therapies.



*Corresponding author: Mehdi Yousefi, Email: Yousefime@tbzmed.ac.ir



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Research Highlights

What is the current knowledge?

- RIF is a complex issue in the field of infertility, influenced by several kinds of variables such as anatomical, genetic, and immunological factors.
- Assessing the endometrial immune profile and classifying patients accordingly for personalized treatment is highly beneficial.
- Endometrial biopsy is an invasive procedure for this purpose that has various limitations.

What is new here?

- RIF patients can be classified according to variables present in their peripheral blood.
- Immunologic and metabolic factors in peripheral blood can reflect conditions in the endometrium.
- The use of peripheral blood rather than endometrial tissue provides a less invasive method that is reproducible and appropriate for routine diagnostic and therapeutic procedures.

Introduction

The preimplantation genetic testing (PGT) consortium of European Society of Human Reproduction and Embryology (ESHRE) currently considers recurrent implantation failure (RIF) to be present when implantation does not occur after the transfer of three or more morphologically high-quality embryos in women younger than 40 years, or after fewer transfers in older patients. This complicated condition, which affects 10–15% of couples undergoing in vitro fertilization (IVF), continues to be one of the most challenging problems in assisted reproductive technologies.^{1,2} Although fetal chromosomal abnormalities are the main cause of implantation failure, a significant proportion of them persist despite the transfer of euploid embryos, highlighting the critical role of maternal factors—particularly altered endometrial receptivity and dysregulated local immune regulation—in the pathogenesis of this disorder.³

During the window of implantation (WOI), the endometrium undergoes precisely orchestrated immunological remodeling to establish selective maternal tolerance toward the semi-allogeneic embryo while maintaining effective defense against pathogens.⁴ This finely tuned equilibrium is maintained through dynamic changes in the composition and function of uterine natural killer (uNK) cells, regulatory T (Treg) cells, and various T-cell subsets, together with a complex network of cytokines and chemokines. Both excessive pro-inflammatory states, such as a dominance of T-helper (Th)1 or Th17 cells, and overly immunosuppressive profiles characterized by Th2 deviation or an overrepresentation of Treg cells have consistently been associated with implantation failure and recurrent pregnancy loss (RPL).^{5,6} Accordingly, detailed characterization of the endometrial immune profile has emerged as a powerful diagnostic and therapeutic tool, enabling the identification of

specific immune dysregulations and facilitating tailored immunomodulatory interventions in affected patients.^{7,8}

Recent advances have shifted the focus from static histological assessments to analyzing the immune profile of the endometrium as a strategy to better understand RIF heterogeneity. Notably, using RT-qPCR analysis of CD56+ uNK levels, IL-15/Fn-14 (Interleukin-15/Fibroblast growth factor-inducible molecule) ratios, and IL-18/TWEAK (Interleukin-18/Tumor necrosis factor-like weak inducer of apoptosis) ratios, the researchers have developed an endometrial-based molecular classification system to classify RIF patients into distinct immune endotypes including balanced immune activation (no immune dysregulation), low immune activation (hypofunction), and excessive immune activation (hyperfunction), enabling personalized immune modulatory interventions.^{7,9} While this approach has demonstrated clinical promise, it relies on invasive endometrial biopsy, limiting its feasibility for routine screening, repeated monitoring, or use in early-cycle risk stratification.¹⁰

Given the limitations and challenges associated with invasive methods like endometrial tissue biopsy, there is a clear clinical need for non- or minimally invasive biomarkers that can reflect endometrial immune status and facilitate repeated assessments.^{7,11} In this context, peripheral blood offers a minimally invasive, accessible surrogate for systemic and, to some extent, uterine immune status.¹² Although peripheral NK (pNK) cells and circulating Th subsets do not fully represent their uterine counterparts, emerging evidence indicates that immune and metabolic markers in peripheral blood may reflect endometrial immune activity in RIF patients.¹³ Altered circulating Th1/Th2 ratios, elevated NK-cell cytotoxicity, and abnormal autoantibody profiles such as anti-phospholipid and anti-thyroid antibodies, as well as dysregulation of adipokines and phospholipid metabolism, have all been associated with RIF and RPL.^{14–16} These observations raise the possibility that a comprehensive peripheral blood immune-metabolic signature could serve as a surrogate for assessing local endometrial immune dysregulation.

The present study aimed to develop and validate a minimally invasive classification system for RIF patients based exclusively on peripheral blood immunological and metabolic markers. Accordingly, we sought to identify distinct immune-metabolic clusters in RIF patients and examine their association with previously validated endometrial immune profiles by analyzing the Th1/Th2 ratio, NK cell percentages, a panel of autoantibodies, metabolites, and total phospholipid levels. Ultimately, this blood-based diagnostic approach intends to provide a patient-friendly, repeatable, and clinically actionable tool for personalized management in assisted reproductive technology (ART) for patients with RIF.

Materials and Methods

Participants

Collection and use of all samples were conducted only after

obtaining written informed consent from all participants. Samples were collected during the WOI from 191 women (aged 20–40 years), comprising 163 with RIF and 28 fertile controls (FC). Participants were consecutively recruited over a period spanning February 2024 to November 2025. Concurrently, 10 cc peripheral blood samples were taken from all participants. The FC group consisted of volunteer women who had at least one previous live birth and had not undergone fertility treatment.¹⁷ All participants, recruited from infertility centers in Tabriz (East Azerbaijan, Iran), exhibited normal menstrual cycles, lacked a history of endometrial pathology (verified by ultrasound), and had not used hormonal medications for three months before sampling. Women were included in the RIF cohort after undergoing three or more embryo transfers without achieving a positive pregnancy test.² Participants with underlying conditions such as autoimmune, rheumatologic, or chronic systemic disorders, or those taking immunomodulatory medications (e.g., corticosteroids or disease-modifying antirheumatic drugs [DMARDs]), were excluded wherever this information was available.

Samples collection and analysis

Endometrial tissue and peripheral blood sampling were done by the referring clinicians during the mid-luteal phase, in a simulated substitution cycle after 5 to 9 days of progesterone administration or in a natural cycle under monitoring. In natural cycles, ovulation was monitored by transvaginal ultrasound to track follicle development and confirm ovulation. Endometrial biopsies were obtained in the mid-luteal phase, specifically 7 days after ovulation (equivalent to LH + 7), corresponding to the WOI. The endometrial fragment was carefully aspirated by rotating a Pipelle de Cornier inside the endometrial cavity.¹⁸ We divided the Pipelle-obtained endometrial tissue into two portions. For the histological test to determine the phase of the cycle, one portion was placed in 4% formaldehyde (Merck, Germany; Cat. No. 1.00496), and the other portion was put in RNAlater stabilization solution for immunological analysis (Yekta Tajhiz Azma, Tehran, Iran; Cat. No. YT9085). After confirming the mid-luteal phase through histological analysis and extracting RNA using RNX-PLUS solution (Sina Clone, Tehran, Iran; Cat. No. EX6101), complementary DNA (cDNA) synthesis was performed utilizing the RevertAid First Strand cDNA Synthesis Kit (Yekta Tajhiz Azma, Tehran, Iran; Cat. No. YT4500), according to the manufacturer's protocol. Oligo Primer Analysis Software v. 7.60 (Molecular Biology Insights, Inc., CA, USA) was used to design specific primers (Table S1). Then, five targeted biomarkers (IL-18, IL-15, TWEAK, Fn-14, and CD56), along with the internal control GAPDH, were determined by quantitative real-time PCR with SYBR Green in a Roche Light Cycler[®] 96 Instrument (Roche, Germany).⁷

Diagnosis of endometrial immune profile

The local immune profile definition according to the

balance of the IL-18/ TWEAK and IL-15/Fn-14 ratio, as well as CD56 mRNA expression is a patent untitled “METHOD FOR INCREASING IMPLANTATION SUCCESS IN ASSISTED FERTILIZATION” registered by Lédée et al, which is described as a procedure for determining uterine receptivity profiles to promote the success of implantation in assisted reproduction (PCT/EP2013/065355).⁷

Based on this method, three endometrial immune profiles are distinguished: a balanced profile, characterized by IL-18/TWEAK and IL-15/Fn-14 mRNA ratios as well as CD56 + mRNA expression in the same range as previously defined by Lédée et al. in the FC group; a low activation immune profile, defined by low mRNA ratios for IL-15/Fn-14 (reflecting immature uNK cells) and/or IL-18/TWEAK or an absence of uNK recruitment; and a immune profile with over activation, marked by high mRNA ratios of IL-18/ TWEAK and/or IL-15/Fn-14 and/or a high CD56 + mRNA expression.⁷

Immunologic and metabolic biomarkers

After defining the subgroups of RIF patients based on this patent, we analyzed our designed panel derived from peripheral blood. This panel includes the percentage of NK cells, the Th1/Th2 cell ratio, a range of autoantibodies (such as Anti-Nuclear antibody, Anti-cardiolipin, Anti-dsDNA, Anti-Phospholipid, Anti-TG, Anti-TPO, Anti-TTG, and Anti- β 2-Glycoprotein), and various metabolites (including sphingosine-1-phosphate [S1P], adiponectin, leptin, phosphatidylserine, prostaglandin E2 [PGE2], total phospholipid, and IGF-1) relevant to these subgroups.

Flow cytometric analysis of Th1/Th2 ratio and NK cells

Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation using Ficoll-Paque (Yekta Tajhiz Azma, Tehran, Iran; Cat. No. YT3000) within 4 h of collection. One hundred microliters of whole blood or 1×10^6 PBMCs were stained with the following fluorochrome-conjugated monoclonal antibodies: CD3-PerCP (clone UCHT1), CD4-PerCP (clone RPA-T4), IFN- γ - FITC (clone 4S.B3) for Th1, IL-4-PE (clone MP4-25D2) for Th2, CD56-PE (clone HCD56), and CD16-FITC (clone B73.1).

All antibodies were obtained from Biolegend (San Diego, CA, USA; Cat. No. 300428, 300528, 502506, 500808, 318306, 360716). For intracellular cytokine staining, cells were stimulated for 4–6 h with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μ g/mL ionomycin in the presence of brefeldin A (Biolegend, San Diego, CA, USA; Cat. No. 423303). First, surface staining was performed, followed by fixation and permeabilization using the Fixation/Permeabilization Solution Kit (Biolegend, San Diego, CA, USA; Cat. No. 426803), in accordance with the manufacturer's protocol. On a BD FACSCalibur (BD Biosciences), at least 50,000 lymphocyte events were recorded. Single-colour controls and BD CompBeads were used for Compensation.

Data were analyzed with FlowJo software version 10.10 (Ashland, OR, USA). Th1 cells were defined as CD4⁺IFN- γ ⁺IL-4⁻, Th2 as CD4⁺IFN- γ ⁻IL-4⁺. The Th1/Th2 ratio was calculated as the percentage of Th1 cells divided by the percentage of Th2 cells. NK cells were identified as CD3⁻CD16⁺CD56⁺ and expressed as a percentage.

Measurement of autoantibodies by enzyme-linked immunosorbent assay (ELISA)

Serum autoantibody levels were quantified using commercial ELISA kits following the manufacturer's instructions. The kits for anticardiolipin, anti- β 2-glycoprotein, antiphospholipid, and anti-TTG were provided by MyBioSource (San Diego, California, USA; Cat. No. MBS3804816, MBS3803458, MBS8820989). Additionally, the kits for ANA, anti-dsDNA, anti-TG, and anti-TPO were supplied by Pishtazteb (Tehran, Iran; Cat. No. PT-ANA-SClgG-96, PT-Anti-dsDNA-96, PT-Anti-TG-96, PT-Anti-TPO-96). Samples were assayed in duplicate. Optical density was measured at 450 nm using a microplate ELISA reader (Stat Fax 4200). Concentrations were calculated from the standard curve using four-parameter logistic regression. The cut-off values were based on the recommendations provided by each manufacturer.

Quantification of metabolites and total phospholipids by ELISA and spectrophotometer

Serum concentrations of the metabolites were determined using commercial ELISA kits according to the manufacturers' protocols. The kits for S1P, Adiponectin, Leptin, IGF-1, PGE2, and Phosphatidylserine were provided by MyBioSource (San Diego, California, USA; Cat. No. MBS766091, MBS283065, MBS9425103, MBS726411, MBS721434, MBS731866). Additionally, serum total lipid was extracted by the method of Bligh/Dyer with a 2:1 chloroform:methanol solution and several washes.¹⁹ Then, using 70% perchloric acid, 2.5% ammonium molybdate solution, and 0.5 ml of 10% ascorbic acid, the necessary standards (10 mg/dL, 20 mg/dL, 30 mg/dL, 40 mg/dL) were prepared. After following the specified steps, 2.5% ammonium molybdate solution

and 10% ascorbic acid were added to the standards and samples. Finally, the optical density of the standards and samples was measured using a Pharmacia LKB Novaspec II spectrophotometer at 800 nm wavelength.

Statistical analysis

GraphPad Prism version 10.6.0 (GraphPad Software, La Jolla, California, United States) was utilized in order to carry out the analyses. Normality of continuous variables was assessed using the Shapiro-Wilk test. Data were expressed as mean \pm standard deviation (SD) for normally distributed variables, and differences between groups were analyzed using one-way analysis of variance (ANOVA) and a t-test, depending on the circumstances. The statistical significance of a *P* value less than 0.05 was considered to be met. Power analysis for the primary outcome (Th1/Th2 ratio) was conducted post hoc. Given the observed group means (Balanced: 8.60 \pm 3.54, Low: 6.92 \pm 2.33, Over-activation: 21.03 \pm 5.40) and sample sizes (Balanced: 32, Low: 47, Over: 84), the effect size for one-way ANOVA was very large, indicating a statistical power > 99% at α = 0.05. Therefore, the sample sizes were adequate to detect differences between groups.

Results

Baseline characteristics

Baseline demographic and clinical characteristics of the study participants are presented in Table S2. No statistically significant differences were observed between RIF patients and FC regarding age (*P* = 0.080), BMI (*P* = 0.072), serum FSH (*P* = 0.163), LH (*P* = 0.081), endometrial thickness (*P* = 0.061), AMH levels (*P* = 0.564), or number of smoking patients (*P* = 0.554). However, a significantly higher proportion of smoking partners was observed in the RIF group compared to controls (46% vs. 35.7%, *P* = 0.048).

Determination of the interpretation norm

Based on the previous study protocol (Lédée et al patent)²⁰ and our data, cutoff values were derived from the FC group using the mean \pm SD for each biomarker (Table 1). All biomarkers were normally distributed.

Table 1. Endometrial biomarker comparison in control and RIF subgroups based on their immune profiles

	Endometrial immune profile	Control group		<i>P</i> value	RIF patients' subgroups			
		Normal	Over activation		Low activation	<i>P</i> value	Balanced	<i>P</i> value
	Number of participants	28	84 (51.5%)			47 (28.8%)		32 (19.6%)
IL18/TWEAK	Range of interpretation	0.02-0.135	> 0.135		< 0.02			0.02-0.135
	Mean	0.071	0.494	< 0.0001*	0.045	0.0257*	0.065	0.926
Biomarkers IL15/Fn14	Range of interpretation	0.36-3.4	> 3.4		< 0.36			0.36-3.4
	Mean	1.399	3.058	0.0021*	0.480	0.0002*	1.431	0.998
CD56 ⁺ cells	Range of interpretation	0.5-1.5	> 1.5		< 0.5			0.5-1.5
	Mean	1.008	0.929	0.762	0.210	< 0.0001*	0.984	0.987

A group of 28 fertile women served as the norms for each biomarker. Data for each RIF group were compared to data for the FC and analyzed with ANOVA. A **P* value < 0.05 was considered statistically significant. Abbreviations: RIF, Recurrent implantation failure; TWEAK, Tumor necrosis factor-like weak inducer of apoptosis; Fn14, Fibroblast growth factor-inducible molecule 14; IL, Interleukin; CD, Cluster of differentiation. Percentages indicate the proportion of patients in each RIF subgroup relative to the total RIF cohort (*n* = 163).

The mean \pm SD for the IL-18/TWEAK mRNA ratio was 0.071 ± 0.036 ; values < 0.02 (mean $- 1$ SD) were classified as low-activation and values > 0.135 (mean $+ 1$ SD) as over-activation. For the IL-15/Fn-14 mRNA ratio, the mean \pm SD was 1.39 ± 0.87 ; values < 0.36 (mean $- 1$ SD) were classified as low-activation and values > 3.4 (mean $+ 2$ SD) as over-activation. For CD56⁺ mRNA expression, the mean \pm SD was 1.008 ± 0.303 cells per field; values < 0.5 (mean $- 2$ SD) were classified as low-activation and values > 1.5 (mean $+ 2$ SD) as over-activation. These reference thresholds were subsequently applied to classify RIF patients into balanced, low-activation, and over-activation immune subgroups.

Fig. 1 illustrates the dispersion of the raw data for the mRNA ratios of IL-18/TWEAK and IL-15/Fn-14, as well as the CD56 mRNA expression in both the FC and the RIF group.

The Th1/Th2 ratio and the NK cell percentage were altered in three subgroups of RIF

In the current study, flow cytometry was used to investigate Th1 and Th2 cells by detecting the percentages of CD4⁺IFN- γ ⁺IL-4⁻ and CD4⁺IFN- γ ⁻IL-4⁺ cells, as well as NK cells detecting the percentages of CD3-CD16⁺CD56⁺ in RIF patients' groups. As shown in Fig. 2, patients in three RIF groups had an altered of Th1/Th2 cells ratio compared with each other (balanced: 8.597 ± 3.537 , low: 6.920 ± 2.325 , over: 21.03 ± 5.395). These changes were not significant for balanced vs. low ($P=0.2180$), On the other hand, it was significant for balanced vs. over and low vs. over ($P<0.0001$). We also observed similar changes in NK cells (balanced: 6.553 ± 3.563 , low: 7.445 ± 2.652 , over: 17.56 ± 3.969 ; Fig. 3). Alterations for this cell also just in balanced vs. over and low vs. over ($P<0.0001$) was significant. Table 2 indicate the comparison of flow cytometric parameters in three RIF subgroups.

Autoantibodies measurement in RIF patients

Serum autoantibody levels were determined by ELISA in RIF patients classified into balanced, low, and over-

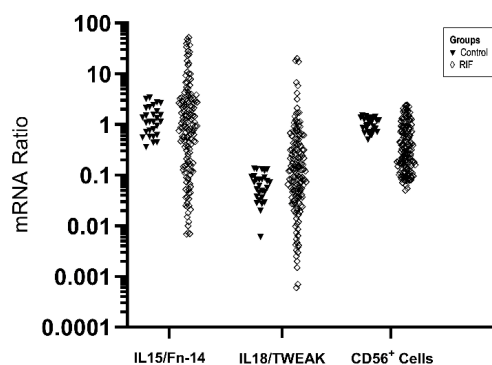


Fig. 1. Distribution of the IL-15/Fn-14 and IL-18/TWEAK ratio, along with CD56 mRNA expression, in the control and the RIF group. The data dispersion is log-transformed. Abbreviations: RIF, Recurrent implantation failure; TWEAK, Tumor necrosis factor-like weak inducer of apoptosis; Fn14, Fibroblast growth factor-inducible molecule 14; IL, Interleukin; CD, Cluster of differentiation.

immune activation groups. No significant differences were observed between the balanced and low-immune activation groups for any of the measured autoantibodies. In contrast, patients in the over group displayed significantly higher levels of several autoantibodies when compared to both the balanced and low-immune activation groups.

Specifically, ANA levels were significantly elevated in the over-immune activation group compared with the balanced ($P=0.0025$) and low-immune activation ($P=0.0152$) groups. Similarly, anti-phospholipid antibody concentrations were markedly increased in the over-immune activation group versus the balanced ($P=0.0002$) and low-immune activation ($P=0.0048$) groups. Anti- β -2-glycoprotein I antibodies were also significantly higher in the over-immune activation group than in the balanced ($P=0.0036$) and low-immune activation ($P=0.0032$) groups. Thyroid-related autoantibodies followed the same pattern: both anti-TG and anti-TPO levels were significantly greater in the over-immune activation group compared with the balanced ($P=0.0126$ and $P=0.0092$, respectively) and low-immune activation ($P=0.0305$ and $P=0.0251$, respectively) groups.

In contrast, serum levels of anti-cardiolipin, anti-dsDNA, and anti-TTG antibodies showed no significant differences across any of the pairwise comparisons (all p-values were greater than 0.05 and were not considered statistically significant). Table 3 illustrates the comparison of autoantibody measurement results in the RIF subgroups.

Serum metabolite levels

Serum concentrations of a panel of metabolites and lipids were assessed in peripheral blood samples from patients with RIF, categorized into three immunological subgroups. Adiponectin, leptin, S1P, PGE2, phosphatidylserine, and IGF-1 were quantified using ELISA, while total phospholipid levels were confirmed by spectrophotometric analysis. Serum concentrations were reported in ng/mL for S1P, adiponectin, leptin, phosphatidylserine, and IGF-1; in pg/mL for PGE2; and in mg/dL for total phospholipids.

Statistical analysis revealed significant alterations in the serum concentrations of several metabolites across the experimental groups. S1P levels showed a highly significant variation across the three groups (balanced: 75.62 ± 6.689 ; low: 137.5 ± 10.15 ; over: 17.44 ± 3.093 ; $P<0.0001$), with each group differing significantly from the others. A similar pattern was observed for adiponectin (balanced: 14.16 ± 4.88 ; low: 45.28 ± 3.11 ; over: 1.461 ± 0.76 ; $P<0.0001$) and leptin (balanced: 11.90 ± 1.83 ; low: 0.96 ± 0.65 ; over: 37.13 ± 2.77 ; $P<0.0001$), where all pairwise comparisons demonstrated statistically significant differences. PGE2 levels also differed significantly between all groups (balanced: 287.1 ± 23.48 ; low: 475.7 ± 23.31 ; over: 87.12 ± 10.21 ; $P<0.0001$). Additionally, total phospholipid concentration exhibited a strong group-dependent variation (balanced:

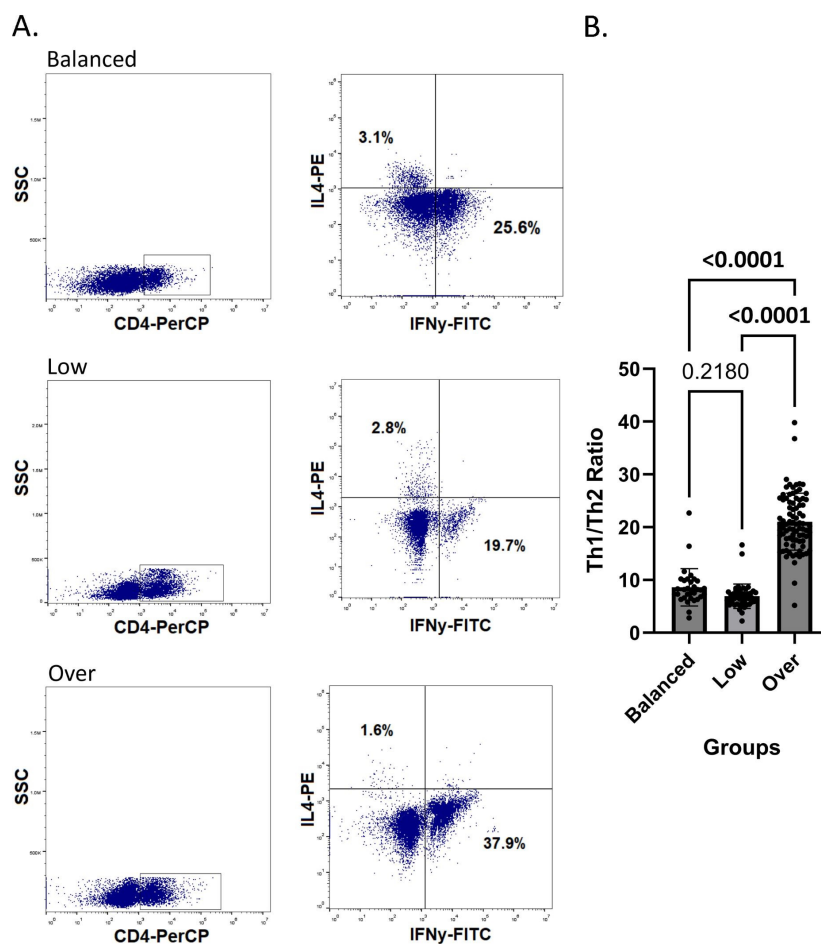


Fig. 2. Cell frequency by flow cytometry technique in RIF subgroups. A. Dot blot of lymphocytes gated to identify Th1/Th2 ratio (IFN- γ + /IL-4 +) in three RIF patients' subgroups (balanced, low, and over). B. Comparing the Th1/Th2 ratio in the three groups considered. $P < 0.05$ considered as significant. (N=32 for balanced, N=47 for low and N=84 for over-immune activation group). Abbreviations: Th, T-helper lymphocytes; CD, Cluster of differentiation; SSC, Side scatter; IL, Interleukin; FITC, Fluorescein isothiocyanate; PE, Phycoerythrin; IFN γ , Interferon gamma; PerCP, Peridinin-chlorophyll-protein.

215.4 \pm 22.29; low: 76.76 \pm 12.55; over: 454.4 \pm 22.23; $P < 0.0001$), with significant differences observed among all comparisons. In contrast, phosphatidylserine (balanced: 66.20 \pm 9.42; low: 60.29 \pm 11.35; over: 69.77 \pm 15.26) did not show significant differences between balanced vs. low ($P = 0.5317$), balanced vs. over ($P = 0.8018$), or low vs. over ($P = 0.1619$). Similarly, IGF-1 (balanced: 254.7 \pm 29.12; low: 227.6 \pm 59.21; over: 265.8 \pm 91.80) demonstrated no statistically significant changes among the groups ($P = 0.4034$, 0.8268, and 0.1898, respectively) (Table 4). Data are presented as mean \pm SD. Fig. 4 shows the serum level of metabolites difference between the three RIF subgroups.

Discussion

RIF continues to be a highly challenging condition in assisted reproduction, with live-birth rates per cycle frequently falling below 25–35% even with improved laboratory and clinical protocols.²¹ Despite advancements in embryo selection, particularly with preimplantation genetic testing for aneuploidy (PGT-A), a significant number of RIF cases continue to occur even after the transfer of euploid embryos.²² This suggests that maternal factors, particularly endometrial and systemic immune dysregulation, can play a crucial role in these

failures.^{23,24} Although the classification of RIF into immune endotypes—Balanced, Low, and Over—has been made possible by endometrial immune profiling using molecular biomarkers (IL-15/Fn-14, IL-18/TWEAK ratios, and CD56 expression), this method necessitates an invasive endometrial biopsy, which presents several limitations.^{7,25} However, this method, introduced by Lédée et al. for classifying RIF patients, has proven to be highly beneficial for developing personalized treatment plans.^{7,20} Treatment approaches vary based on the type of endometrial immune profile identified.²⁶ For instance, patients categorized with low-immune activation may receive treatments such as endometrial scratching and luteal hCG.^{27,28} In contrast, those with excessive immune activation may be treated with high concentrations of estrogens during the proliferative phase, along with immunotherapy.^{29,30} It is noteworthy that patients classified as having balanced immune activation (no dysregulation) are unlikely to benefit from therapies based on immune system regulation.⁷ therefore, a comprehensive evaluation of other factors is essential to determine the most appropriate treatment options. Considering the usefulness of the method of classifying RIF patients based on endometrial immune profiling, in this study, we sought to develop a practical, minimally

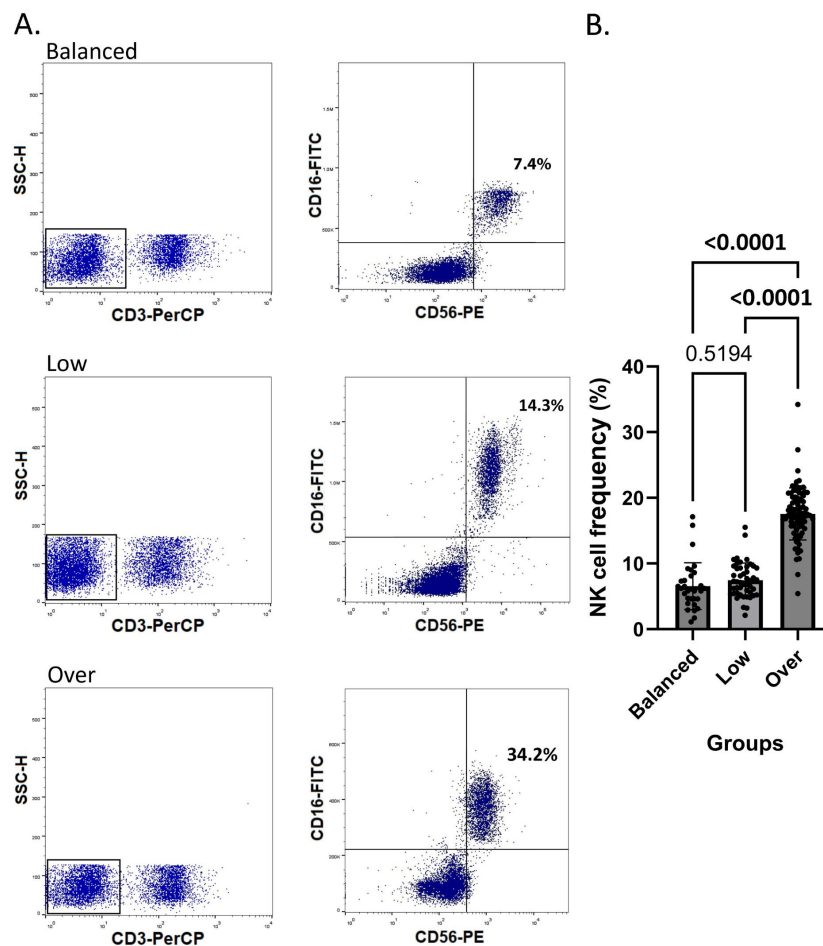


Fig. 3. NK cell frequency by flow cytometry technique in RIF subgroups. A. Dot blot of lymphocytes gated to identify NK cells (CD3⁺CD16⁺CD56⁺) in three RIF patients' subgroups (balanced, low, and over). B. Comparing the NK cells frequency in the three groups considered. $P < 0.05$ considered as significant. (N=32 for balanced, N=47 for low and N=84 for over-immune activation group). Abbreviations: NK cell, Natural killer cell; CD, Cluster of differentiation; SSC-H, Side scatter histogram; FITC, Fluorescein isothiocyanate; PE, Phycoerythrin; PerCP, Peridinin-chlorophyll-protein.

Table 2. The comparison of flow cytometric parameters in three RIF subgroups

Variables	Balanced group (Mean \pm SD)	Low activation group (Mean \pm SD)	Over activation group (Mean \pm SD)	Balanced vs. Low (P value)	Balanced vs. Over (P value)	Low vs. Over (P value)
NK cells (CD3 ⁺ CD16 ⁺ D56 ⁺)	6.553 \pm 3.563	7.445 \pm 2.652	17.56 \pm 3.969	0.5194	< 0.0001	< 0.0001
Th1/Th2 (CD4 ⁺ IFN- γ ⁺ /CD4 ⁺ IL4 ⁺)	8.597 \pm 3.537	6.920 \pm 2.325	21.03 \pm 5.395	0.2180	< 0.0001	< 0.0001

Abbreviations: RIF, Recurrent implantation failure; NK cells, natural killer cells; IL, Interleukin; IFN- γ , Interferon gamma; Th 1, T helper 1 lymphocytes; Th 2, T helper 2 lymphocytes; CD, Cluster of differentiation. Data are presented as mean \pm SD. A P value < 0.05 was considered statistically significant.

Table 3. The comparison of autoantibody measurement results in the three RIF subgroups

Variables	Balanced group Pos/Total (%)	Low activation group Pos/Total (%)	Over activation group Pos/Total (%)	Balanced vs. Low (P value)	Balanced vs. Over (P value)	Low vs. Over (P value)
ANA	0/32 (0%)	3/47 (6.4%)	24/84 (28.6%)	0.9989	0.0025	0.0152
Anti-cardiolipin	0/32 (0%)	3/47 (6.4%)	4/84 (4.76%)	0.7243	0.7307	0.9996
Anti-dsDNA	0/32 (0%)	6/47 (12.8%)	4/84 (4.76%)	0.6851	0.5598	0.9998
Anti-Phospholipid	0/32 (0%)	3/47 (6.4%)	28/84 (33.3%)	0.9845	0.0002	0.0048
Anti-TG	0/32 (0%)	0/47 (0%)	20/84 (23.8%)	0.9999	0.0126	0.0305
Anti-TPO	0/32 (0%)	0/47 (0%)	32/84 (38.09%)	> 0.9999	0.0092	0.0251
Anti-TTG	1/32 (3.1%)	3/47 (6.4%)	8/84 (9.52%)	0.9981	0.3110	0.3359
Anti- β 2GPI	0/32 (0%)	0/47 (0%)	20/84 (23.8%)	0.9586	0.0036	0.0032

Abbreviations: RIF, Recurrent implantation failure; ANA, Anti-nuclear antibody; Anti-dsDNA, anti-double stranded DNA antibody; Anti-TG, anti-thyroglobulin antibody; Anti-TPO, anti-thyroid peroxidase antibody; Anti-TTG, anti-tissue transglutaminase antibody; Th 2, T helper 2 lymphocytes; Anti- β 2GPI, Anti- β -glycoprotein I. Pos/Total: Positive cases/Total cases (represents the proportion of individuals who tested positive for a specific autoantibody relative to the total number of individuals tested for that antibody within each group). Immune profile classification: Balanced = gene expression within \pm 1 SD of fertile controls; Low = below this range; Over = above this range (based on IL-15, IL-18, TWEAK, and Fn-14 RT-qPCR analysis). Data are presented as a ratio of the number of positive people in each group (%). A P value < 0.05 was considered statistically significant.

Table 4. The comparison of serum levels of metabolites in RIF subgroups

Variables	Balanced group (Mean ± SD)	Low activation group (Mean ± SD)	Over activation group (Mean ± SD)	Balanced vs. Low (P value)	Balanced vs. Over (P value)	Low vs. Over (P value)
S1P	75.62 ± 6.689	137.5 ± 10.15	17.44 ± 3.093	< 0.0001	< 0.0001	< 0.0001
Adiponectin	14.16 ± 4.88	45.28 ± 3.11	1.461 ± 0.76	< 0.0001	< 0.0001	< 0.0001
Leptin	11.90 ± 1.831	0.9605 ± 0.6492	37.13 ± 2.773	< 0.0001	< 0.0001	< 0.0001
Phosphatidylserine	66.20 ± 9.424	60.29 ± 11.35	69.77 ± 15.26	0.5317	0.8018	0.1619
PGE2	287.1 ± 23.48	475.7 ± 23.31	87.12 ± 10.21	< 0.0001	< 0.0001	< 0.0001
Total phospholipid	215.4 ± 22.29	76.76 ± 12.55	454.4 ± 22.23	< 0.0001	< 0.0001	< 0.0001
IGF1	254.7 ± 29.12	227.6 ± 59.21	265.8 ± 91.80	0.4034	0.8268	0.1898

Abbreviations: RIF, Recurrent implantation failure; IGF-1, insulin-like growth factor 1; PGE2, prostaglandin E2; S1P, sphingosine 1 phosphate. Immune profile classification: Balanced = gene expression within ± 1 SD of fertile controls; Low = below this range; Over = above this range (based on IL-15, IL-18, TWEAK, and Fn-14 RT-qPCR analysis). All values are expressed in the following units: S1P (ng/mL), Adiponectin (ng/mL), Leptin (ng/mL), Phosphatidylserine (ng/mL), PGE2 (pg/mL), Total phospholipid (mg/dL), and IGF-1 (ng/mL). Data are presented as mean \pm SD. A *P* value < 0.05 was considered statistically significant.

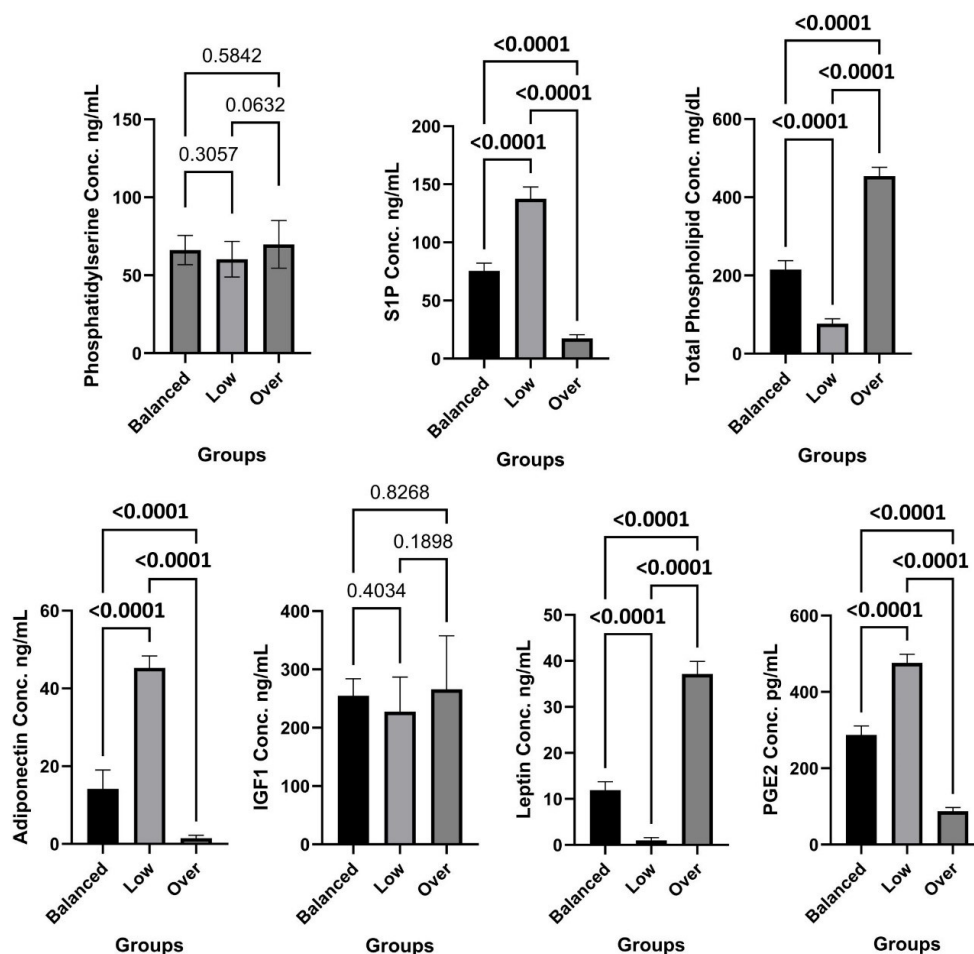


Fig. 4. The serum level of metabolites in three RIF subgroups. Abbreviations: RIF, Recurrent implantation failure; IGF-1, insulin-like growth factor 1; PGE2, prostaglandin E2; S1P, sphingosine 1 phosphate; Conc, concentration. *P* < 0.05 considered as significant. (N = 32 for balanced, N = 47 for low and N = 84 for over-immune activation group).

invasive peripheral blood-based classification system capable of mirroring the established endometrial immune profiles previously defined by molecular biomarkers in biopsied tissue.¹² By incorporating peripheral Th1/Th2 balance, NK-cell expression, autoantibody signatures, and a panel of circulating metabolites, we aimed to capture an integrated systemic reflective of endometrial immune misregulation.^{31,32} The results demonstrate that peripheral blood parameters are highly informative for distinguishing

low, balanced, and over-active immune endotypes in RIF patients, providing a compelling foundation for the development of an accessible diagnostic tool to guide personalized clinical intervention.

Our data suggest a tripartite systemic immune-metabolic perspective that could correspond to three types of endometrial immune activation (low, balanced, and over). The over-immune activation group exhibited a pronounced pro-inflammatory and autoimmune

phenotype.³³ This pattern is evidenced by a significantly increased Th1/Th2 ratio, a higher percentage of NK cells, and elevated levels of various autoimmune antibodies, including ANA, antiphospholipid, anti- β 2-glycoprotein I, anti-TG, and anti-TPO. The presence of these autoimmune antibodies indicates an expanded autoreactive phenotype commonly linked to thrombophilia, uterine vascular dysfunction, and inflammatory activation, all of which are recognized as contributing factors in implantation failure.^{34,35} These findings align with existing literature linking excessive Th1 polarization and autoimmunity to impaired endometrial receptivity and embryo rejection.³⁶ Increased Th1-dominance has previously been reported in women with RIF and RPL, reflecting a skew toward pro-inflammatory cytokines such as IFN- γ and TNF- α that disrupt implantation-associated tolerance.^{33,37,38} Similarly, heightened systemic NK-cell cytotoxicity has been linked to inadequate remodeling of spiral arteries, insufficient trophoblast invasion, and increased risk of reproductive failure.^{39,40} Our data confirm that these immune characteristics are not only present locally in the endometrium—as shown by the elevated IL-15/Fn-14 and IL-18/TWEAK ratios in this subgroup—but are also significantly detectable in peripheral circulation. Notably, the absence of significant differences in anti-cardiolipin, anti-dsDNA, and anti-TTG levels suggests a selective, rather than generalized, autoimmune activation in RIF—likely reflecting localized immune perturbations rather than systemic autoimmune disease.³³ This selectivity reinforces the clinical relevance of our panel: it discriminates pathogenic autoimmunity relevant to implantation from incidental seropositivity.

In parallel, the Over-immune activation group displayed a striking metabolic signature: elevated leptin, and total phospholipids, alongside markedly reduced adiponectin, S1P, and PGE2. S1P, a bioactive sphingolipid, is a potent regulator of immune cell trafficking, vascular permeability, and endothelial barrier function.⁴¹ Its dramatic elevation in the low-immune activation group may promote excessive leukocyte infiltration into the endometrium, disrupt vascular remodeling, and foster a pro-thrombotic milieu—all detrimental to embryo implantation.⁴² The high leptin-to-adiponectin ratio mirrors a state of chronic low-grade inflammation and insulin resistance, both of which are known to impair endometrial decidualization and embryo–endometrial dialogue.⁴³ The suppression of PGE2—a key lipid mediator that supports implantation by modulating uNK cell function, vascular tone, and immunosuppressive Treg differentiation—further exacerbates this hostile endometrial environment.⁴⁴ Together, these metabolic alterations suggest a feed-forward loop between immune hyperactivation and metabolic dysregulation.

The Low-immune activation group displayed noticeably reduced Th1/Th2 ratios, consistent with insufficient inflammatory priming and impaired recruitment or maturation of uNK cells, both of which are critical for the orchestration of decidualization and angiogenesis

during the WOI.⁴⁵ This finding aligns with earlier reports indicating that overly immunosuppressed endometrial states are equally detrimental for implantation as excessive inflammation.⁴⁶ The differences in most autoantibodies and flow cytometric parameters between the balanced and low subgroups did not reach statistical significance. This suggests that metabolic biomarkers may be particularly important for distinguishing these endotypes. Additionally, the similarity in autoantibody profiles between the balanced and low subgroups supports the idea that reduced immune activation is mechanistically different from hyperactivation and does not involve overt autoreactivity.⁴⁷

While Th1/Th2 ratios and NK-cell percentages were comparable to the balanced group, so the results for this group exhibited significantly lower leptin and total phospholipids, but strikingly elevated adiponectin, S1P, and PGE2. Adiponectin, typically viewed as anti-inflammatory and insulin-sensitizing, may, in this context, reflect an excessively immunosuppressive state that fails to mount the controlled inflammatory response required for successful implantation.^{48,49} Similarly, while PGE2 is generally pro-implantation, its extreme elevation in the absence of adequate Th1-driven cytokine signaling (e.g., IFN- γ) may disrupt the delicate cytokine balance necessary for trophoblast invasion and vascular transformation.⁵⁰ The lack of significant differences in phosphatidylserine and IGF-1 across groups further refines our understanding. Phosphatidylserine, an “eat-me” signal exposed on apoptotic cells, did not vary, suggesting that systemic apoptotic tone is not a major discriminator in RIF subtypes.⁵¹ Similarly, stable IGF-1 levels imply that major disruptions in the growth hormone–IGF axis are not central to the immune endotypes we describe, redirecting focus toward adipokines, and bioactive lipids.⁵²

Direct research on total phospholipid levels and their singular effect on trophoblast invasion is limited, as most studies focus on specific phospholipid classes, metabolites, or signaling pathways.⁵³ However, a strong and clear mechanistic link exists: disruptions in phospholipid metabolism—whether through overall depletion, imbalance, or specific alterations—can significantly impair trophoblast invasion.⁵⁴ In this study, Total phospholipid levels displayed a similarly strong triphasic separation, supporting a broader lipidomic disruption underlying implantation failure. These lipids contribute directly to membrane fluidity, cell signaling, and trophoblast invasion capacity, suggesting a structural and biochemical dimension of dysfunction in both low and over-immune states.^{53,55} This paradox—where both excessive and insufficient immune-metabolic activity impair implantation—underscores the “Goldilocks principle” of endometrial receptivity: successful implantation requires immune and metabolic responses that are neither too strong nor too weak, but precisely calibrated.⁵⁶

These insights strengthen the rationale for applying targeted, mechanism-specific interventions rather than

empirical, one-size-fits-all treatment strategies in ART patients. The convergence of immune, autoimmune, and metabolic abnormalities observed exclusively in the over-immune activation group highlights an especially high-risk endotype that may benefit most from aggressive immunomodulatory treatment strategies.⁷ In contrast, patients in the low-immune activation group would be more appropriately treated with immune-activating approaches (e.g., endometrial scratching, hCG-triggered immune activation, or intralipid/PRP strategies) rather than the suppressive interventions sometimes used empirically in RIF.⁷ The noteworthy point is that, balanced-profile patients—who are likely to be in the normal situation in terms of their immune system profile—may avoid unnecessary and potentially harmful immunotherapies entirely.^{7,57} Thus, a non-invasive blood-based algorithm could not only reduce the need for endometrial biopsies but also streamline clinical decision-making by enabling earlier, safer, and repeated profiling throughout treatment cycles.

Our study can be significant in several respects. First, it integrates multi-omic peripheral markers—immune cells, autoantibodies, and metabolites—into a unified classification framework validated against a pre-registered endometrial molecular assay. On the other hand, the use of standardized, commercially available assays enhances reproducibility and clinical translatability. Furthermore, the segregation of RIF subtypes by systemic signatures supports the biological plausibility of endometrial–systemic immune crosstalk, likely mediated by shared cytokine networks, hormonal signals, and circulating immune cells.

However, several limitations warrant consideration. First of all, while peripheral blood partially reflects uterine immune function, discordance may occur due to the tissue-specific specialization of uNK and endometrial T cells. Additionally, our cross-sectional design only captures a single point in time during the mid-luteal phase; longitudinal studies examining both pre-implantation and peri-implantation periods would enhance predictive biological resolution. Moreover, including additional controls—such as women facing unexplained infertility without a history of repeated implantation failures—would bolster the validity of our findings. Information on participants' concurrent medications and comorbid conditions, including autoimmune and rheumatologic disorders, was considered as part of the study exclusion criteria wherever possible; however, complete data were not available for all participants, which can be considered a study limitation. Lastly, expanding our approach to include multiple omics analyses, such as detailed lipidomics, proteomics, and cytokine profiling, could further refine subgroup classifications.

Future studies should investigate whether blood-based immune-metabolic signatures can predict ART outcomes prospectively and evaluate whether targeted immunotherapy guided by this classification improves live-birth rates. Validation in larger, independent cohorts

and across centres is also necessary before broad clinical implementation.

Conclusion

In conclusion, this study, in line with previous research, demonstrates that RIF is not a single entity but a syndrome comprising distinct immune-metabolic endotypes, each identifiable through a minimally invasive peripheral blood test. The over phenotype is marked by autoimmune activation, Th1 skewing, and a pro-inflammatory metabolic milieu, whereas the low phenotype exhibits insufficient immune activation coupled with an exaggerated anti-inflammatory metabolic state. These findings support the development of a non-invasive diagnostic platform for personalized management of RIF, with the potential to transform clinical decision-making and reduce unnecessary biopsy procedures. Furthermore, by classifying patients based on peripheral blood biomarkers, clinicians can move beyond empiric, one-size-fits-all protocols toward rationally targeted, mechanism-based therapies that address the root cause of implantation failure in each individual.

Authors' Contribution

Conceptualization: Mehdi Yousefi, Sina Baharaghdam.

Data curation: Sina Baharaghdam, Shima Karimi, Mohammad Esfani Farahani.

Formal analysis: Özkan Yildirim, Mohammadbagher Pirouzpanah, Reza Mousavi Ardehaie.

Funding acquisition: Mehdi Yousefi.

Investigation: Sina Baharaghdam, Ali Hassanzadeh, Amin Kamrani, Amir Mehdizadeh.

Methodology: Mehdi Yousefi, Leili Aghebati-Maleki, Sina Baharaghdam.

Project administration: Mehdi Yousefi, Leili Aghebati-Maleki.

Resources: Parvin Hakimi, Javad Ahmadian Heris.

Software: Özkan Yildirim, Mohammadbagher Pirouzpanah, Danial Abbas Kabiri, Amirhossein Aghapour.

Supervision: Mehdi Yousefi.

Validation: Mehdi Yousefi, Leili Aghebati-Maleki, Parvin Hakimi, Javad Ahmadian Heris.

Visualization: Mehdi Yousefi, Sina Baharaghdam.

Writing—original draft: Sina Baharaghdam, Shima Karimi, Mohammad Esfani Farahani.

Writing—review & editing: Özkan Yildirim, Mohammadbagher Pirouzpanah, Danial Abbas Kabiri, Reza Mousavi Ardehaie.

Competing Interests

None to be declared.

Ethical Approval

Collection and use of all samples were approved by the Ethics Committee of the Research Council of the Faculty of Medicine, Tabriz University of Medical Sciences (IR.TBZMED.REC.1403.158).

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Supplementary Files

Supplementary file 1 contains Tables S1 and S2.

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