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# Lifestyle and environmental factors and human fertility

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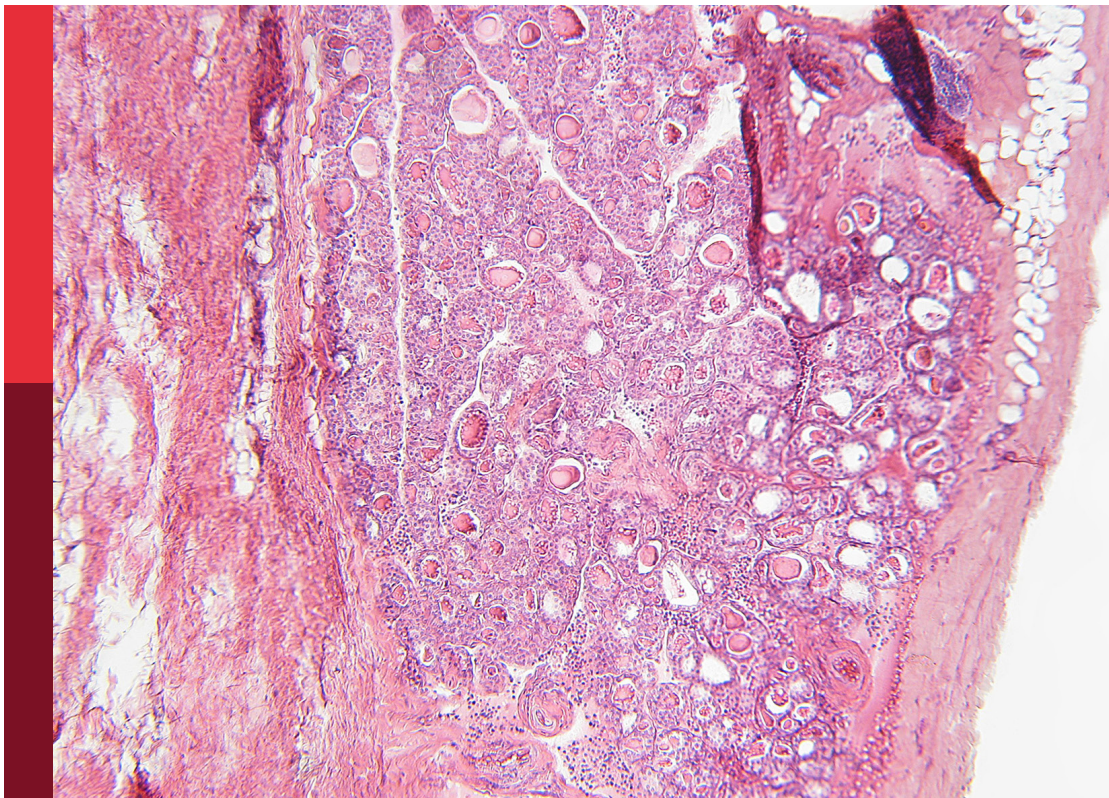

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# Lifestyle and environmental factors and human fertility

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# Editorial: Lifestyle and environmental factors and human fertility

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Editorial on the Research Topic
Lifestyle and environmental factors and human fertility

## Introduction

Human fertility is a complex and multifactorial process influenced by genetic, hormonal, physiological, behavioral, and environmental determinants. A growing body of evidence has highlighted a concerning global trend: declining fertility rates and an increasing prevalence of reproductive disorders in both men and women.

Fertility rates have declined worldwide over the past several decades, with most countries now below the replacement level of 2.1 children per woman. This trend is especially pronounced in industrialized and high-income regions, but is increasingly observed globally (1–7). Recent estimates show over 55 million men and 110 million women affected globally, with the burden increasing steadily from 1990 to 2021 and projected to continue rising through 2040 (5–9). Male reproductive health is deteriorating, evidenced by declining sperm counts, increased testicular cancer, and more reproductive system abnormalities (4, 8, 10). Female infertility is also increasing, with the highest burden among women aged 35-39, and a trend toward earlier onset (1, 9, 11). High-income countries show declining fertility and, in some cases, a decrease in primary infertility, but secondary infertility and reproductive disorders remain concerns (1, 2, 7). Low- and middle-income regions are experiencing a rapid rise in infertility, often linked to infections, limited healthcare access, and environmental exposures (5, 6, 8, 9). Aging populations and delayed parenthood further exacerbate infertility risks, especially for women (1, 9, 11).

While part of this decline can be attributed to socio-demographic and cultural changes -such as delayed parenthood, economic pressures, and access to contraception- there is mounting concern that modifiable factors related to lifestyle and environmental exposures play a crucial role in shaping reproductive health outcomes. Lifestyle factors including diet, physical activity, alcohol consumption, tobacco use, stress, and sleep quality are now recognized as key modulators of reproductive function (12–17).

At the same time, environmental influences -ranging from air pollution and industrial contaminants to occupational exposures and climate change- have become increasingly relevant in the context of fertility (18–20). Pollutants such as heavy metals, pesticides, and

microplastics can interfere with hormone signaling, oxidative stress pathways, and DNA integrity, ultimately affecting both gamete quality and embryonic development.

Importantly, the impact of these factors may extend across generations. There is strong evidence -especially from animal models- that environmental and lifestyle factors can induce epigenetic changes in the germline, which are transmitted across generations and increase reproductive risk (21, 22). While human data are less direct, the potential for transgenerational inheritance of reproductive disorders is a growing concern, warranting further research and preventive action. Given this complex interplay between biological systems, lifestyle behaviors, and environmental conditions, interdisciplinary research has become essential. The integration of epidemiological data, clinical observations, molecular insights, and experimental models would allow for a comprehensive understanding of how modern living environments influence human reproductive capacity.

## Insights from this Research Topic

The Frontiers in Endocrinology Research Topic "Lifestyle and Environmental Factors and Human Fertility" brings together contemporary research addressing this multifaceted challenge. The Topic provides a platform that collectively advances our understanding of the intricate relationships between lifestyle, environment, and reproductive health with the goal of fostering a multidisciplinary dialogue among scientists and clinicians investigating the diverse determinants of human reproductive health.

Recognizing that fertility decline cannot be fully explained by demographic or genetic factors alone, the Topic sought to integrate perspectives from: 1) lifestyle factors and metabolic health, 2) environmental exposures and pollution, 3) machine learning, omics and predictive models, 4) nutrition, diet and endocrine regulation, 5) clinical and reproductive outcomes, and 6) socio-environmental and behavioral dimensions (Figure 1). By doing so, it aimed to capture the multifactorial nature of reproductive impairment and to identify actionable strategies for prevention and intervention. Collectively, these contributions explore the effects of dietary habits, physical activity, body composition, environmental pollutants, occupational exposures, psychosocial stressors, and circadian rhythm disturbances on human fertility (Table 1). Across diverse methodologies -from machine learning prediction models and large-scale epidemiological analyses to experimental and clinical investigations- these studies reveal converging evidence that metabolic health, oxidative balance, environmental toxicants, and psychosocial well-being are deeply intertwined with fertility outcomes in both women and men. Research exploring the Life's Essential 8 and Life's Crucial 9 indices (Gu et al., Cui et al., Huang et al., Zhang et al., Pang et al.) highlights the value of integrative clinical metrics in predicting infertility risk, while investigations into arsenic exposure (Su et al.), air pollution (Chen et al.), pesticides (Liu et al.), and endocrine disruptors (Tricotteaux-Zarqaoui et al., Sciorio et al.) reinforce the reproductive consequences of environmental contaminants. Studies addressing sleep quality, body composition, and inflammatory dietary patterns (Xie et al., Liu et al., Liang et al., Xia et al., Ding et al.) further emphasize the complex behavioral and metabolic mediators of reproductive potential. Complementary research on assisted reproductive technologies, vaccination, and seasonality (Wei et al., Ouyang et al., Li et al., Han et al., Shui et al., Li et al.) reflects how clinical and environmental variables jointly shape reproductive outcomes. Experimental findings from animal models (De la Cruz Borthiry et al.) and nutritional and physical interventions (Najdgholami et al., Chen et al.) provide mechanistic

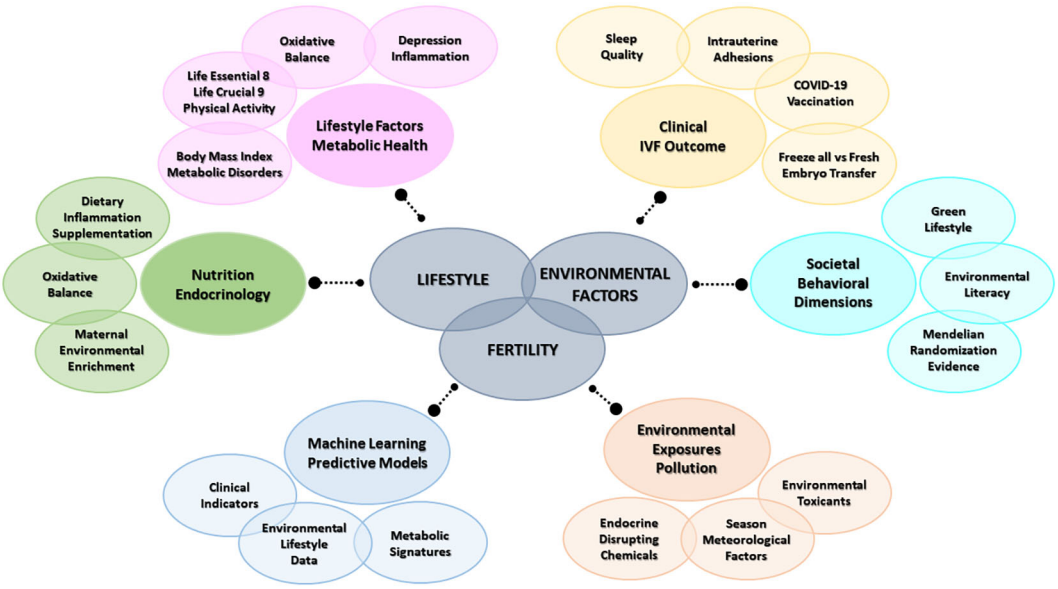


FIGURE 1
Topic integrative perspectives.

TABLE 1 Topic contributions.

| | Authors | Study Type/Data Source | Main Findings |
|---|---|---|---|
| Lifestyle Factors and Metabolic Health | Gu X et al. | Machine learning model (NHANES, SHAP method) | Combined Life´s Essential 8 (LE8) and heavy metal exposure predict female infertility risk |
| | Cui S et al. | Observational and mediation analyses (LE8) | Depression, inflammation and metabolic disorders mediates LE8´s effect on infertility |
| | Liu M et al. | Cross-sectional (NHANES) | Body roundness index independently linked to females infertility |
| | Huang S et al. | Epidemiology study | Life´s Crucial 9 (an expanded LE8) protects against infertility among U.S. women |
| | Xia P et al. | Population-based study | |
| | Chen P et al. | Logistic regression and mediation analyses (NHANES) | Physical activity mitigates infertility risk via lower HbA1c |
| Environmental Exposures and Pollution | Su L et al. | Cross-sectional (NHANES) | Urinary arsenic positively associated with endometriosis prevalence |
| | Chen M et al. | Retrospective IVF cohort | Maternal PM2.5 exposure linked to preterm birth and miscarriage in IVF patients |
| | Liu F et al. | Popultaion-based cross-sectional | Maternal pesticide exposure increases risk of birth defects |
| | Li H et al. | Cohort (IVF outcomes) | Season and meteorological factors influence embryo transfer outcomes |
| | Sciorio R et al. | Narrative review | Environmental toxicants impair sperm quality and fertility potential |
| | Tricotteaux-Zarqaoui S et al. | Review | Endocrine-disrupting chemicals (EDCs) alter fertility via epigenetic mechanims |
| Machine Learning, Omics and Predictive Models | Gu X et al. | ML model (SHAP) | Combined environmental and lifestyle data yield robust infertility prediction |
| | Zhang R et al. | Machine learning model | Clinical indicators accurately predict infertility and pregnancy loss |
| | Ding N et al. | Metabolomics profiling | Distinct metabolic signatures identify women at risk of pregnancy loss |
| Nutrition, Diet and Endocrine Regulation | Liang M et al. | Mediation analyses (NHANES) | Dietary inflammatory and oxidative balance scores predict reproductive function |
| | Najdgholami Z et al. | RCT (PCOS) | Flaxseed supplementation improves reproductive endocrine profiles |
| | De la Cruz Borthiry F et al. | Environmental enrichment | Maternal environmental enrichment increases corpora lutea and progesterone levels |
| Clinical and Reproductive Outcomes | Xie Y et al. | Prospective birth cohort | Sleep quality differs between ART and naturally conceiving women |
| | Ouyang et al. | IVF cohort study | Intrauterine adhesions impair endometrial receptivity and IVF outcomes |
| | Wei J et al. | Cohort | COVID-19 vaccination does not negatively affect IVF outcomes |
| | Han Y et al. | Systematic review and meta-analyses | Freeze-all vs fresh embryo transfer outcomes in adenomyosis and endometriosis |

*(Continued)*

TABLE 1 Continued

| | Authors | Study Type/Data Source | Main Findings |
|---|---|---|---|
| Socio – environmental and Behavioral Dimensions | Li Y et al. | Survey study | Identified determinants promoting green lifestyles among Chinese residents |
| | Shui Y et al. | Cross-sectional (rural Sichuan) | Environmental literacy cprrelates with better health outcomes |
| | Pang Q et al. | Review of Mendelian randomization studies | Summarized MR-evidence on common male-specific diseases affecting fertility |

insights into hormonal regulation and reproductive resilience. Taken together, this Research Topic advances a holistic understanding of fertility as a reflection of both biological integrity and environmental stewardship, calling for interdisciplinary approaches that integrate public health, environmental science, and reproductive medicine.

Importantly, the Topic sought to underscore the interconnected nature of these influences. Rather than acting independently, lifestyle and environmental exposures often converge to amplify reproductive risk. This integrative approach reflects a paradigm shift in reproductive endocrinology: from studying isolated risk factors toward a systems-level understanding of fertility as an indicator of overall health. The assembled articles collectively reinforce the idea that human fertility can serve as a sensitive biomarker of broader physiological and ecological well-being.

In summary, this Research Topic represents a comprehensive, evidence-based synthesis of how modern living conditions affect reproductive potential. By bringing together observational, mechanistic, and clinical insights, it lays the groundwork for future translational research aimed at mitigating reproductive risks through lifestyle modification, environmental policy, and public health action. The 24 articles published in this Research Topic collectively address how lifestyle patterns and environmental conditions interact to modulate human reproductive capacity. Across the categories investigated, a unifying message emerges: fertility reflects systemic health. Whether the trigger is metabolic imbalance, environmental pollution, or psychosocial strain, the reproductive axis responds as a sensitive indicator of physiological stability. The evidence gathered collectively supports the notion that reproductive endocrinology is inseparable from broader lifestyle and environmental sciences.

## Scientific and societal relevance

The cumulative findings of this Research Topic highlight the profound intersection between human reproductive health and broader societal and environmental contexts. Fertility is not merely a clinical endpoint; it is a sensitive biomarker reflecting systemic physiological integrity, environmental exposures, and lifestyle patterns. Understanding these interconnections carries both scientific significance and societal urgency.

From a scientific perspective, the articles collectively advance knowledge in several domains. First, they provide mechanistic evidence linking metabolic and nutritional factors to gamete quality and reproductive outcomes. This reinforces the concept that metabolic health is a modifiable determinant of fertility, opening opportunities for targeted interventions. Second, research on environmental pollutants underscores the vulnerability of the reproductive system to even low-level exposures, highlighting the need for stringent monitoring and regulatory policies. Third, studies addressing psychosocial stress, circadian disruption, and physical activity emphasize that lifestyle behaviors operate through complex endocrine and epigenetic pathways, offering additional avenues for preventive strategies.

The societal relevance of these findings is equally compelling. Globally, fertility rates are declining in many regions, and the burden of infertility is increasingly recognized as both a public health challenge and a social concern (4). Its consequences extend far beyond individual health to impact families, communities, and national development. By identifying modifiable lifestyle and environmental risk factors, the Research Topic contributes actionable knowledge that can inform health promotion, reproductive counseling, and population-level interventions. For instance, promoting balanced diets, regular yet moderate physical activity, and adequate sleep, alongside policies to reduce exposure to air pollution, could enhance fertility outcomes and improve overall health.

Moreover, these studies illuminate the interdisciplinary nature of reproductive science. Fertility research no longer resides solely within endocrinology or gynecology; it spans toxicology, epidemiology, nutrition, behavioral science, and environmental policy. This integrated perspective enriches scientific understanding and strengthens the evidence base for translational and preventive strategies that can benefit individuals and populations alike.

Finally, the Research Topic underscores the importance of equity and access. Environmental exposures and lifestyle-related risks are often unevenly distributed across socio-economic strata. Recognizing and addressing these disparities is essential for achieving reproductive justice, ensuring that all individuals have the opportunity to maintain reproductive health regardless of geographic, economic, or social constraints. The 24 articles collectively demonstrate that human fertility is both a marker of personal health and a reflection of societal and environmental conditions. They provide a compelling case for integrating lifestyle optimization and environmental stewardship into public

health agendas, while simultaneously expanding the scientific horizons of reproductive endocrinology.

## Future directions and concluding remarks

The research compiled in this Research Topic provides a comprehensive snapshot of how lifestyle and environmental factors influence human fertility, yet it also highlights the complexity and remaining gaps in our understanding. Despite substantial progress, many mechanisms linking metabolic status, environmental exposures, and psychosocial stress to reproductive function remain incompletely elucidated. For instance, the interplay between multiple exposures -metabolic, chemical, and behavioral- requires integrative models capable of capturing synergistic or antagonistic effects. Future studies employing systems biology approaches, longitudinal cohorts, and advanced biomarker analyses will be critical to untangle these relationships.

Another promising avenue lies in translational and preventive research. While clinical studies increasingly incorporate lifestyle counseling, robust evidence-based guidelines are still limited. Interventions targeting diet, physical activity, sleep, and environmental risk reduction could be standardized and tested across diverse populations. Similarly, elucidating the molecular signatures of environmental and lifestyle exposures may allow the development of early biomarkers of reproductive risk, enabling timely intervention before fertility is compromised.

From a societal perspective, research must continue to address health disparities and environmental justice. Many populations disproportionately experience high environmental exposures or limited access to reproductive care, emphasizing the need for inclusive studies and policies that prioritize vulnerable groups. Global collaboration, open data sharing, and interdisciplinary partnerships will be essential to translate scientific insights into equitable solutions.

For young investigators entering this field, the Research Topic offers both inspiration and guidance. Fertility research is no longer confined to isolated laboratories or clinical silos; it intersects endocrinology, toxicology, epidemiology, nutrition, behavioral science, and public health. Embracing this interdisciplinary mindset offers opportunities to make meaningful contributions that resonate far beyond the lab, influencing policy, health outcomes, and societal well-being.

In conclusion, lifestyle and environmental factors are powerful determinants of human reproductive health, reflecting both individual choices and broader ecological conditions. The studies presented here demonstrate that interventions at multiple levels -molecular, clinical, behavioral, and societal- have the potential to preserve and enhance fertility. As the field moves forward, a combination of rigorous science, innovative methodologies, and a commitment to public health and equity will be crucial. By integrating these perspectives, the next generation of researchers can not only deepen scientific understanding but also foster a future in which reproductive health is safeguarded as a vital component of human well-being.

## Author contributions

MR: Conceptualization, Investigation, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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# Maternal pesticide exposure and risk of birth defects: a population-based cross-sectional study in China

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**Objective:** This study aimed to examine the association between maternal pesticide exposure during the periconceptional period and birth defects in their offspring.

**Methods:** A survey was conducted among 29,204 women with infants born between 2010 and 2013 in Shaanxi Province, Northwest China. All cases of birth defects were diagnosed using the International Classification of Diseases, Tenth Revision (ICD-10). Given the multistage sampling design, the generalized estimating equation (GEE) binomial regression models with log link and exchangeable correlation structures were used to analyze the association between maternal pesticide exposures and birth defects.

**Results:** Among the 29,204 subjects, 562 mothers had children with birth defects, resulting in an incidence rate of 192.44 per 10,000 live births. The incidence of birth defects was higher in the pesticide-exposed group compared to the control group (737.46/10,000 vs. 186.04/10,000). After adjusting for baseline demographic characteristics, fertility status, nutritional factors, and environmental factors in the GEE model, the results indicated that the risk of birth defects and cardiovascular system defects in mothers exposed to pesticides during the periconceptional period was 2.39 times (95% CI: 1.84–3.10) and 3.14 times (95% CI: 1.73–5.71) higher, respectively, compared to the control group.

**Conclusion:** This study demonstrated that maternal exposure to pesticides during the periconceptional period was associated with an increased risk of birth defects, particularly cardiovascular system defects in offspring. Consequently, it would be beneficial to avoid pesticide exposure from three months before pregnancy through the first trimester to lower birth defects in infants.

KEYWORDS

**pesticides, birth defects, perinatal pregnancy, cardiovascular system defects, crosssectional survey**

## 1 Introduction

Congenital abnormalities, commonly known as birth defects, include a wide range of anatomical and functional irregularities that develop during embryonic or fetal growth. The International Classification of Diseases, Tenth Revision (ICD-10), identifies approximately 10 systems in which birth defects can occur. Over 20 significant birth defects are frequently

encountered in clinical practice, including congenital heart disease, neural tube defects, limb shortening, cleft lip and palate, and trisomy 21 syndrome. In China, the prevalence of birth defects was 123 per 10,000 perinatal births during 2000–2021 (1). These defects contribute significantly to early abortion, stillbirth, perinatal death, infant mortality, and congenital disabilities (2).

A growing body of evidence suggests that the risk of birth defects is influenced by a complex interaction of genetic and environmental factors. The widespread use of pesticides, especially in agriculture, has been shown through experimental research to produce endocrine disruptors, neurodevelopmental toxicants, immunotoxicants, and carcinogens (3, 4). Numerous studies, both domestic and international, indicate that prenatal exposure to pesticides may increase the risk of adverse birth outcomes, including miscarriage, premature delivery, stillbirth, and birth defects (5–9). These exposures may also impact the long-term cognitive and physical development of offspring (10–13). A case–control study in Northern Netherlands identified maternal occupational exposure to pesticides and dust as potential risk factors for oral clefts in offspring (14). Additionally, exposure to personal, household, and agricultural pesticides during pregnancy may raise the risk of holoprosencephaly (15). An animal study provides new insights into the harmful effects of pesticide and environmental chemical exposure on craniofacial skeletal development in zebrafish embryos (16).

However, some studies have produced conflicting results regarding the correlation between prenatal pesticide exposure and birth defects. For instance, a case–control study in California found no evidence of a correlation between maternal residential exposure to agricultural pesticides and specific congenital heart defects in children (17). Moreover, most pesticides analyzed did not show a statistically significant relationship with an increased risk of hypospadias in male infants (18). These conflicts might result from differences in pesticide exposure assessment methods and study population heterogeneity (19). Different agricultural practices, pesticide types, genetic backgrounds in various regions, as well as small sample sizes in some studies and possible protective genetic factors in certain areas with low pesticide usage, can make the exposure birth defect association less obvious and reduce statistical power (20, 21).

The period extending from 3 months prior to conception through the first trimester of pregnancy is critical for both conception and fetal development. Notably, during the first trimester, pregnant women are particularly susceptible to the effects of pesticides due to physiological changes and the immature state of the placenta (22). In many agricultural regions of low- and middle-income countries, pesticide use coincides with the periconceptional stage, increasing exposure risk (23). Pesticide exposure in the early stage of life can also lead to long term issues like developmental delays, autism spectrum disorder and learning disabilities (24). Given the current understanding of the link between maternal pesticide exposure during early pregnancy and birth defects in offspring, further research is needed.

Therefore, this study used data from a large population-based survey of birth defects conducted in Shaanxi Province, Northwest China, to explore the potential connection between maternal pesticide exposure during the periconceptional period and the occurrence of birth defects in offspring. The findings of this study could help inform strategies for the prevention and management of birth defects.

# 2 Methods

## 2.1 Study design and participants

This study used data from a population-based cross-sectional survey on birth defects conducted in Shaanxi Province, Northwest China, between July and November 2013. Mothers with documented pregnancy outcomes and their infants born between 2010 and 2013 were selected using a stratified multistage sampling approach. The detailed sampling methodology has been previously published (25). The study was approved by the Human Research Ethics Committee of Xi'an Jiaotong University (approval number: 2012008). Written informed consent was obtained from all participants. After excluding 823 subjects due to unclear pregnancy outcomes or incomplete questionnaires, the final analysis included 29,204 women.

## 2.2 Definition and diagnosis of birth defects

Birth defects were classified according to ICD-10 codes, covering malformations of various body systems, including defects of the nervous system; eyes, ears, face, and neck; circulatory system; respiratory system; cleft lip and palate; digestive system; reproductive organs; urinary system; musculoskeletal system; other congenital malformations; and chromosomal abnormalities not classified elsewhere.

An expert team, including senior medical technicians from the departments of congenital heart surgery, ultrasound, obstetrics, and gynecology at the First Affiliated Hospital of Xi'an Jiaotong University, was assembled for the diagnosis of birth defects. The diagnosis of congenital anomalies was determined by this team using the ICD-10. For pediatric patients with external anomalies, medical records were reviewed, and malformations were documented with photographic evidence to aid in diagnosis. For congenital internal malformations, including cardiovascular anomalies, medical records were reviewed, and supplementary ultrasound examinations were conducted at the First Affiliated Hospital of Xi'an Jiaotong University to establish a definitive diagnosis.

## 2.3 Definition of pesticide exposure and the periconceptional period

Pesticides included various chemical classes such as pyrethroids, carbamates, organophosphorus compounds, organofluorides, organochlorines, avermectin, carbendazim, dafenazine, sofril, rodenticides, and herbicides, among others. The periconceptional period was defined as the span from 3 months before pregnancy to the first 3 months of pregnancy.

## 2.4 Definitions of covariates

Previous research (26–28) has identified several factors correlated with birth defects, including maternal socio-demographic characteristics, fertility status, nutritional factors, and environmental factors. Specifically, these variables included maternal age (<30

and ≥ 30 years), education level (college degree and above, high school or technical secondary school, junior high school, primary school and below), ethnicity (Han, other), residence (rural, urban), household wealth index (poor, moderate, rich), first pregnancy (yes, no), history of abortions (yes, no), type of pregnancy (singleton, multiple), family history of birth defects (yes, no), folic acid supplementation (yes, no), infections (yes, no), tobacco exposure (yes, no), occupational risk exposure (yes, no), and industrial exposure (yes, no).

The folic acid supplementation regimen was defined as the consistent intake of at least 400 μg of folic acid per day during the periconceptional period, sustained for more than three consecutive months. Infection was characterized by febrile episodes (temperature exceeding 38°C) and symptoms related with influenza or the common cold, including influenza-like symptoms and seasonal influenza during pregnancy. Tobacco exposure during pregnancy was categorized into two groups: active smoking, defined as the consumption of at least one cigarette per week for 3 consecutive months, and passive smoking, defined as inhaling secondhand smoke for a minimum of 15 min per day over one consecutive month. Occupational exposure was defined by the presence of biological, physical, chemical, or psychosocial risk factors in the workplace. Industrial exposure was defined as residing within a one-kilometer radius of mines, cement factories, power plants, fertilizer factories, or major traffic thoroughfares during pregnancy.

## 2.5 Data collection

A standardized questionnaire was developed by the Department of Epidemiology and Health Statistics at the School of Public Health, Xi'an Jiaotong University Health Science Center. This questionnaire gathered information on maternal socio-demographic characteristics, fertility status, nutritional factors, and environmental factors during pregnancy, and child birth outcomes as reported by the mothers. Trained field staff conducted face-to-face interviews to administer the questionnaire. Additionally, diagnostic information regarding birth outcomes, including the timing of diagnosis and types of birth defects, was collected from local hospitals. Data on perinatal pesticide exposure were self-reported by mothers, detailing contact with pesticides via skin or respiratory routes during this period.

## 2.6 Statistical analysis

Categorical variables were summarized as frequencies and percentages, and intergroup comparisons were performed using the chi-squared test, corrected chi-squared test, or Fisher's exact test, as appropriate. Continuous variables were reported as mean and standard deviation, with group comparisons conducted using the *t*-test for normally distributed variables or the Wilcoxon rank-sum test for non-normally distributed variables. The generalized estimating equation (GEE) extends the generalized linear model, specifically addressing the analysis of non-independent data. The GEE is adept at fitting suitable statistical models to dependent variables that conform to a variety of distributions, such as normal, binomial, and Poisson. This statistical method effectively mitigates the issue of correlation among dependent variables in longitudinal datasets, thereby enhancing the reliability of the estimates obtained. Given the multistage sampling design, the GEE was used to analyze the association between maternal pesticide exposures during the periconceptional period and the incidence of birth defects, aiming to mitigate intra-group correlations. Additionally, to explore the potential heterogeneity in the effects of pesticide exposures on birth defects, effects were estimated within subgroups defined by various maternal characteristics.

The data entry process was conducted in duplicate with error checking using EpiData 3.1 software. Statistical analysis was performed using SAS 9.4 software (Statistics Analysis System, Inc., Cary, North Carolina, United States), with statistical significance set at a two-tailed *p*-value of less than 0.05.

# 3 Results

## 3.1 Participants' characteristics

Table 1 and Supplementary Table S1 presents the baseline characteristics of the 29,204 pregnant women included in the study. The mean age of the participants was 27.09 ± 4.76 years. Most participants had a junior high school education level (49.60%), were of Han ethnicity (99.36%), and resided in rural areas (67.24%). Among the participants, 339 women were in the pesticide exposure group, while 28,865 were in the non-exposure group. Compared to the non-exposure group, women in the pesticide exposure group were more likely to be older, live in rural areas, have a history of abortion and infection, be exposed to tobacco, and encounter occupational and environmental risk factors. They also tended to have lower education levels and household wealth index and were less likely to take folic acid supplements. No significant differences were observed between the two groups in terms of maternal ethnicity, type of pregnancy and family history of birth defects.

## 3.2 Prevalence of birth defects

Table 2 shows the prevalence of birth defects among the 29,204 participants. A total of 562 women gave birth to children with birth defects, resulting in an overall incidence rate of 192.44 per 10,000 live births. The prevalence of birth defects (737.46/10,000 vs. 186.04/10,000), congenital malformations of the circulatory system (324.48/10,000 vs. 60.28/10,000), and other congenital malformations (147.49/10,000 vs. 29.79/10,000) was significantly higher in the pesticide-exposed group compared to the unexposed group.

## 3.3 Pesticide exposure and birth defects

Due to the hierarchical structure of the data, with aggregation at the county level, a GEE model was used to account for the county (district) as a random effect in the multifactor analysis of pesticide exposure and birth defects (Table 3). In the unadjusted model (model 1), the risks of birth defects and cardiovascular system defects in the offspring of mothers exposed to pesticides were 4.20 times (95% CI: 3.16–5.59) and 5.53 times (95% CI: 3.14–9.73) higher, respectively, compared to the control group. After adjusting for baseline

TABLE 1 Baseline characteristics of participants.

| Variables | Total | Pesticide exposure group | Non-pesticide exposure group | t/$\chi^2$/Z | *p* value |
|---|---|---|---|---|---|
| Maternal age, Mean ± SD | 27.09 ± 4.76 | 30.05 ± 5.63 | 27.05 ± 4.74 | 9.907 | <0.001[a] |
| Maternal age, *n* (%) | | | | | |
| <30 years | 22,309 (76.39) | 172 (50.74) | 22,137 (76.69) | 125.144 | <0.001 |
| ≥30 years | 6,895 (23.61) | 167 (49.26) | 6,728 (23.31) | | |
| Maternal education level, *n* (%) | | | | | |
| Junior college and undergraduate or above | 5,326 (18.24) | 15 (4.42) | 5,311 (18.40) | 98.615 | <0.001 |
| High school | 5,835 (19.98) | 35 (10.32) | 5,800 (20.09) | | |
| Middle school | 14,485 (49.60) | 209 (61.65) | 14,276 (49.46) | | |
| Primary school or below | 3,558 (12.18) | 80 (23.60) | 3,478 (12.05) | | |
| Residence, *n* (%) | | | | | |
| Rural | 19,637 (67.24) | 314 (92.63) | 19,323 (66.94) | 100.333 | <0.001 |
| Urban | 9,567 (32.76) | 25 (7.37) | 9,542 (33.06) | | |
| Household wealth index, *n* (%) | | | | | |
| Poor | 9,743 (33.36) | 159 (46.90) | 9,584 (33.20) | 34.489 | <0.001 |
| Moderate | 9,773 (33.46) | 109 (32.15) | 9,664 (33.48) | | |
| Rich | 9,688 (33.17) | 71 (20.94) | 9,617 (33.32) | | |
| First pregnancy, *n* (%) | | | | | |
| Yes | 14,926 (51.11) | 68 (20.06) | 14,858 (51.47) | 132.337 | <0.001 |
| No | 14,278 (48.89) | 271 (79.94) | 14,007 (48.53) | | |
| History of abortions, *n* (%) | | | | | |
| Yes | 4,057 (13.89) | 91 (26.84) | 3,966 (13.74) | 48.097 | <0.001 |
| No | 25,147 (86.11) | 248 (73.16) | 24,899 (86.26) | | |
| Maternal folic acid supplementation, *n* (%) | | | | | |
| Yes | 10,593 (36.27) | 70 (20.65) | 10,523 (36.46) | 36.218 | <0.001 |
| No | 18,611 (63.73) | 269 (79.35) | 18,342 (63.54) | | |
| Maternal infections, *n* (%) | | | | | |
| Yes | 3,821 (13.08) | 77 (22.71) | 3,744 (12.97) | 27.970 | <0.001 |
| No | 25,383 (86.92) | 262 (77.29) | 25,121 (87.03) | | |
| Tobacco exposure, *n* (%) | | | | | |
| Yes | 17,359 (59.44) | 242 (71.39) | 17,117 (59.30) | 20.302 | <0.001 |
| No | 11,845 (40.56) | 97 (28.61) | 11,748 (40.70) | | |
| Occupational risk exposure, *n* (%) | | | | | |
| Yes | 2,414 (8.27) | 110 (32.45) | 2,304 (7.98) | 264.511 | <0.001 |
| No | 26,790 (91.73) | 229 (67.55) | 26,561 (92.02) | | |
| Industrial exposure, *n* (%) | | | | | |
| Yes | 7,766 (26.59) | 122 (35.99) | 7,644 (26.48) | 15.512 | <0.001 |
| No | 21,438 (73.41) | 217 (64.01) | 21,221 (73.52) | | |

[a]Wilcoxon rank test.

demographic characteristics (model 2), these risks decreased to 3.45 times (95% CI: 2.55–4.65) and 5.07 times (95% CI: 2.75–9.35) higher, respectively. Further adjustment for fertility status (model 3) reduced the risks to 3.23 times (95% CI: 2.40–4.33) and 4.70 times (95% CI: 2.52–8.77) higher, respectively. Finally, after adjusting for baseline demographic characteristics, fertility status, nutritional factors, and environmental factors (model 4), the risks of birth defects and cardiovascular system defects were 2.39 times (95% CI: 1.84–3.10) and 3.14 times (95% CI: 1.73–5.71) higher, respectively, compared to the control group.

TABLE 2 The prevalence of birth defects (1/10,000).

| Types of birth defects | Total | Pesticide exposure group | Non-pesticide exposure group | $\chi^2$/Z | *p* value |
|---|---|---|---|---|---|
| Nervous system defect | 20 (6.85) | 1 (29.50) | 19 (6.58) | | 0.208[a] |
| Eyes, ears, face and neck defect | 70 (23.97) | 2 (59.00) | 68 (23.56) | | 0.195[a] |
| Circulatory system defect | 185 (63.35) | 11 (324.48) | 174 (60.28) | | <0.001[b] |
| Respiratory system defect | 11 (3.77) | 1 (29.50) | 10 (3.46) | | 0.121[a] |
| Cleft lip and palate | 34 (11.64) | 2 (59.00) | 32 (11.09) | | 0.059[a] |
| Digestive system defect | 25 (8.56) | 0 (0.00) | 25 (8.66) | | 1.000[a] |
| Reproductive organs defect | 17 (5.82) | 1 (29.50) | 16 (5.43) | | 0.180[a] |
| Urinary system defect | 6 (2.05) | 0 (0.00) | 6 (2.08) | | 1.000[a] |
| Malformations and deformations of the musculoskeletal system | 101 (34.58) | 2 (59.00) | 99 (27.38) | | 0.760[b] |
| Other congenital malformations | 91 (31.16) | 5 (147.49) | 86 (29.79) | | 0.001[b] |
| Chromosomal abnormalities | 2 (0.68) | 0 (0.00) | 2 (0.69) | | 1.000[a] |
| Birth defects (overall) | 562 (192.44) | 25 (737.46) | 537 (186.04) | 53.982 | <0.001 |

[a]Fisher exact test.
[b]Continuity corrected $\chi^2$ test.

TABLE 3 Association between pesticide exposure and birth defects.

| | Model 1 | | Model 2[a] | | Model 3[b] | | Model 4[c] | |
|---|---|---|---|---|---|---|---|---|
| | Crude OR (95% CI) | *p* value | Adjusted OR (95% CI) | *p* value | Adjusted OR (95% CI) | *p* value | Adjusted OR (95% CI) | *p* value |
| Birth defects | 4.20 (3.16–5.59) | <0.001 | 3.45 (2.55–4.65) | <0.001 | 3.23 (2.40–4.33) | <0.001 | 2.39 (1.84–3.10) | <0.001 |
| Cardiovascular system defect | 5.53 (3.14–9.73) | <0.001 | 5.07 (2.75–9.35) | <0.001 | 4.70 (2.52–8.77) | <0.001 | 3.14 (1.73–5.71) | <0.001 |

[a]Model 2 adjusted baseline demographic characteristics (maternal age, education level, ethnic, residence and household wealth index).
[b]Model 3 adjusted baseline demographic characteristics and fertility status (first pregnancy, history of abortions, type of pregnancy, and family history of birth defects).
[c]Model 4 adjusted baseline demographic characteristics, fertility status, nutritional factor (folic acid supplement), and environmental factors (infection, smoke or passive smoke, occupational exposure, and industrial exposure).

## 3.4 Sensitivity analyses

The relationship between pesticide exposure and birth defects was further examined across various demographic subgroups. Pesticide exposure was consistently associated with an increased risk of birth defects in all subgroups. This association was statistically significant for women under 30 years of age, women 30 years or older, those with a high school education or above, those with a middle school education or below, those living in rural areas, and those experiencing a non-first pregnancy. No significant interaction was observed between pesticide exposure and any covariates for birth defects (Supplementary Table S2).

# 4 Discussion

Drawing on a comprehensive population-based survey conducted in Shaanxi Province, Northwest China, this study investigated the relationship between pesticide exposure during the periconceptional period and the risk of birth defects. Using a GEE model, we adjusted for baseline demographic characteristics, fertility status, nutritional factors, and environmental factors. Our findings indicated that maternal pesticide exposure during the periconceptional period was significantly associated with an increased risk of birth defects, particularly congenital circulatory malformations, in offspring.

This study identified a correlation between maternal pesticide exposure during the periconceptional period and birth defects based on data from a cross-sectional survey conducted across 20 counties and 10 districts in Shaanxi Province, China. Our results were aligned with international findings in previous reports. For instance, a systematic review found that maternal exposure to pesticides during pregnancy is associated with a significantly increased risk of birth defects, with an odds ratio of 4.44 (95% CI: 2.61–7.57) compared to unexposed pregnant women (29). Evidence from a nested case–control study also suggested that pregnant women in southwest China were ubiquitously exposed to low-level pyrethroid pesticide (PYRs), and maternal household pesticides use was related to congenital anomalies (30). Similarly, a previous case control study conducted in a rural area of Northern China suggested that prenatal exposure to Organochlorine pesticides (OCPs) was associated with increased risk for neural tube defects (NTDs), the OCPs also had a joint effect on NTD as its risk increased almost linearly with concentrations of the 16 OCPs as a mixture (31). Another birth cohort study also revealed that exposure to pesticides in maternal residences increased the likelihood of congenital heart disease, reproductive system malformations, and skeletal muscle malformations in offspring (32).

Unlike previous studies, this research specifically focused on pesticide exposure during the 3 months preceding pregnancy and the first trimester—a critical period for fetal development. The odds ratios of similar international studies were basically like those of this study, with case—control studies as the main research methods and maternal self-reporting often used for exposure assessment. Therefore, it is of great necessity to conduct large sample prospective studies and quantitatively measure the level of maternal pesticide exposure in the future to reveal the causal relationship between pesticides and birth defects.

In addition to the acute toxic effects of cholinergic toxicity, organophosphorus pesticides have been shown to cause genetic toxicity, including DNA damage, gene mutations, chromosome aberrations, and the induction of cell carcinogenesis. Moreover, evidence suggests that these pesticides disrupt embryogenesis and development, have long-term impacts on the structural and functional integrity of the nervous system, and may increase the risk of neurodegenerative diseases (33, 34). Organochlorine pesticides, which are commonly used in agriculture, can accumulate in the maternal body and be transmitted to the fetus through the placental barrier, potentially leading to birth defects (35).

This study identified a correlation between maternal pesticide exposure during the periconceptional period and cardiovascular system defects, consistent with prior research. A birth cohort study conducted in North Carolina found a significant association between maternal exposure to pendimethalin and an increased risk of atrial septal defect in offspring (OR = 2.09, 95% CI = 1.63–2.68), as well as an elevated risk of patent ductus arteriosus (OR = 1.64, 95% CI = 1.29–2.08) (36). Similarly, results from a nested case–control study suggested a trend toward a higher risk of septal defects (ORs ranged from 1.80 to 2.36) with greater serum neonicotinoid pesticides (NEOs), especially nitro-containing NEOs represented by imidacloprid (IMI) (37). Most animal studies have used nitrofen to induce congenital heart malformations in mouse embryos (38, 39), with the primary mechanism involving the impact of toxic substances on the proliferation or apoptosis of cells involved in heart development. Due to poor perceptions of biological control and lack of capacity, many low- and middle-income countries are reluctant to pursue biological control, increasing a dependency on pesticides, and increases the risk of pesticide exposure for mothers during pregnancy (40). In addition, since biopesticides can promote the increase of crop production without compromising human health, they have already received widespread attention (41). However, there are very few studies on the relationship between exposure to new pesticides during pregnancy and cardiovascular system defects, further research is needed to confirm its safety to mothers and children.

This research represented a large-scale, population-based cross-sectional survey conducted in Northwest China, focusing on the correlation between maternal pesticide exposure during the periconceptional period and the incidence of birth defects. However, several limitations should be acknowledged. Firstly, pesticide exposure was assessed retrospectively through self-reporting, which might introduce recall bias due to the time elapsed between pregnancy and the survey. To mitigate this bias, we established a rigorous investigative protocol and selected clear indices pertinent to pesticide exposure, thereby enhancing participants' ability to accurately recall long-term exposure histories. For example, the indices encompassed commonly used local pesticides and their corresponding application seasons. Secondly, the low incidence of birth defects and the limited number of cases of malformations outside the cardiovascular system prevented an analysis of other types of birth defects. Thirdly, the study did not differentiate between specific types of pesticide exposures, limiting our ability to perform sensitivity analyses. Finally, despite adjusting for potential confounders using multivariable regression, unmeasured or latent confounders, such as pregestational diabetes mellitus, genetics, environmental toxins and other environmental risk factors, might still influence the results.

## 5 Conclusion

Our findings demonstrated that maternal exposure to pesticides during the periconceptional period was associated with an increased risk of birth defects, particularly those affecting the cardiovascular system in offspring. This association is particularly pronounced among individuals residing in rural areas and those undergoing subsequent pregnancies. Therefore, in order to prevent birth defects in infants, it may be beneficial to pesticide exposure from 3 months before conception through the first trimester of pregnancy. These findings carry significant policy implications for maternal health interventions in China.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

## Ethics statement

The studies involving humans were approved by the Human Research Ethics Committee of Xi'an Jiaotong University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

## Author contributions

FL: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. XL: Data curation, Formal analysis, Writing – original draft. JC: Data curation, Formal analysis, Writing – original draft. YH: Data curation, Formal analysis, Writing – original draft. SD: Conceptualization, Funding acquisition, Investigation, Project administration, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2024.1489365/full#supplementary-material

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# Endocrine disruptor chemicals exposure and female fertility declining: from pathophysiology to epigenetic risks

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Over the last decades, human infertility has become a major concern in public health, with severe societal and health consequences. Growing evidence shows that endocrine disruptors chemicals (EDCs) have been considered as risk factors of infertility. Their presence in our everyday life has become ubiquitous because of their universal use in food and beverage containers, personal care products, cosmetics, phytosanitary products. Exposure to these products has an impact on human reproductive health. Recent studies suggest that women are more exposed to EDCs than men due to higher chemical products use. The aim of this review is to understand the possible link between reproductive disorders and EDCs such as phthalates, bisphenol, dioxins, and pesticides. In women, the loss of endocrine balance leads to altered oocyte maturation, competency, anovulation and uterine disorders, endometriosis, premature ovarian insufficiency (POI) or embryonic defect and decreases the *in vitro* fertilization outcomes. In this review, we consider EDCs effects on the women's reproductive system, embryogenesis, with a focus on associated reproductive pathologies.

KEYWORDS

**endocrine disrupting chemicals, female fertility, epigenetic, endometriosis, pesticides, female reproductive disorder, infertility**

## 1 Introduction

Founding a family, deciding the number, timing and spacing of their children are essential and inalienable human rights (1). However, the realization of these rights can be severely negated by infertility. Even though infertility is an important component of sexual and reproductive health and rights (SRHR), it remains largely neglected and underfunded worldwide (2). Infertility has devastating societal and health consequences, including severe social stigma, financial hardships, and gender-based violence, with as well as adverse physical and mental health outcomes (2, 3). Thus, addressing infertility is critical to improve the lives of individuals and couples afflicted by infertility and reach the Sustainable Development Goal 3 set by the United Nations (2, 4).

Infertility, unlike most other types of conditions, is defined by the absence of an event (i.e., not getting pregnant). The World Health Organization (WHO) defines it as "a disease of the male or female reproductive system defined by the failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse" (5). It affects a substantial proportion of the

world's population with approximately one in six people affected. Infertility can be primary or secondary. Primary infertility refers to the inability to achieve a first pregnancy, while secondary infertility occurs after at least one prior pregnancy has been achieved. Infertility rates are comparable for high and low-income countries (17.8 and 16.5%, respectively) and affects both men and women equally (6, 7).

Over the last 70 years, global fertility has been constantly in decline due to behavioral and societal changes (8). In fact, the number of women pursuing education and participation in the workforce has increased. Besides, the cost of raising children has become more expensive, impacting family planning decisions and leading to a late age of mothering and fathering. This delay in childbearing, which became more possible by better access to contraception, has made infertility-related disorders more difficult to treat. Literature has described the association of multiple factors with infertility such as lifestyle changing, food habits, body mass index (BMI), hormonal dysfunction, stress and late age of mothering and fathering (9, 10). Moreover, emerging evidence has shown that infertility incidence is linked to exposure to environmental factors such as tobacco, alcohol, and a wide range of endocrine disrupting chemicals (EDCs) including pesticides (Chlorpyrifos, glyphosate, dichlorodiphenyltrichloroethane and methoxychlor), phthalates, polychlorinated biphenyls (PCB), dioxins, and bisphenols (11). The association between environmental and professional EDCs exposure and male infertility has been well documented in literature and our previous published work (12). In this review, we hypothesized the link between environmental and professional EDCs exposure and women fertility declining as well as other related diseases such as endometriosis, premature ovarian insufficiency (POI) and endocrine axis dysregulation.

EDCs exposure has been reported to impact sperm and induce DNA damage and hormonal disorders in males (12). Similarly, EDCs exposure may affect female fertility at multiple levels through various mechanisms. One of the most described mechanisms is when EDCs mimic hormones such as estrogen and bind to their receptors leading to hormonal disruption (13). By interfering with the release and timing of Luteinizing Hormone (LH), EDCs can alter the ovulation process (13). Additionally, oxidative stress in ovarian tissues, that damages cells and impairs their function, is induced by several EDCs. Thus, EDCs disrupt the development of ovarian follicles and can be directly toxic to gametes, decreasing their numbers and quality (14). These molecules can also affect the epigenome causing modifications in gene expression of ovulation regulators without altering their DNA sequence (15). These mechanisms all together lead to abnormalities such as blocked Fallopian tubes, ovarian disorder, uterine disorders, failure to produce an oocyte, abnormal oocyte quality, local inflammation, and endocrine disorders in women may be linked to EDCs exposure. Failure of any or more of these conception factors can result in infertility (16). The association between exposure to EDCs and the development of potential and chronic diseases is thus a significant concern for public health, especially when fertility, a sign of health, is affected. When an individual is in subhealth, the reproductive system is more likely the first affected. This prompts us to delve in this major health problem and find a better understanding of the link between exposure to EDCs and women infertility.

The primary aim of the present review is to comprehensively summarize the current knowledge on the impact of EDCs on female fertility. This includes understanding the mechanisms by which EDCs cause ovarian aging, folliculogenesis, decrease of oocyte quality, ovulation disorders, development and receptivity of endometrium, endometriosis, fetal development abnormalities, and epigenetics modulation. Highlighting these targeted disorders by EDCs helps us shed light on taking into consideration the levels of EDCS exposure in the treatment of women's infertility. To enhance the focus of this review, we addressed research questions about how primary mechanisms of EDCs disrupt female reproductive health in particular the impact on ovarian and follicle health, endometrium development, placenta, fetus and epigenetics. By addressing these questions, this review aims to provide a comprehensive understanding of the link between EDCs exposure and female infertility, thereby informing future research and public health strategies.

## 2 Endocrine disruptor chemicals exposure: who is concerned?

EDCs are defined by the European Chemical Agency as "chemicals that may interfere with the hormonal system and thereby produce harmful effects in both humans and wildlife" (17). EDCs are either natural or human-made chemicals that may disrupt the endocrine system, by mimicking, blocking, or interfering with the body's hormones and may cause adverse health effects the organism or its progeny (17, 18). In fact, they alter their biosynthesis, secretion, transport, or by reducing their effects. However, the most common mechanism of action of EDCs is to imitate endogenous hormones and compete with their nuclear receptors as agonists or antagonists. EDCs have been released into the environment through human activity without prior knowledge of their impact on ecosystems or human health. Consequently, different national or supranational agencies established lists such as REACH of known or suspected EDCs, chemicals exhibiting effects on the endocrine system (19). Chemicals considered as potential EDCs will be subject to further evaluation and regulation to reduce their use and ultimately replace them with safer alternatives. Over the years, public concerns grew toward EDCs and recent studies have suggested that EDCs are associated with cancer

Abbreviations: AhR, Aryl hydrocarbon receptor; ART, Assisted Reproductive Technology; ATR, Atrazine; BBP, Butylbenzyl phthalate; BMI, Body Mass Index; BPA, Bisphenol A; cAMP, Cyclic Adenosine MonoPhosphate; DBP, Dibutyl phthalates; DDE, Dichlorodiphenyldichloroethylene; DDT, Dichlorodiphenyltrichloroethane; DEHP, Di-(2-ethylhexyl) phthalate; DES, diethylstilbesterol; DNA, Deoxyribonucleic acid; DNMTs, Deoxyribonucleic acid methyltransferase; DOHaD, Developmental Origins of Health and Disease; $E_2$, Estradiol; EDCs, Endocrine disrupting chemicals; EGs, Estrogens; ESR1, Estrogen receptor 1; FSH, Follicle-stimulating hormone; GLUT1, Glucose type 1 transporter; GnRH, Gonadotropin-releasing hormone; GPX, Glutathione peroxidase; HOXA10, Homeobox A10; HPG, Hypothalamic–pituitary-gonadal; KISS, Kisspeptin; LH, Luteinizing hormone; MEHP, Mono-(2-ethylhexyl) phthalate; mRNA, Messenger ribonucleic acid; MUC1, Mucin 1; MXC, Methoxychlor; $P_4$, Progesterone; PFASs, Per- and polyfluoroalkyl substances; POI, Premature ovarian insufficiency; ROS, Reactive oxygen species; SGK1, Serine/threonine-protein kinase 1; SOD1, Superoxide dismutase 1; SRHR, Sexual and reproductive health and rights; StAR, Steroidogenic acute regulatory protein; TCDD, 2,3,7,8-Tetrachlorodibenzo-p-dioxin; WHO, World Health Organization; VEGF, Vascular endothelial growth factor; hCG, Human chorionic gonadotropin; DEPH, Diethylhexyl phthalate; PPAR$\gamma$, Peroxisome proliferator-activated receptor $\gamma$; miRNA, Micro ribonucleic acid; Tet1, Ten-eleven translocation methylcytosine dioxygenase 1.

(20), cardiovascular risk, behavioral disorders, autoimmune abnormalities (21), and reproductive disorders (18, 22).

Humans are chronically exposed to a wide range of EDCs. Their exposure is related to their lifestyle and profession. In high producers and pesticide consumer countries, mostly in United States and Europe, chemical industries workers and especially farmers are highly exposed to pesticides that have been identified as EDCs. These cases are mostly detected in regions with high agriculture activities. As an example, in the Haut De France region in northern France, known for its extensive use of pesticides to maintain high agricultural output, studies of the impact of EDCs showed that altered sperm parameters (23) and oocyte dysmorphism (24) are associated with exposure to pesticides in risk zones. EDCs exposure does not only concern professionals. By contaminating food, soil, and the air, these substances reach the general population (by ingestion, inhalation, and skin contact) and expand to an environmental exposure. As food itself contains ECDs residues, many other everyday items, such as plastic bottles, food containers, the liners of metal food cans, detergents, flame retardants, gadgets, cosmetics, contains chemicals such as PCB, bisphenol A (BPA), phthalates and their metabolites (dibutyl phthalates (DBP) and di-(2-ethylhexyl) phthalate (DEHP)), and alkyl phenols, that are also classified as EDCs (25) (Figure 1).

However, exposure degree differs between individuals due to different lifestyle, food habits, and habitat location. In addition, the dose, the route of exposure, and the individual's susceptibility are also key factors to determine the impact of EDCs on reproductive health. Moreover, the timing of exposure in an important parameter to evaluate the effect of EDCs. As the DOHaD concept explains, when the exposure occurs in the first 1,000 days of life, the risk of developing pathologies in adulthood is higher than exposure occurring during adult age (26, 27). A prime example is diethylstilbesterol (DES) a potent synthetic estrogen, prescribed to pregnant women to prevent miscarriage. *In utero* exposure to DES has been associated with reproductive tract abnormalities and the development of vaginal clear-cell adenocarcinoma in young female offspring (28) and breast cancer (29). The effects of these chemicals on the endocrine system had devastating consequences on individuals. However, almost all major classes of EDCs can target the estrogen pathways as many EDCs display estrogenic activity (30, 31), and can affect both genetic (32) and epigenetic levels. Thus, the hypothalamo-pituitary-gonadal (HPG) axis in women is a prime target of EDCs.

The HPG axis is a hormone-regulating structure crucial for reproduction. There are noticeable distinctions in how the HPG operates in males and females. Unlike its male counterpart, the female reproductive system shows regular cyclic changes that can be regarded as preparation for fertilization and pregnancy. Two cycles occur at the same time: the ovarian cycle and the uterine cycle. The ovarian cycle features the series of changes happening within the ovary with follicles recruitment, the oocyte maturation and release, and the *corpus luteum* development before regressing. The uterine cycle features endometrial thickening in preparation for embryo implantation, as well as the shedding of the uterine lining following unsuccessful implantation. They are both regrouped into a single cycle describing all these physiological changes that occur, the menstrual cycle (33). The menstrual cycle is tightly controlled by four structures that communicate with each other's and orchestrate this cycle: the hypothalamus, the pituitary, the ovaries, and the uterus. The key hormones of this system are the ovarian hormones estradiol (E2) and progesterone (P4), the gonadotropin-releasing hormone-I (GnRH) secreted within the hypothalamus, the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secreted in the pituitary. P4 and E2 will modulate the activity of the neuronal network in the hypothalamus controlling the release of GnRH that will be secreted into the pituitary portal circulation in an intermittent manner. This intermittent release of GnRH will generate very distinct pulses of LH and FSH, allowing the appropriate development of the recruited follicles during the follicular phase (34). The frequency and amplitude of GnRH pulses are determined by multiple neuroendocrine modulators including kisspeptin (KISS), leptin, ovarian steroids, and peptides providing a link between the environment and reproductive status. Recently, emerging studies have shed light on a key reproductive neuroendocrine modulator KISS and EDCs exposure. KISS stimulates endogenous GnRH secretions and is involved in both E2 positive and negative feedback (35). A French study using animal experimentation assessed the impact of maternal exposure to a pesticide chlorpyrifos (CPF) and a high-fat diet (HFD) on the reproductive function of rat offspring. The study outcome revealed that pesticide exposure disrupts the normal expression of KISS and GnRH, in the testis, potentially leading to reproductive health issues. Additionally, histological analysis showed a significant increase in atretic follicles and abnormal testis structures with germ cell desquamation in the CPF-exposed group. This suggests the role of kisspeptin in reproductive disorders (36). These signals provide a link between the environment and reproductive status. The HPG plays a crucial role in the regulation of the reproductive function in women. Any alteration of the functionality or imbalance on this beautifully orchestrated system will have adverse or deleterious effects on women's reproductive health and thus may lead to infertility.

# 3 Effect of EDC on ovarian aging and decrease of oocyte quality

## 3.1 Ovarian aging

It is generally accepted that women are born with a finite follicle pool, called the ovarian reserve, that will go through a constant decline as they age and will ultimately lead them to the final step of their fertile life, the menopause. Ovarian aging is a natural and physiological process, which encompasses a progressive and gradual decline in both quantity and quality of oocytes within primordial follicles of the ovarian cortex until exhaustion of ovarian function (37, 38). This gradual decline will impair the reproductive ability of women as they age by demonstrating diminished fertility early in their life (about 35 years old) due to decay in oocyte quality and will cause endocrine dysfunction and raises their risks of cardiovascular diseases once they reach the final stage of ovarian aging (39, 40).

POI is a serious medical condition characterized by very low blood levels of anti-Müllerian hormone, where the ovarian reserve is depleted before the age of 40, leading to a premature loss of ovarian function and early menopause. This acceleration of follicular atresia often results in subfertility or infertility associated with menstrual irregularities and high pregnancy failure (41, 42). This condition affects 1% of women worldwide and alters severely their quality of life. Even though genetic abnormalities have been associated with POI, its etiology remains undetermined in more than 75% of cases. However,

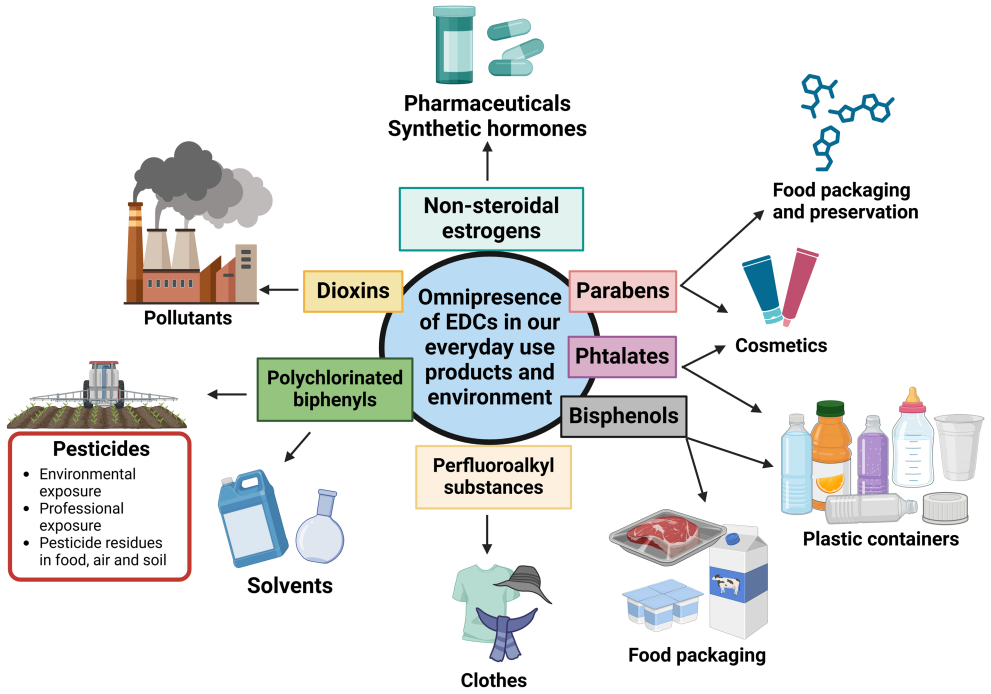


FIGURE 1
Various groups of endocrine-disrupting chemicals present in daily life. Adapted from Yan et al. (25).

the environmental factor seems to be the main cause beyond the genetic reason (43).

Over the last few decades, evidence has confirmed that exposure to EDCs was a major environmental risk factor for ovarian function, ovarian aging, and POI. Indeed, several studies have linked exposure to persistent EDCs, such as dioxins, organochlorine pesticides (e.g., DDT) (44), PCBs (45), perfluoroalkyl and polyfluoroalkyl substances (PFASs) (46), and non-persistent EDCs, phthalates, phytoestrogens, BPA, and parabens to ovarian aging. A French study associated pesticides exposure with an endocrine-disrupting action in agricultural region with syndromes linked with fertility decline, poor IVF outcomes, such as POI, polycystic ovarian syndrome, and endometriosis (47).

The case of phthalates is remarkable concerning ovarian aging. Phthalates are groups of synthetic chemicals widespread in everyday products such as plastic containers, drug coating, building, and personal care products that lead to ubiquitous human exposure via oral ingestion, inhalation, and direct contact. Furthermore, women are often exposed to higher levels of phthalates than men through their extensive use of personal care and cosmetic products (48). Phthalates are described in many studies as being endocrine disruptors altering ovarian function. One way that phthalates can exert ovarian toxicity rests on one of the most commonly found active metabolites of phthalates, the mono-(2-ethylhexyl) phthalate (MEHP). MEHP induces an oxidative stress [imbalance of pro- and antioxidants in a system mostly induced by raises the physiological level of reactive oxygen species (ROS)] within antral follicles, increasing the expression of proapoptotic factors such as *Bax*, which inhibit the follicle growth. It also reduces the expression of cell-cycle regulators (*Ccnd2*, *Ccne1* and *Cdk4*), the antiapoptotic factor (*Bcl-2*) and thus, reducing estradiol ($E_2$) production by inducing atresia (14).

BPA, another domestic EDC mostly find in food and beverage packaging, is a known estrogen mimicker binding on the estrogen receptor $\alpha$ (49). Studies in mouse models pointed out that exposure to BPA is associated with a decrease in ovarian reserve regardless of the exposure window whether it is prenatal, neonatal or adult age (50). Indeed, by inhibiting steroidogenesis, BPA decreases crucial hormone production in antral follicles, leading to an inhibition of follicle growth and induces atresia. In the granulosa and theca cells, BPA down-regulates the steroidogenic acute regulatory protein (*StAR*) and the *cP450* sidechain cleavage mRNA expression preventing cholesterol transport into the mitochondria and impairs the $E_2$ biosynthetic pathway altering the HPG normal function (Figure 2) (51). Normally EGs, such as $E_2$, stimulate follicle growth and protect the follicle from atresia but it is no longer the case in presence of BPA (52).

## 3.2 Impact on folliculogenesis and oocyte quality

In addition to affecting ovarian aging mechanisms, EDCs can exert ovarian toxicity through targeting granulosa and theca cells, which are crucial for oocyte development. Some broad-spectrum pesticides are also known as EDCs, such as the renowned DDT, it's subsequent dehydrochlorination the DDE, and methoxychlor (MXC) will impact the folliculogenesis. DTT and DDE, have been well studied from the 90s since Guillette et al. report about altered sex steroid hormone concentrations in alligators (45). DDT and DDE both shown alter progesterone biosynthesis in a dose-dependent manner and impact $E_2$ production by decreasing cAMP synthesis, thus interfering with gonadotropin receptor signaling pathways in granulosa and theca cells. Many studies have

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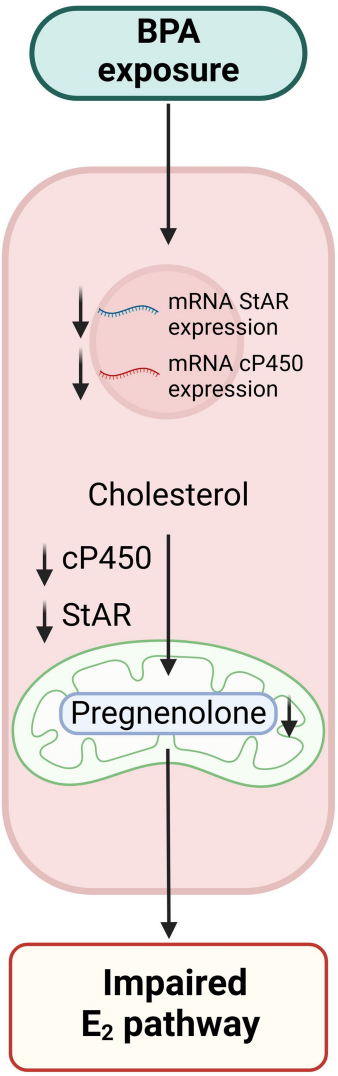


FIGURE 2
Suggested mechanisms of Bisphenol A impairing decidualization. Exposure to BPA leads to downregulation of steroidogenic acute regulatory protein (*StAR*) and cP450 expression in theca and granulosa cells. This downregulation hinders the transport of cholesterol into the mitochondria for conversion into pregnenolone, thereby disrupting the E2 biosynthetic pathway and impairing the normal function of the HPG axis.

demonstrated that their replacement, MXC and its metabolite, have also adverse effects on progesterone synthesis, $E_2$'s FSH-stimulated biosynthesis and increase $E_2$ catabolism in granulosa and theca cells leading the either follicular atresia or to meiotic aberrations (aneuploidy) in oocytes impairing its quality (47).

Another way for EDC to impact oocyte quality and development is via DNA damage through oxidative stress. Oocyte quality is defined as the competence of an oocyte to develop a genetically undamaged embryo. Liu et al. (53) suggested that MXC exposure induces oxidative stress and affects mouse oocyte meiotic maturation via the accumulation of superoxide radicals and other ROS by decreasing the enzymatic activity and expression of antioxidants SOD1, GPX and catalase.

The mitochondria, the main source of ROS, is also a target for EDCs. Growing evidence shows that mitochondrial imbalances are associated with impaired oogenesis, poor oocyte quality and ovarian aging. The loss of energy balance and damage to the mitochondria induce mitochondrial stress increasing ROS level that leads to apoptosis and thus, depleting the ovarian reserve (54, 55).

As a result, this evidence emphasizes that the dysfunction of the HPG axis by altering the ovarian function is a contributing factor to ovarian aging and oocyte quality loss in humans.

## 4 Effect of EDC on ovulation

EDC can influence the fertilization process in women by different means. If the oocyte cannot mature, due to an early ovarian aging, impaired folliculogenesis or cannot be expelled due to anovulation, the fertilization cannot occur. Moreover, if the released oocyte quality is impaired due to altered maturation, the fertilization can occur, but oocytes with cytoplasmic anomalies will lead to significantly lower pregnancy rates (56).

The WHO has developed a classification standard in 1973 for anovulation diseases with 3 categories.

Group I (Hypothalamus–Pituitary Failure). This situation may be caused by low LH and FSH concentration and with reduced $E_2$ levels. In this case the development of follicles in the ovary is blocked, which in turn leads to anovulation and amenorrhea.

Group II (Hypothalamic–Pituitary Dysfunction). This is the most common type of anovulation, accounting for about 90% of all anovulatory patients. In this type of anovulation, women's serum gonadotropin and $E_2$ levels are normal, but LH/FSH are often abnormally elevated. The occurrence and growth of follicles can be observed, but the follicles cannot mature and cannot spontaneously ovulate.

Group III (Ovarian Failure). This condition is mainly due to severe lack of primordial follicles with increased serum FSH and LH, and decreased $E_2$. The clinical manifestations are characterized by a poor response to induced ovulation, and the ovarian function has deteriorated. The patients suffer from amenorrhea, infertility, and low estrogen-induced perimenopausal symptoms (57, 58).

While anovulatory disorders may be caused by drug uses, genetic factors, or chronic diseases, they are one of the main manifestations of EDC on the women's reproductive health. Ovulation is a biological process that is so finely tuned, any imbalance in the HPG axis and the endocrine system surrounding it may lead to ovulatory blockade. Thus, impaired folliculogenesis induced by BPA, a high aneuploidy rate induced by MXC, or the acceleration of follicular atresia as described above, will lead to anovulation due to the failure of the oocyte to reach maturity.

Atrazine (ATR) is a widely used herbicide used for agricultural purposes in the United States, China, and Africa and a common environmental contaminant known with EDC effect and reproductive toxicity (59). Its anovulatory effects have been demonstrated throughout different animal studies. In female rats, ATR reduced the number of oocytes released, the effect of LH surge by suppressing FSH-induced LH receptor expression and as well as the length of estrus cycle. In FSH-stimulated rat granulosa cells, ATR reduced serum $E_2$ serum levels and reduce LH receptor mRNA levels, resulting in ovulation blockade (60). Studies in human granulosa cells have also

demonstrated that ATR reduces the levels of $E_2$ and (progesterone) $P_4$ and prevent LH-dependent expression of ovulation genes (61).

Hypothalamic hormones are also important factors responsible for a normal ovulation. Thus, any disturbance on the GnRH production or pulse frequency caused by EDC that incurs a slower or faster frequency than the physiological cycle of 60–90 min, altering follicles development in the ovary which in turn leads to anovulation (62).

## 5 Effect of EDC on uterine development, endometrial receptivity, and implantation

The endometrium is a dynamic layer that undergoes proliferation, secretion and shedding phases during the menstrual cycle and involves a balanced yet complex interaction between $E_2$ and $P_4$. EDCs by altering the endocrine equilibrium within the HPG axis can affect this crucial uterine layer and evidence has confirmed that exposure to EDCs was a major environmental risk factor for uterine disorders. Studies have shown that, the process of decidualization is sensitive to EDCs and may pose a hazard to embryo attachment and implantation (63). BPA induces impairment of decidualization through the dysregulation of EG and $P_4$ receptors in endometrial cells. At low BPA exposure, decidualization is impaired by oxidative damages due to an increased level of ROS. By upregulating EG receptors and $P_4$ receptors, antioxidant enzymes such as SOD, GPx, HO, and catalase have reduced activities, and nitric oxides see their production increased. At high levels of BPA exposure, EG and $P_4$ receptors are down-regulated and impairs decidualization by increasing ERG1 expression and decreasing SGK1 serum level (64) (Figure 3). The disruption of their function could lead to implantation and/or placentation failure leading to pregnancy complications, loss of pregnancy, or infertility (65, 66).

Other EDCs such as endosulfan reduces the expression of the primary endometrial markers of receptivity (such as MUC1, HOXA10, Inn and E-cadherin) and affect the normal endometrial receptivity impairing the adhesion and the implantation process of the blastocyst (67). Emerging evidence shows that EDCs can also affect the receptivity of the endometrium by disturbing the immune system that plays a critical role in the events of implantation, early placentation and alter the endocrine signaling required for maternal immune tolerance toward implantation and thus altering the timing of the implantation window (68). A successful implantation requires synchronized actions of both the blastocyst and the endometrium to attach and initiate the implantation.

## 6 EDC and endometriosis risks

Endometriosis is a chronic estrogen-dependent inflammatory systemic gynecological disease, where alterations involving both the endocrine and immune systems have been observed. Clinical symptoms are often life-impairing pain and infertility and affects approximately 10% of women in reproductive age worldwide. This common yet complex disease is difficult to diagnose as most of the time the disease is found during a diagnostic work-up for infertility. Furthermore, laparoscopy or surgery is required in order to make the definitive diagnosis. Even though endometriosis is currently actively researched, its etiology is still today poorly understood. A combination of hormonal, metabolic, genetic, epigenetic and environmental factors are currently being investigated. However, growing evidence suggests that EDCs exposure may be a co-causal factor that could play a role in disease etiology. By their ability to alter steroidogenesis, EDCs could affect endometriosis. In fact, suspicions about ECDs involvement first arose from a study conducted by Rier and his coworkers in 1993 (69), where they observed that the severity of endometriosis in Rhesus monkey was directly correlated with TCDD exposure. TCDD is known to disturb EG and $P_4$ pathways, and as an estrogen-dependent disease, $E_2$ production and $P_4$ resistance play a crucial role in the disease progression and/or maintenance. TCDD also unbalance the pro/anti-inflammatory balance and activates an inflammation-like pattern using the AhR pathways that could be involved in the development and progression of endometriosis by increasing vascularization, cell proliferation, and VEGF levels (47, 70). Increased local production of $E_2$ can also promote endometriosis in combination with TCDD inhibition on $P_4$. Furthermore, women afflicted by endometriosis demonstrate decreased responses to $P_4$. Short term exposure to TCDD reduced endometriotic lesions due to its estrogen antagonist proprieties (71), highlighting the effect of long chronical exposure to develop the disease.

## 7 Effect of EDC in fetal development

### 7.1 Impact on the placenta

The placenta is an ephemeral yet vital organ that performs various endocrine, immunological, and physiological processes throughout pregnancy. It develops from the trophectoderm layer of a blastocyst through placentation (72). Its role is essential for the normal development and growth of the fetus as it produces hCG to maintain pregnancy, is the primary site of the transport of nutrients, gases, and waste materials between the fetus and the mother, and acts as a barrier protecting the fetus during pregnancy. Its failure has devastating consequences. Placenta anomalies lead to pre-eclampsia, abnormal fetal development, miscarriage, and placental disruption. The placenta is vulnerable to endocrine disruption due to a large presence of hormone receptors and a lack of enzymatic machinery to guard against EDCs (25). Evidence suggests that phthalates such as DEPH, alter placental endocrine function, and inhibits placental cell proliferation by decreasing the expression of trophoblast differentiation markers (PPARγ, AhR, hCG) (73, 74). Furthermore, phthalate exposure disrupts the vascularization of the placenta, induces apoptosis of placenta cells reducing placental size and shape (75). BPA exposure alters nutrient transport, particularly glucose transporter by affecting GLUT1 expression level (76–78) and exhibit trophoblast cell invasion, and abnormal placental vessel remodeling in mice (79). Other EDCs such as organochlorine pesticides can impair placenta ability to produce and release hormones and enzymes, transport nutrients, or produce waste. They contribute to preterm birth by disrupting the balance between $P_4$ and $E_2$ during pregnancy (25).

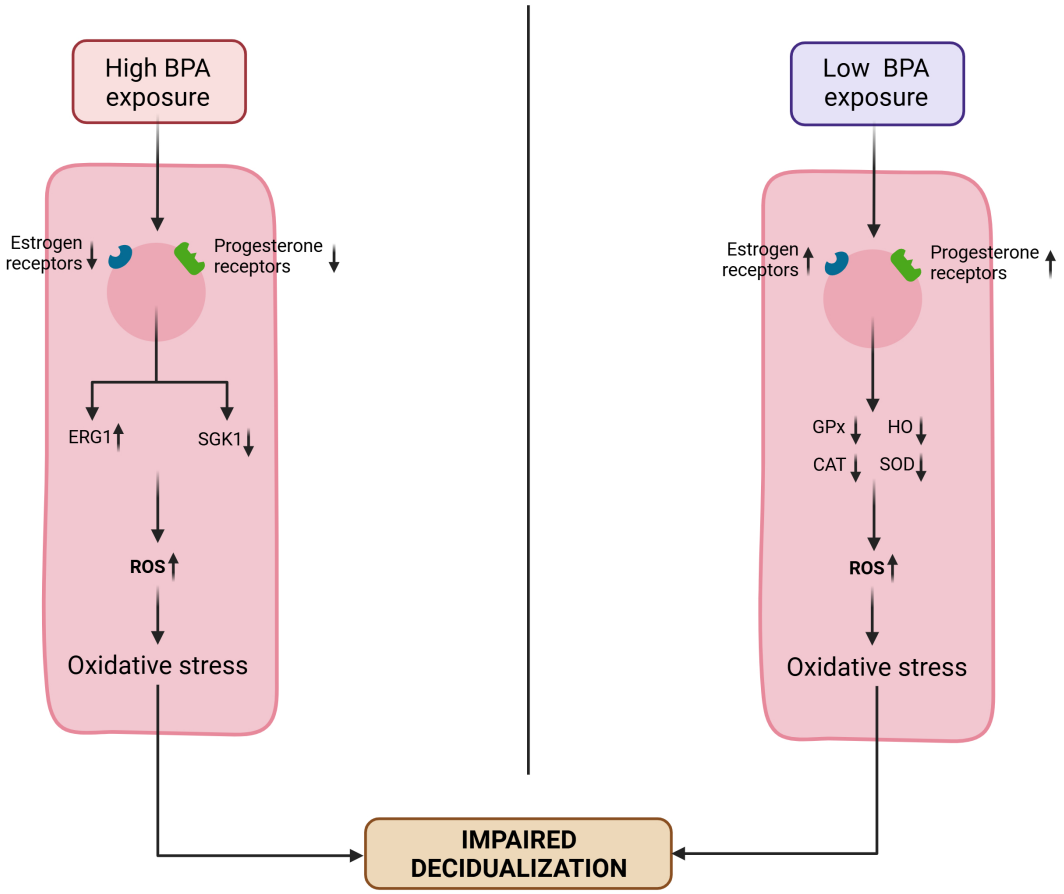


FIGURE 3
Suggested mechanisms of Bisphenol A impairing decidualization. Exposure to low doses of BPA leads to an upregulation of estrogen (ER) and progesterone (PR) receptors. This reduces the levels and activities of antioxidant enzymes (superoxide dismutase [SOD], glutathione peroxidase [GPx], heme oxygenase [HO], and catalase [CAT]), and thus increasing ROS level leading to an impaired decidualization. Exposure to high level of BPA downregulates the ER and PR, increase the expression of early growth response-1 (EGR1) and reduce the expression of glucocorticoid-induced kinase-1 (SGK1), resulting in elevated ROS levels, which in turn impair decidualization. Adapted from Nelson et al. (64).

## 7.2 Impact on fetuses/embryo

Impact of EDCs can go beyond the placenta and reach the fetus. Immature fetuses are vulnerable to EDCs exposure because different EDCs such as BPA, dioxins, and organochlorine pesticides can breach the placental barrier using passive diffusion and can be transferred from the mother to the fetus. As EDCs perturb the endocrine system, EDCs exposure in humans is highly harmful during these critical life stages as early development requires dose and accurate timing of hormone action to endorse proper tissues and organs growth and development. However, just like the placenta, fetuses typically lack the enzymatic machinery to guard against EDCs effects as elimination processes are not fully developed (80, 81). During early embryonic development, EDCs such as dioxins (TCDD) can target cell cleavage, differentiation, or organogenesis (82). Other EDCs, such as DDT and BPA were linked to impaired fetal growth, and metabolic dysfunction which can possibly lead to miscarriage (25, 83).

The impact of EDCs extends beyond lowering the rate of a successful pregnancy and increasing the risk of miscarriage in women; they also impair the future reproductive health of the fetus. EDCs exposure during this critical developmental window can have lasting health effects on the fetus, extending into adulthood. Thus, EDCs such as BPA exhibits pseudo-estrogenic and anti-androgenic effects, which may disrupt the developmental program of the male reproductive system. In mice, BPA exposure during pregnancy, resulted in decreased testosterone secretion, sperm motility and sperm count in male offspring (12, 84, 85). Studies on female offspring have shown that *in-utero* exposure to BPA impaired early oogenesis and follicle formation in the fetal ovary (86, 87).

# 8 Impact of EDC on epigenetic risks

Embryo development stages are part of the prenatal period that is a sensitive window for potential environmental factors negative effects. During this period, vulnerability to pollutants is higher than in adults. Exposure to toxic molecules during this sensitive window is a risk factor to chronic disease in adulthood. In fact, the embryo is deprived of any detoxification or excretion mechanism (88). As a result, the reach of EDCs to the embryo by transplacental transfer can lead to genome alteration during embryonic germ cell precursors

reprogramming. As DNA has evolved to build resistance against external threats to preserve the stability of the genome, EDCs mainly affect the epigenome (89). Thus, genetics and environmental pollutants are indissociable risk factors of infertility which is clearly a complex multifactorial disorder (90). Epigenome alterations by EDCs include DNA methylation patterns (at the fifth carbon of the cytosine base, 5-methycytosine), histone modification and miRNA regulation, as previously detailed in our published work regarding epigenetic modifications and male infertility (12). EDCs can deregulate epigenetic patterns by altering methylation enzymes (DNMTs) and DNA demethylation enzymes (e.g., ten-eleven translocation TET proteins) (15). Resulting transcriptional changes may explain all the reproductive disorders mentioned above and the negative influence on human fertility. In fact, the establishment of the DNA methylome is crucial for the beginning of the ovarian cycle during embryo development stages since DNA methylation regulates gene expression to control cell differentiation (91). Research has shown that exposure to the synthetic estrogen DES can cause epigenetic modifications that affect not only the exposed individuals but also their offspring. Reproductive tract abnormalities in women have been linked to alterations in DNA methylation patterns that control several estrogen-responsive genes in addition to histone modifications (89), after exposure to DES. Furthermore, DES prenatal exposure induced a decrease in *Tet1* mRNA expression in mouse uterus while BPA exposure leaded to its down regulation in zebrafish gonads. Interestingly, studies suggest that BPA effect can be sex-specific: prenatal exposure led to DNA hypermethylation in male offspring but DNA hypomethylation in female (92). Altered DNA methylation patterns by BPA lead to impaired folliculogenesis and reduced oocyte quality. These changes can persist into adulthood, leading to long-term fertility issues (92). In addition to their impact on methylation and demethylation enzymes, DES and BPA can also affect cofactor (methyl donor) of these enzymes (15). In parallel, DES and BPA have been shown to change histone-modifying enzymes (93). Epigenetic changes linked to BPA exposure have been associated with early puberty in girls (94). Another consequence of DNA methylation changes is inhibition of mammalian oocytes maturation. Moreover, epigenetic modifications by dioxins and phthalates decrease antral follicle counts and lead to aberrant folliculogenesis (89). Exposure of mice to phthalates doses close to human exposure caused multigenerational effects on ovaries (94).

Many biological processes depend on differentially methylated regions known as imprinted control regions (ICRs) that are established during gametogenesis (89). Modification of methylation pattern of these regulatory sequences can prevent silencing of potentially dangerous transposons and proper chromosome segregation. Exposure to BPA has been linked to 1,251 human transposon regions that were differentially methylated (95). Further differential DNA methylation caused by EDCs (PCBs DEHP, methyl mercury and pesticides) has been detected in many locus/genes (96). Notably, phthalates exposure has been linked to differentially methylated regions of genes involved in early growth and metabolic processes. It has been demonstrated that phthalate BBP (butyl benzyl phthalate) can modulate the methylation of the estrogen receptor (ESR1) promoter site, altering the transcriptional expression (97). Moreover, they can modulate microRNAs, which means that these substances are able to interfere with more than one epigenetic process and have transgenerational effects at F1 and F3 generations (98, 99). Exposure to the phthalate DEHP during the prenatal period induced dysregulation in hormone levels and folliculogenesis and led to a multigenerational increase in ovarian cysts. Exposure to DEHP in ancestors accelerates the onset of puberty and reproductive aging in the F3 generation of female mice (100). Similarly, methoxychlor and DDT pesticide exposure during gestation was linked to incidence of ovary diseases in the F1 and F3 (101, 102). Even though these pesticides have been banned, differentially methylated regions they induced expanded their effect to the next generations: sperm epimutations in parent resulted in obesity and ovarian dysfunctions in female offspring (103). These epigenetic modulations by EDCs suggest serious long-term effects on human reproductive health and fertility decline.

The clinical and research communities have lately given great interest in the monitoring and reversing of epigenetic changes induced by EDCs. DNA methylation can be monitored using bisulfite sequencing and methylation-specific PCR while histone modifications can be detected using chromatin immunoprecipitation (ChIP) assays. In addition, RNA sequencing and microarray analysis can measure the expression of non-coding RNA affected by EDCs (104). Notably, pharmacological interventions, including the use of DNA methyltransferase inhibitors and histone deacetylase inhibitors, have demonstrated potential in reversing epigenetic modifications (105). Additionally, lifestyle and dietary changes, such as the intake of specific nutrients and antioxidants, have been suggested by some studies to help mitigate the epigenetic effects of EDCs (106, 107).

Today, EDCs are ubiquitous and exposure to these substances is now nearly impossible to avoid. Highly used and produced for the effect they were intended for, these environmental pollutants are now everywhere, and the general population is exposed through food and beverages, air, water and ground. Some populations are more exposed to these pollutants than others. Indeed, farmers, hairdressers, cashiers, to quote a few, are not only exposed in their private life but they are also exposed through their professional life. With the decline of global fertility, the impact of exposure to various EDCs on reproduction is now a public health concern. Studies showed that they are associated with male and female fertility decline, low ongoing pregnancy rates, and elevated risk of miscarriage. However, a lot of crosstalk toward EDCs still happens. It's not rare to encounter situations where known EDCs are still in use because the advantages, they offer exceed the health risks associated. Many EDCs can interact with the female reproductive system in early development, in puberty but also during their adulthood and may lead to various reproductive disorders due to endocrine disruption. EDCs effect has more deleterious effect on women than men, as severe endocrine disorders may lead to an early end of their reproductive life or can severely impair their ability to carry out a pregnancy. Their reproductive system is especially vulnerable to compounds that mimic estrogen or alter the estrogen pathway. Scientific research on the impact of EDCs on the reproductive system and fertility has yielded complex and sometimes conflicting evidence due to the very diverse mechanisms of action of EDCs and the challenges of studying real-life mixtures. Furthermore, limitations in current studies, including variability in exposure levels and the complexity of human reproductive systems, make it challenging to fully understand EDCs' impact on human fertility. However, the negative impact of EDCs on female fertility is

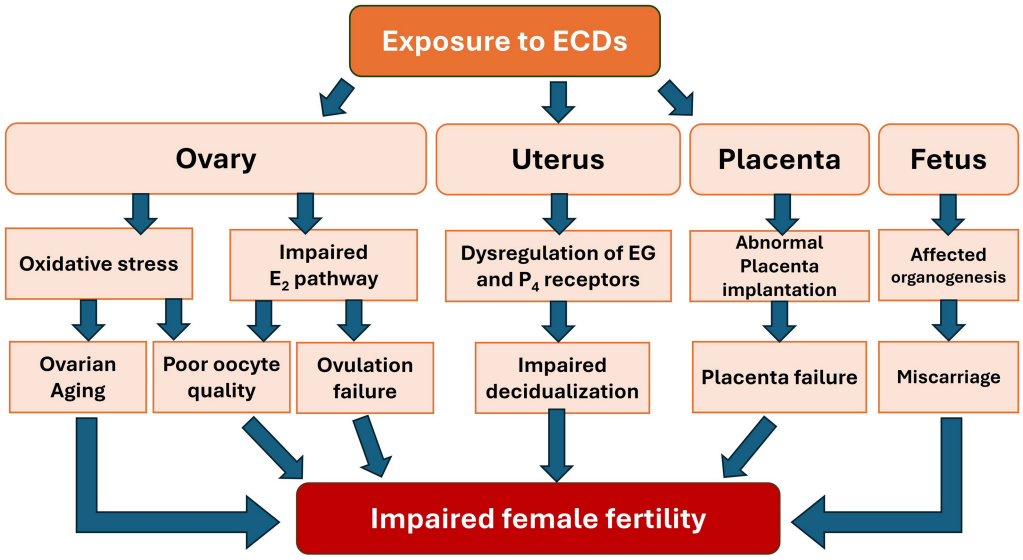


FIGURE 4
Impact of EDCs exposure on the reproductive system and fetal development associated with impaired female fertility.

now a global health concern that must be addressed to improve the fertility of affected individuals and couples. Even though today ART techniques are quite developed, they are not miracle techniques able to resolve any female infertility caused by EDCs. Thus, from a clinical perspective, evaluating EDCs exposure, particularly professional exposure, and screening for these chemicals could assist practitioners in more effectively managing couples seeking infertility treatment. On the other hand, future research on EDCs should aim to collect multiple exposure windows, consider the presence of EDC mixtures in order to fully comprehend the impact of EDCs on female fertility and take in account the professional exposure of these couple coming for infertility treatment.

## 9 Conclusion

In summary, the impact of EDCs on female reproductive health is multifaceted, affecting various processes from ovarian aging and folliculogenesis to endometrial receptivity, ovulation, and implantation. Our review highlights how EDCs disrupt these key reproductive events through mechanisms such as interference with hormonal signaling pathways, the hypothalamic–pituitary-gonadal (HPG) axis and epigenetic modifications. The detailed examination of these mechanisms underscores the complexity of EDCs' effects, linking them to clinical outcomes like reduced IVF success rates. Additionally, our discussion on fetal development and epigenetic risks underscores the critical importance of these areas in understanding the full scope of EDCs' impact. By expanding on these topics, we aimed to provide a more comprehensive view of how EDCs influence not only immediate reproductive processes but also long-term generational health. This integrated discussion underscores the importance of considering the cumulative and interconnected effects of EDCs on female fertility, offering a holistic perspective that bridges the detailed sections of our review (Figure 4).

## Author contributions

ST-Z: Writing – original draft, Writing – review & editing. ML: Writing – original draft, Writing – review & editing. MAD: Writing – original draft, Writing – review & editing. AC: Writing – review & editing. PC: Writing – review & editing. RC: Writing – review & editing. VB: Writing – review & editing. HK-C: Writing – original draft, Writing – review & editing. MB: Writing – original draft, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# The impact of environmental literacy on the health level of rural residents: evidence from the mountainous areas of Sichuan, China

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**Introduction:** Environmental pollution and health issues are hot topics of discussion in modern society. However, there is a lack of research from the perspective of subjective factors such as environmental protection to study the impact of environmental literacy on health, especially in rural areas.

**Methods:** First, through field research in the mountainous rural areas of Sichuan Province, 396 data points were collected. Based on the KAP model, we constructed an interactive impact mechanism model for the health of rural residents. We used chi-square tests and t-tests to examine the relationship between the background characteristics of rural residents and environmental protection. All environmental literacy variables were classified into strong and weak observational variables, and a multiple linear regression model was employed to explore the impact mechanism of environmental literacy (divided into environmental protection awareness, attitudes, behaviors, and perceptions of environmental pollution) on the health of rural residents.

**Results and discussion:** (1) Young village officials with higher education levels are more likely to have stronger environmental literacy. (2) The health status of rural residents is positively affected by their positive attitude towards environmental protection and negatively affected by their environmental protection behaviors. (3) Age, gender, marital status, and party membership have a significant impact on the health of rural residents. The results of this study can enhance the attention to the living environment and health in rural areas, and provide a scientific basis for improving the environmental literacy and health level of rural residents.

KEYWORDS

**environmental literacy, rural residents, health level, mountainous areas, China**

## 1 Introduction

Under the pressing concerns of global environmental degradation, governmental bodies and associated institutions are progressively intensifying environmental propaganda, thereby further advocating for the adoption of energy-saving and emission-reduction concepts by the general public. In 2021, China formally proposed the strategic goals of "carbon peak and carbon neutrality." The "dual carbon" strategy requires the participation of all people, which naturally imposes higher demands on the environmental literacy of the public (1). Therefore, against the backdrop of the "dual carbon" strategy, the status of environmental literacy among Chinese residents becomes particularly noteworthy. Because humans are inextricably linked

to the environment, social knowledge, as well as that of environmentally significant behavior (53, 54) and avenues for addressing environmental issues, is necessary for environmental programming to achieve its goals. Given this general situation, most residents have developed a considerable degree of environmental literacy. An increasing number of rural residents recognize the importance of the ecological environment and choose to recycle waste as fertilizer, adopting green and sustainable methods for agricultural planting (2, 3). Under the relatively isolated conditions of mountainous areas, the level of environmental awareness among rural residents varies, as does the amount and choice of chemical fertilizers and pesticides, resulting in different levels of pollution (4). If this continues long-term, it will further exacerbate pollution levels and produce harmful consequences. Therefore, it is of practical significance to place emphasis on the awareness of environmental protection, lifestyle, and health status of rural residents.

In 1968, the American scholar Roszak first proposed the concept of "environmental literacy (EL)." As research has deepened, the scope of the field has continuously expanded. In addition to measuring environmental literacy, the complex relationships between the components of environmental literacy have increasingly become the focus of research (55, 56). We define EL as knowledge of and dispositions (e.g., environmental identity and self-efficacy, connection to nature) towards environmental (social-ecological) systems, practices (e.g., identifying issues, creating possible solutions) one uses while engaging with those systems, and the behavior that results (5). Environmental literacy includes multiple elements such as environmental knowledge, environmental awareness, environmental attitudes, environmental behaviors, and so on (6–10). Recent research has indicated that environmental literacy has the potential to influence various aspects of rural residents' livelihoods and productivity (11). For instance, a heightened awareness of environmental protection positively impacts rural residents' responses to policies, while also influencing their environmental behaviors in a positive manner. Scholars have presented three dimensions of rural residents' environmental literacy, namely: awareness of environmental issues, attitudes towards environmental protection, and tolerance for environmental pollution. Through studying the relationship between rural residents' environmental literacy and the enhancement of their living environment, researchers have discovered a significant influence of environmental literacy on their improvement behaviors (12). Teng et al. (13) found that farmers' energy saving emotion and ecological values were the main factors affecting energy saving behavior. However, it should be noted that certain studies have identified a discrepancy between environmental literacy and actual pro-environmental behaviors among rural residents (14, 15). Therefore, further in-depth studies are required to fully comprehend the tangible impact of environmental literacy on the production-related activities of rural residents.

Currently, the literature pertaining to the health of rural residents predominantly adopts a medical standpoint, focusing primarily on the determinants influencing their health status. Within this domain, subjective psychological factors have gradually garnered attention. Broadly speaking, health concerns are increasingly situated within the purview of natural, economic, and social systems, and analytical approaches are characterized by a more holistic and systemic utilization of concepts and methodologies (16). Both domestic and international scholars dedicate their attention to the determinants of health, whereby foreign scholars generally regard that income and wealth bear no direct association with health. Instead, marital status, employment, proficiency in health-related knowledge, environmental conditions, social relations, educational quality, community standing, and self-awareness exhibit stronger correlations with health status (17, 18). The study into factors impacting the health degree of rural residents has been explored from diverse angles, chiefly encompassing regional economic and social development, the economic and cultural circumstances of rural residents, and the capacity of regional health services (19, 20). Notably, certain scholars have delved into the factors influencing self-reported ailments among residents residing in impoverished rural areas, deducing that health consciousness, economic circumstances, and psychological stress serve as predominant determinants (21, 22). Existing research on the health of rural residents primarily emanates from a medical perspective, with a conspicuous dearth of an encompassing empirical analytical framework (23).

In addition, the measurement of the health degree in the research has also changed from a single physiological or psychological indicator to comprehensive physiological, social and psychological indicators, and it will be more scientific to transform these indicators into a relatively objective health index for health research. The prevailing quantitative research on the health status of rural households primarily employs health degree as independent variables rather than dependent variables. In addition, the utilization of distinct models and indicators has led to substantial variations in the derived conclusions. Moreover, the factors under consideration have predominantly encompassed objective variables, such as age, gender, and the natural and economic circumstances of the rural community. Conversely, scant attention has been given to investigating the influence of environmental literacy factors, including ecological awareness and environmental protection, on the health of rural residents. Furthermore, a theoretical framework for such analysis is notably lacking. Consequently, this study has the potential to address gaps in the existing literature and advance the research framework pertaining to the health degree and lifestyles of rural residents.

The Knowledge-Attitude-Practice (KAP) model, which originated from learning theory and diffusion of innovation theory, provides a theoretical framework for this study (24). In this study, the KAP model was used to examine the environmental literacy of rural residents. The KAP model suggests that knowledge is an antecedent to attitude formation and drives practical behaviors through attitudes. In this context, knowledge is the awareness or understanding of information, attitude indicates a positive or negative evaluation of a goal, and behavior refers to regular activities carried out in the face of different problems. The model has been widely used in social research fields such as family planning, public health, education, sports, etc., and its applicability in identifying cognitive differences, attitudinal barriers and individual behaviors has been well tested. As the model continues to mature, the KAP model has also been expanded to green sustainable development fields (such as sustainable community development), land use change and rural land policy practice fields (such as rural industrial land), and other research fields (24, 25).

This paper combines the KAP model and existing domestic and international studies to improve the theoretical analytical framework from the specific situation of rural residents in mountainous areas of China in order to elucidate the interactive influence mechanism of their healthiness (Figure 1). Subsequently, this study conducted research and analyses based on this framework, dividing the investigated influencing factors into environmental literacy and

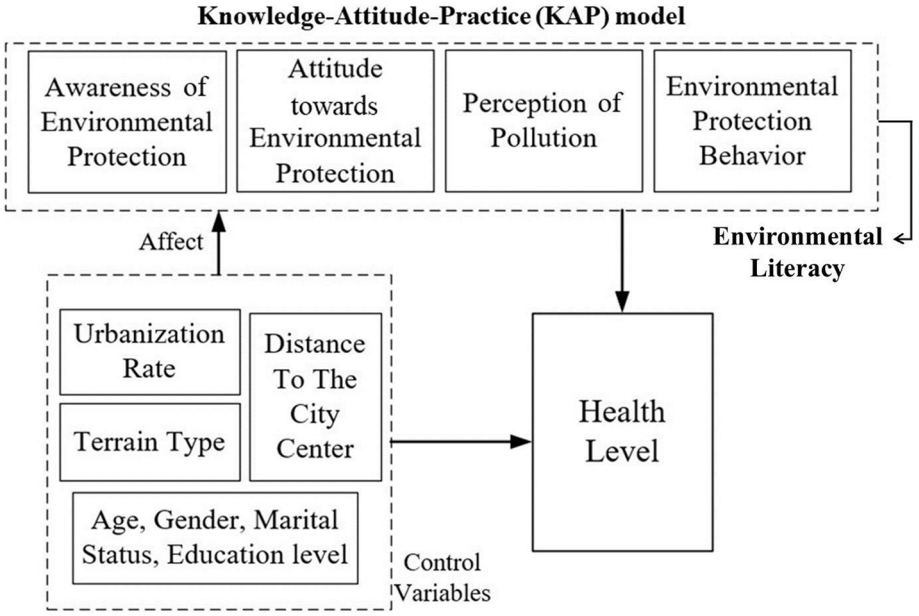


FIGURE 1
Theoretical analysis framework of the influence mechanism of farmers' health degree.

control variables. The environmental literacy variable adds "pollution perception" to the KAP model, which consists of four main aspects: rural residents' knowledge of environmental protection (In this study, we use environmental protection awareness to summarize this content), attitudes towards environmental protection, perception of pollution, and participation in environmental protection behaviors. On the other hand, the control variables predominantly encompass individual factors of rural residents (e.g., age, gender, marital status, and educational level) as well as aspects related to the villages they inhabit, such as terrain type, urbanization rate, and distance to the city center. The control variables exhibit interplay and mutual reinforcement, collectively influencing both the environmental literacy and the health degree of rural residents. Simultaneously, the environmental literacy also exert a certain impact on health degree. This paper seeks to appraise the correlation between the environmental literacy of rural residents in mountainous areas with their health degree. Therefore, it establishes a theoretical analysis framework for exploring the research on health degree in relation to these factors.

## 2 Data and methods

### 2.1 Research area and data source

The data used by the paper institute comes from the questionnaire survey conducted by the research group in Sichuan Province in 2019, which covers the basic situation of farmers' families, family production, family income and expenditure, etc. The reasons for using this data are as follows: Sichuan Province, situated in southwestern China, encompasses a total area measuring 485,000 square kilometers. Representing a prototypical mountainous province in the western part of the country, approximately 76.83% of its overall territory comprises mountainous counties (57). Simultaneously, there exists a rural population of 36.209 million individuals within Sichuan Province, accounting for 43.27% of the total populace, thus portraying it as a paradigmatic agricultural province. For the purpose of this study, the focus area encompasses the rural regions within the mountainous locales of Sichuan Province. These rural areas possess distinctive spatial attributes, including small size, elevated terrain, dispersed settlements, remote locations, seclusion, and bordering proximity. They feature a substantial proportion of illiterate and semi-literate individuals, a considerable prevalence of rural settlements lacking road connectivity, as well as a notable scarcity of medical resources available to the population (26). Due to geographical constraints, these rural areas experience relative isolation, potentially resulting in significant variations in the residents' environmental awareness compared to other regions.

Given the considerable variations in economic and social development both across different counties in Sichuan Province and within each individual county, a rigorous and systematic approach was employed to ensure the representativeness and diversity of regional characteristics (27). The survey adopts stratified sampling and equal probability random sampling to select respondents and the data is detailed and reliable (28). First, the 183 counties within Sichuan Province were classified into five groups based on per capita industrial output value. A portion of counties was randomly selected from each group, resulting in the identification of five sample areas representing varying degrees of economic development. Subsequently, employing the same methodology, each sample area was further divided into two groups. From each group, one township was randomly selected, yielding a total of two townships and ten sample townships. To ensure a balance between socioeconomic development levels and geographical distribution, two administrative villages were randomly chosen from each sample township, resulting in a total of 20 sample villages. Finally, employing the village roster and a random number table, 20 households were randomly selected from each village, resulting in a total of 400 household samples, and the corresponding questionnaire was administered. The geographical distribution of the

sample villages is presented in Figure 2. Following thorough data screening and cleaning procedures, a final dataset of 396 samples was obtained.

In the sampled area, the mountainous counties were classified into two categories based on the proportion of their mountainous and hilly areas (mountain counties: mountain area ≥ 40% and hill area < 50%; hill counties: mountain area ≥ 40% and hill area ≥ 50%, or mountain area < 40%), and each sampled village was assigned a terrain type based on the classification of its respective county (29).

## 2.2 Research methods

### 2.2.1 Measure of rural residents' environmental literacy

With reference to the study by various researchers (12, 30) a questionnaire was meticulously devised, encompassing a comprehensive set of 15 inquiries. These questions were intended to understanding the cognizance of rural inhabitants with respect to environmental preservation, their disposition towards environmental conservation, their understanding of pollution, and their corresponding environmental protection practices (Table 1).

We take environmental protection awareness as an example to elaborate on how we reduce the dimensionality of multiple items from the original scale. The definition of awareness of environmental protection is subject to varying interpretations among scholars. According to some, it encompasses a comprehensive range of social thoughts, theories, emotions, willpower, and perceptions. This multifaceted construct reflects the intricate connection between humanity and nature, embodying a novel set of values that promote the harmonious development of human and natural environment interactions (30). In contrast, alternative perspectives suggest that environmental awareness involves the acquisition and mastery of knowledge pertaining to environmental protection, as well as the cultivation of conscientiousness in guiding environmentally friendly behaviors (31). Additionally, it has been argued that environmental awareness is the subjective perception and behavioral tendency of individual social members to the relationship between human and environment, and is the result of a series of complex psychological processes (32). In accordance with related research, this paper defines that the rural residents' awareness of environmental protection as the extent to which individuals understand the environment and the principles of safeguarding it within the context of their livelihood and production activities. It encapsulates residents' cognizance and ongoing commitment to adjusting their production practices and social conduct in order to preserve the environment, while simultaneously fostering a harmonious equilibrium between humans and their surroundings, as well as within themselves. Environmental attitude pertains to the willingness of rural residents to actively engage in environmentally friendly practices in their daily lives, serving as an indicator of their emphasis on environmental protection. Perception of pollution corresponds to the degree of perceptiveness exhibited by rural residents towards environmental issues that result in pollution, thereby elucidating the extent of their concern regarding such matters. Lastly, environmental behavior encompasses the proactive involvement of rural residents in past environmental conservation

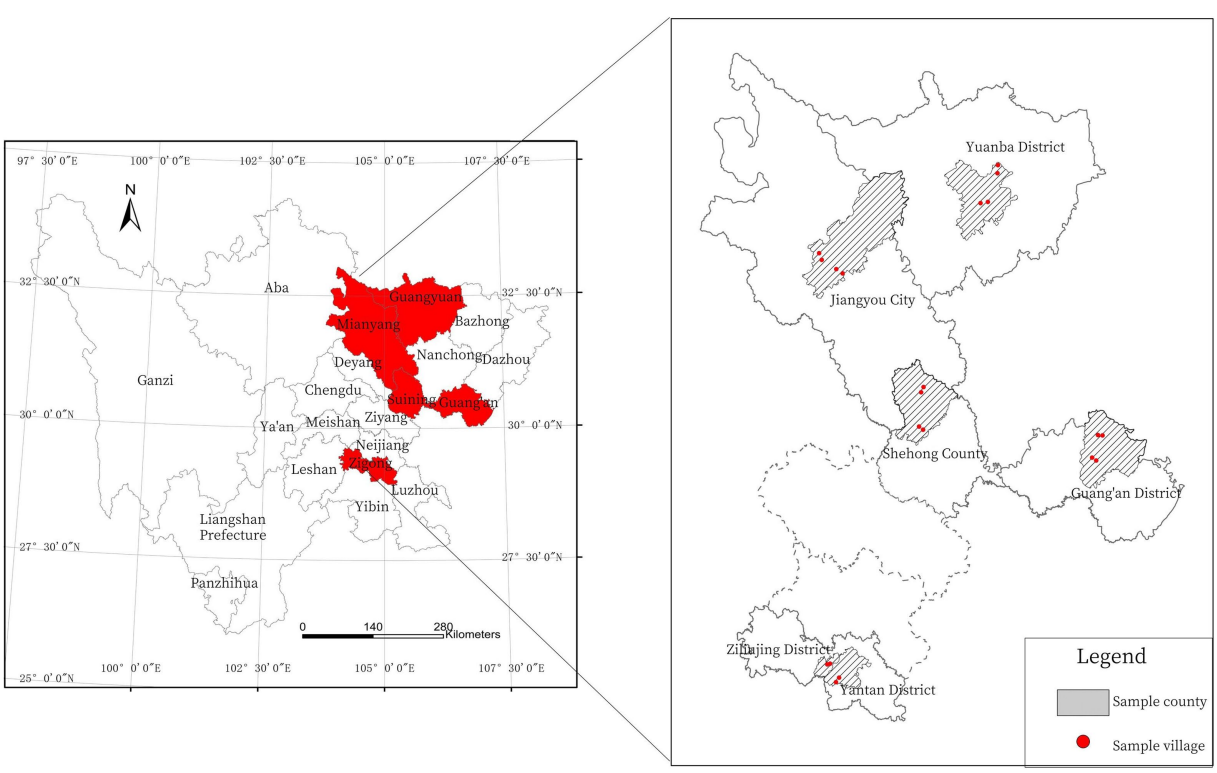


FIGURE 2
Study area (52).

TABLE 1 Scale of rural residents' environmental literacy.

| | No. | Questions | Choices |
|---|---|---|---|
| Awareness of environmental protection | 1. | Do you think pesticides pollute farmland? | 1 = Yes; 2 = No; 3 = No idea |
| | 2. | Do you believe that the disposal of pesticide bottles pollutes farmland? | 1 = Yes; 2 = No; 3 = No idea |
| | 3. | Do you believe that chemical fertilizers cause pollution to farmland? | 1 = Yes; 2 = No; 3 = No idea |
| | 4. | Do you believe that organic fertilizers pollute farmland? | 1 = Yes; 2 = No; 3 = No idea |
| | 5. | Do you believe that farm manure pollutes farmland? | 1 = Yes; 2 = No; 3 = No idea |
| | 6. | Do you believe that plastic mulch pollutes farmland? | 1 = Yes; 2 = No; 3 = No idea |
| | 7. | Do you believe that returning straw to the field pollutes farmland? | 1 = Yes; 2 = No; 3 = No idea |
| | 8. | Do you believe that irrigation pollutes farmland? | 1 = Yes; 2 = No; 3 = No idea |
| Attitude towards environmental protection | 1. | Are you willing to spend time to protect the environment from pollution? | 1 = Yes; 2 = No |
| | 2. | Are you willing to spend money to protect the environment? | 1 = Yes; 2 = No |
| | 3. | Are you willing to take actions to reduce environmental pollution? | 1 = Yes; 2 = No |
| Perception on pollution | 1. | Has there been an increasing trend of man-made environmental pollution in your area in recent years? | 1 = No; 2 = No idea; 3 = Yes |
| | 2. | What are the trends in the pollution of rivers, streams, and other water bodies in your area? | 1 = Mitigation; 2 = Unchanged; 3 = More serious |
| Environmental protection behavior | 1. | Have you watched or read any TV programs or books about environmental protection? | 1 = Yes; 2 = No |
| | 2. | Whether to recycle rigid plastics/plastic bags/paper waste paper? | 1 = Yes; 2 = No |
| | 3. | The frequency of Garbage classification and processing. | 1 = high; 2 = low |

endeavors, as well as their willingness to undertake precautionary measures to address future environmental challenges.

For the analysis of the data pertaining to the awareness of environmental protection, a factor analysis approach was employed based on the principal component analysis model. In accordance with the criterion stipulating an eigenvalue exceeding unity, two distinct common factors were identified and subsequently extracted. Subsequently, to effectuate factor rotation, the Varimax method, complemented by Kaiser Normalization, was implemented.

This study employs factor analysis to derive two factors associated with the awareness of environmental protection, as illustrated in the remaining variables presented in Table 1. To ensure accurate results, the K-means clustering technique is utilized. It should be noted that different approaches for determining the initial values of the centroids may yield dissimilar results. To minimize random classification, the class average method is first employed within the systematic clustering approach, employing Euclidean distance for clustering. Subsequently, the sample numbers represented by the leaves in the dendrogram are arranged in ascending order (from left to right). By utilizing the K-means clustering method, all variables in the questionnaire are classified into two categories pertaining to rural residents. Importantly, the mean differences between the two identified rural resident types, based on each factor and variable, have achieved statistical significance at the 99% confidence level.

The average factor scores reveal that the first type of awareness of environmental protection is predominantly characterized by negative scores (indicating a greater inclination to choose "yes" to a larger number of questions in the awareness of environmental protection scale). Consequently, this type is classified as rural residents with a "strong awareness of environmental protection." Conversely, the factors associated with the second type predominantly exhibit positive scores, leading to their classification as rural residents with a "weak awareness of environmental protection." By the same token, rural residents are further categorized into groups based on strong and weak environmental attitudes, strong and weak perceptions of environmental pollution, as well as strong and weak environmental behaviors.

### 2.2.2 Measure of rural residents' health level

The research used three indicators of self-rated health, whether you have chronic diseases diagnosed by doctors, and whether you were sick and hospitalized in the past year to measure health degree. Use factor analysis to check whether the three indicator variables are measuring a unique factor variable (health degree). The method of extracting factors is principal component factor method.

To be specific, only a strong correlation can be used for principal component analysis, and the correlation test of various indicators can be carried out. The results are shown that the value of K in the table is $0.602 > 0.5$, $p$ value is 0. Through the significance test, it can be the principal component analysis.

Table 2 shows the results obtained by the principal component factor method. It shows that a common factor with an eigenvalue greater than 1 is extracted, and its cumulative variance contribution rate is 55.297%, indicating that the three indicator variables are indeed measuring the only factor variable (health degree). The result of factor analysis shows that the selected three index variables can measure the "health degree" well.

This paper uses the weighted mean method to calculate the "health degree" of rural residents in mountainous areas of Sichuan province. The weight is the factor load of factor analysis. According to the intervals and weights of the three indicator variables, the health degree ($Y_1$) obtained is calculated as shown in Equation 1.

$$Y_1 = 0.472 * X_1 + 0.413 * X_2 + 0.458 * X_3 \quad (1)$$

## 2.3 The regression model

In this study, the dependent variable under scrutiny is the "health degree of rural residents," which constitutes a subjective and objective evaluation of the health status among this population. Considering that the dependent variable and most of the independent variables in this study are continuous variables, a multiple linear regression model is employed to examine the association between the environmental literacy, control variables, and the health degree of rural residents. A multiple linear regression model is a statistical technique used to describe the relationship between two or more independent variables and a single dependent variable. It is an extension of simple linear regression, which involves only one independent variable. The equation is as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 ... + \beta_j X_j + \mu \quad (2)$$

In Equation 2, $X_1, X_2, \ldots, X_j$ constitutes the independent variables of interest in the model, encompassing both the control variable and the environmental literacy $\beta_0, \beta_1, \ldots, \beta_j$ refers to the coefficient estimate of the influencing factor, and $\propto$ is a random error.

# 3 Result analyses

## 3.1 Descriptive statistical results

The individual and social backgrounds of rural residents encompasses nine distinct categories: gender, age, marital status, level of education, whether they are party members or village cadres, urbanization rate of the village, terrain type, and distance to the township center. Analysis of the results (Table 3) reveals that the male population constitutes the majority of rural residents, spanning from 20 to 85 years of age. A significant proportion of the populace consists of older adults individuals. Marital unions encompass 87% of the rural inhabitants, with an average educational attainment of 5.67 years, approximately corresponding to the primary school level, thus indicating a limited educational background. Membership in political parties is observed among only a small segment of rural residents, and an even smaller fraction assumes roles as village officials. In addition, concerning the control variables, 75% of the sampled rural residents reside in hilly terrain. The villages exhibit an average urbanization rate of 52.87%, denoting an overall favourable development.

Simultaneously, the table reveals a noteworthy pattern regarding the disposition and conduct of the residents concerning environmental protection. A majority of the residents exhibit a resolute attitude and behavior towards safeguarding the environment, while half of them display a relatively feeble awareness of environmental protection. Additionally, a considerable number of rural residents perceive a decline in pollution levels. Among the four pivotal variables under observation, the perception of pollution by rural residents emerges as the most pronounced, with a substantial portion acknowledging a decrease in pollution. On the whole, the rural residents demonstrate a certain level of environmental concern, albeit leaving room for advancement in terms of enhancing their consciousness regarding environmental protection (33).

## 3.2 The influence of rural residents' background on the environmental literacy

To provide a comprehensive overview of the sampled rural residents and elucidate the disparities in the environmental literacy, a significant difference test was conducted on all explanatory variables. The relationship between categorical control variables and environmental literacy variables was examined using the Pearson chi-square test ($\chi^2$), while the association between continuous control variables and environmental variables was assessed through an independent sample *t*-test (T). The results of these statistical tests (Table 4) reveal noteworthy disparities in various aspects, including the awareness of environmental protection, among rural residents belonging to different age groups, educational backgrounds, and roles as village officials. Notably, age exhibits a significant negative correlation with awareness of environmental protection. Older rural residents encounter relatively limited avenues for accessing diverse forms of information compared to their younger counterparts, which may impede their development of a heightened consciousness towards environmental protection. Moreover, rural residents serving as village officials demonstrate significantly higher levels of awareness regarding environmental protection and perception of pollution compared to those who do not hold such positions, presumably due to the nature of their daily responsibilities. Moreover, there exists a notable positive correlation between the level of education and both awareness of environmental protection and attitude. Marital status and party membership also exert a significant positive influence on environmental behavior and perception of pollution, respectively. Unmarried rural residents display a greater inclination toward engaging in environmental practices, while farmers who hold party membership exhibit heightened concerns regarding environmental pollution, coupled with a heightened level of perception. These findings collectively underscore the noteworthy impact of rural residents' backgrounds on the environmental literacy variables pertaining to environmental protection, a phenomenon that agrees with the theoretical framework outlined in this article.

TABLE 2 Principal component analysis of health level.

| | Eigenvalue | Factor loading | Variance (%) | Cumulative variance (%) |
|---|---|---|---|---|
| $X_1$ (Self-rated health) | 1.659 | 0.472 | 55.297 | 55.297 |
| $X_2$ (Whether you were sick and hospitalized) | 0.743 | 0.413 | 24.763 | 80.060 |
| $X_3$ (Whether you have chronic diseases diagnosed by doctors) | 0.598 | 0.458 | 19.940 | 100.000 |

## 3.3 Impact of environmental literacy on health degree

To delve deeper into the influences exerted on the health status of rural inhabitants, an examination is conducted in this study, wherein individual background and the rural situation are introduced as control variables. Subsequently, the comprehensive effects of key observables, namely the awareness of environmental protection, attitude towards environmental protection, perception of pollution, and environmental protection behavior, on the Health degree of rural residents are explored. To accomplish this, a multiple linear regression (MLR) model is employed (Table 5).

The regression results for the impact environmental literacy variables on the health degree of rural residents are presented in Model 1. Model 2 encompasses the regression outcomes of both the environmental literacy variables and the control variables pertaining to the characteristics of rural residents. Model 3, which combines Models 1 and 2, includes all control variables and environmental literacy variables for regression analysis. Evaluation of the pseudo R-square values reveals that Model 3 exhibits a significantly better fit than Models 1 and 2, thereby establishing it as the primary model employed for analysing the regression results in this study. Moreover, the Variance Inflation Factor (VIF) test is conducted to assess the presence of severe multicollinearity among the independent variables across different models. The VIF results demonstrate that all variables have values below 10, indicating the absence of severe multicollinearity. Furthermore, the chi-square test statistics for the models indicate that all models have successfully passed the overall significance test. This suggests that in each model, there exists a significant relationship between at least one independent variable and the dependent variable.

The analysis of Model 3 regression results (Table 5) reveals a nuanced impact of the observational variables on the health degree of rural residents. And the significant influence relationship among variables is in Figure 3. A summary analysis shows that attitude towards environmental protection and being party members have a significant positive impact on the health degree. Gender, age and marital status have a significant negative impact on the health degree. However, awareness of environmental protection, perception of pollution, environmental protection behavior, educational level, assuming the role of a village official, urbanization rate, and distance to the city center do not have an impact on the health degree.

Notably, the regression coefficient value of the attitudes towards environmental protection is 0.164 ($t = 2.277$, $p = 0.023 < 0.05$), attitudes towards environmental protection exhibit highly significant positive impact on the health degree of rural residents. This suggests that a stronger attitudes towards environmental protection is associated with higher health degree. This implies that a one-unit increase in attitudes towards environmental protection raises the probability of rural residents opting for an average health degree by a factor of 0.164.

Consequently, rural residents who are more inclined to allocate time and financial resources towards environmental matters tend to exhibit better health conditions. Conversely, individuals who are

TABLE 3 Descriptive statistics for scales.

| | | Variable | Abbreviation | Definition | Number | Mean | Standard deviation | Min | Max |
|---|---|---|---|---|---|---|---|---|---|
| Dependent variables | | Health degree | HEA | Comprehensive calculation indicators | 396 | 2.28 | 0.62 | 1.34 | 3.63 |
| Independent variables | Environmental literacy | Awareness of environmental protection | AWA | 1 = Strong; 0 = Weak | 396 | 0.50 | 0.50 | 0.00 | 1 |
| | | Environmental protection behavior | BEH | 1 = Strong; 0 = Weak | 396 | 0.83 | 0.37 | 0.00 | 1 |
| | | Attitude towards environmental protection | ATT | 1 = Strong; 0 = Weak | 394 | 0.76 | 0.43 | 0.00 | 1 |
| | | Perception of pollution | PER | 1 = Strong; 0 = Weak | 392 | 0.25 | 0.44 | 0.00 | 1 |
| | Control variables | Age | AGE | Year | 396 | 61.54 | 10.82 | 21.00 | 86 |
| | | Gender | GEN | 1 = Man; 2 = Woman | 396 | 1.45 | 0.50 | 1.00 | 2 |
| | | Marital status | MAR | 1 = Unmarried; 2 = Married | 396 | 1.88 | 0.33 | 1.00 | 2 |
| | | Educational level | EDU | Year | 396 | 5.67 | 3.58 | 0.00 | 15 |
| | | Party member | PAR | 1 = Yes; 0 = No | 396 | 0.15 | 0.35 | 0.00 | 1 |
| | | Village official | VIL | 1 = Yes; 0 = No | 396 | 0.08 | 0.28 | 0.00 | 1 |
| | | Terrain type | TER | 1 = Mountain; 2 = Hill | 396 | 1.75 | 0.43 | 1.00 | 2 |
| | | Urbanization rate | URB | Percentage | 396 | 52.87 | 15.01 | 36.20 | 92.08 |
| | | Distance to the township center | DIS | km | 396 | 4.80 | 3.50 | 0.50 | 14 |

unwilling to dedicate their time and effort to addressing environmental pollution tend to have poorer health. One possible explanation for this discovery is that rural residents with stronger environmental attitudes may be more concerned about the harmful effects of environmental pollution on their well-being, leading to higher levels of health. On the other hand, those with poorer health may exhibit lower awareness of the health consequences associated with environmental pollution. This observation aligns with existing research, which highlights that individuals dealing with health issues are often highly concerned about environmental matters (34). Similarly, awareness of environmental protection and perception of pollution also exert certain influences on the health degree of rural residents.

Among the control variables, the influence of gender, age, marital status, and being a party member on the health status of rural inhabitants is noteworthy. The impacts associated with these variables can be defined as follows: With regard to gender, male individuals residing in rural areas tend to report higher health ratings compared to those who self-assess their health as poor. Previous research findings consistently indicate that, regardless of mental or physical well-being, men generally perceive themselves as being in better health than women (35, 36). Age exhibits a significantly adverse effect on the health status of rural residents. This phenomenon can be attributed to the fact that individuals in their middle age constitute the primary workforce within their families, engaging in extensive labour and participating in numerous social activities, thereby increasing their susceptibility to injuries and illnesses. Meanwhile, the physiological functions of the older adults experience a gradual decline, accompanied by a decrease in immune response, a heightened susceptibility to functional and organic ailments, and an increased probability of accidents such as falls (37). As individuals age, their physical health progressively diminishes (38). Regarding marital status, unmarried rural inhabitants exhibit higher self-assessed health degree in comparison to those who perceive their health as poor. Moreover, assuming the role of being a party member exerts a significantly positive influence on the health status of rural residents, as they tend to report higher health ratings. In terms of the variable of education level, the data showed no significant relationship with health. The possible reason for this result is that the education level of the subjects surveyed in this paper is relatively low, which is not enough to reflect the conclusion of previous studies that the higher the education level, the more concerned about their health (39).

From Table 5, it can be seen that taking the awareness of environmental protection, attitude towards environmental protection, perception of pollution, environmental protection behavior and other control variables as independent variables, and health degree as the dependent variable for linear regression analysis, the estimated model can be:

Health Degree =3.874 + 0.001*AWA+ 0.164*ATT + 0.090*PER − 0.080*BEH − 0.008*EDU − 0.133*GEN − 0.020*AGE − 0.175*MAR + 0.023*VIL + 0.318*PAR − 0.088*LAN+ 0.003*URB − 0.000*DIS. The R-squared value of the model is 0.148, indicating that all independent and control variables can explain the 14.8% change in rural residents' health degree.

# 4 Discussions, conclusions and implications

## 4.1 Discussions

In comparison to previous relevant studies, this study exhibits certain similarities and disparities. The selection of participants in this study predominantly focused on rural mountainous regions, and a Chinese rural resident-oriented environmental protection scale was devised. The results of this research present a more accurately depiction of the prevailing circumstances in rural mountainous areas of China.

This study highlights the positive impact of environmental protection attitudes on the health status of rural residents. Relevant studies have found that Wellbeing and quality of life are linked to a positive attitude, which makes people satisfied and healthy in their lives (40). Previous studies have shown that environmental attitude plays an important role in KAP and is the driving force of environmental behavior, but there is still a certain gap with the occurrence of behavior, which also proves the importance of environmental attitude in this result (41). At the same time, studies have proved that in the case of poor environmental awareness, there can still be a high environmental attitude, and attitude is closely related to health (42). Roy et al. (43) confirmed that the defensive attitude of the people may reduce the severity of landfill exposure, improved that defensive attitude near landfill significantly affects residents' health status. These findings are similar to those of this study. Previous studies have often believed that psychological factors such as attitude cognition can affect health levels through behavior as a mediating variable. However, further exploration in this study found that

TABLE 4 Cross-correlations of scales.

| Variable | Pearson chi-square value/T value | | | | Significance | | | |
|---|---|---|---|---|---|---|---|---|
| | AWA | ATT | PER | BEH | AWA | ATT | PER | BEH |
| Gender | 1.4695 | 1.900 | 0.069 | 1.379 | 0.225 | 0.168 | 0.792 | 0.24 |
| Age | −1.624 | 0.221 | −0.448 | 1.384 | 0.034** | 0.774 | 0.655 | 0.471 |
| Marital status | 0.2096 | −0.202 | −0.315 | 1.111 | 0.647 | 0.498 | 0.247 | 0.080** |
| Educational level | 2.298 | −0.351 | 0.496 | −0.099 | 0.015** | 0.058* | 0.620 | 0.122 |
| Health degree | 1.264 | 2.753 | 0.711 | −1.044 | 0.738 | 0.263 | 0.322 | 0.1995 |
| Village official | 7.438 | 1.427 | 5.823 | 0.954 | 0.006*** | 0.232 | 0.016** | 0.329 |
| Party member | 1.3413 | 1.866 | 4.921 | 1.791 | 0.247 | 0.393 | 0.085** | 0.408 |

*** means significant at the level of 1%, ** means significant at the level of 5%, * means significant at the level of 10%.

TABLE 5 Regression results of environmental literacy's impact on health degree.

| Independent variable | | Health degree | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Model I | | Model II | | Model III | | |
| | | Coef. (St. Err.) | 95% CI | Coef. (St. Err.) | 95% CI | Coef. (St. Err.) | Standardized Coef. | 95% CI |
| Environmental literacy | Awareness of environmental protection | 0.055 (0.868) | −0.069 ~ 0.178 | −0.003 (−0.049) | −0.121 ~ 0.115 | 0.001 (0.014) | 0.001 | −0.118 ~ 0.120 |
| | Attitude towards environmental protection | 0.160** (2.124) | 0.012 ~ 0.307 | 0.161** (2.251) | 0.021 ~ 0.301 | 0.164** (2.277) | 0.112 | 0.023 ~ 0.304 |
| | Perception of pollution | 0.094 (1.287) | −0.049 ~ 0.237 | 0.091 (1.309) | −0.045 ~ 0.227 | 0.090 (1.297) | 0.063 | −0.046 ~ 0.227 |
| | Environmental protection behavior | −0.017 (−0.206) | −0.182 ~ 0.147 | −0.083 (−1.031) | −0.242 ~ 0.075 | −0.080 (−0.984) | −0.048 | −0.240 ~ 0.079 |
| Control variables | Educational level | | | −0.008 (−0.795) | −0.028 ~ 0.012 | −0.008 (−0.780) | −0.046 | −0.028 ~ 0.012 |
| | Gender | | | −0.131** (−1.989) | −0.260 ~ −0.002 | −0.133** (−2.001) | −0.106 | −0.263 ~ −0.003 |
| | Age | | | −0.020*** (−6.025) | −0.027 ~ −0.014 | −0.020*** (−5.879) | −0.349 | −0.027 ~ −0.013 |
| | Marital status | | | −0.169* (−1.839) | −0.349 ~ 0.011 | −0.175* (−1.893) | −0.094 | −0.357 ~ 0.006 |
| | Village official | | | 0.037 (0.322) | −0.190 ~ 0.264 | 0.023 (0.193) | 0.010 | −0.206 ~ 0.251 |
| | Party member | | | 0.315*** (3.305) | 0.128 ~ 0.502 | 0.318*** (3.323) | 0.181 | 0.130 ~ 0.506 |
| | Terrain type | | | | | −0.088 (−0.903) | −0.061 | −0.278 ~ 0.102 |
| | Urbanization rate | | | | | 0.003 (1.298) | 0.071 | −0.001 ~ 0.007 |
| | Distance to the city center | | | | | −0.000 (−0.044) | −0.003 | −0.022 ~ 0.021 |
| | Constant | 2.038*** (16.083) | 1.790 ~ 2.287 | 3.881*** (10.209) | 3.136 ~ 4.626 | 3.874*** (8.946) | - | 3.025 ~ 4.723 |
| Number of samples | | 396 | | 396 | | 396 | | |
| $R^2$ | | 0.022 | | 0.141 | | 0.148 | | |
| *F* | | $F(4,385) = 2.131$, $p = 0.076$ | | $F(10,379) = 6.204$, $p = 0.000$ | | $F(13,376) = 5.008$, p = 0.000 | | |
| D-W | | 2.043 | | 2.115 | | 2.126 | | |

**, * are significant at the levels of 1, 5%.

this effect did not occur, and attitude only directly affects health levels.

In existing studies, many literatures show that attitude has a significant impact on behavior, behavior will affect health, and environment can affect residents' health through behavioral mediators (44). However, in this paper, environmental protection attitudes directly affect health, while behaviors have no significant effect. It is speculated that this effect may be caused by the choice of behavioral variables, or it may be influenced by the complex relationship between attitude and behavior. Therefore, it is necessary to further study the relationship between attitude factors at the psychological level, behavior factors at the objective level and both subjective and objective health factors. In terms of the fundamental variables observed among rural residents, this study further reveals a robust inclination towards environmental protection, perceptiveness of pollution, and engagement in environmental behaviors. Nevertheless, the overall consciousness pertaining to environmental protection is relatively limited, which may be due to poor environmental knowledge, especially about agricultural sources of pollution (45, 46, 57). Concurrently, preceding studies have primarily emphasized the influence of factors such as age, gender, family income, educational attainment, and occupation on the awareness of environmental protection among residents (47–49). While these studies generally assert that age positively affects environmental awareness, this study also reveals a positive impact of educational level on such awareness. However, it also uncovers a negative association between age and environmental consciousness. This could potentially be attributed to the generally low educational attainment of the older adults population within the rural sample of this study, thereby resulting in an inadequate comprehension of environmental issues. Moreover, the study identifies that rural residents assuming roles as village officials exhibit a heightened awareness of environmental protection.

Previous studies on the determinants of health among rural mountain residents have predominantly focused on individual factors such as age and gender, as well as social factors such as the regional economic environment, yielding valuable insights. Qualitative studies from Western contexts have also underscored the necessary nature of environmental protection for maintaining good health (50, 51). In the findings of this study, the influence of individual factors, including gender, age, and marital status, on health degree remains notable. Some prior research suggests that a happy marriage exerts a long-lasting and stable protective effect on health. However, given that most unmarried rural residents are younger, this study reveals that they perceive their health degree as higher. The accurate mechanism by which marital status operates necessitates further research. Moreover, the roles of party members also emerge as significant in this study. Notably, this article categorizes the sampled rural areas residing in mountainous regions based on terrain, uncovering substantial disparities in the health degree of residents between mountainous and hilly villages. The terrain factor warrants further exploration in future research.

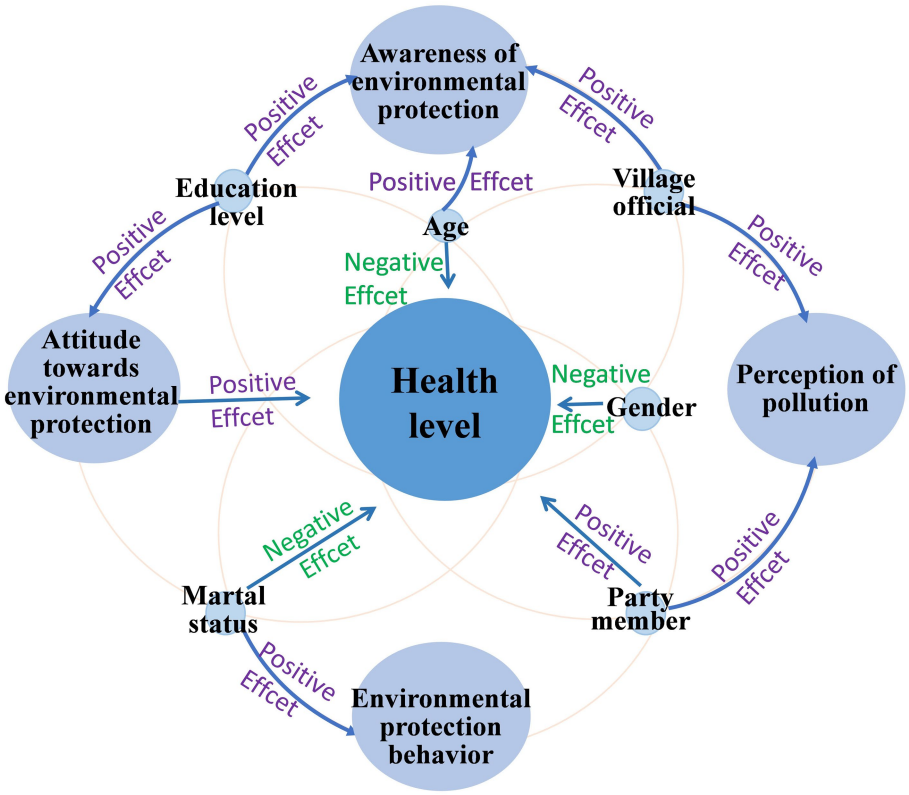


FIGURE 3
Significant influence relationship among variables.

## 4.2 Conclusions and policy recommendations

This study employs field research data to scrutinize the influence of geographical conditions prevalent in mountainous rural regions, the degree of environmental literacy exhibited by rural inhabitants, and various other factors on the overall health status. A comprehensive scale is devised to assess rural residents' environment literacy, which includes cognizance of environmental preservation, their attitudes towards it, corresponding behaviors, and perception of pollution. Additionally, aKAP model, encompassing both environmental literacy and control variables, is constructed to elucidate the relationship between rural resident health and the aforementioned variables. Comparative to extant research, this study accentuates the potential repercussions stemming from environmental literacy and other environmental factors on the health status of rural residents, leading to the formulation of the following conclusions:

1 Environmental literacy of rural residents is significantly influenced by demographic factors. Age shows a negative correlation with environmental protection awareness, while education level and village official status are positively associated. Education is also linked to more positive environmental attitudes, and marital status influences environmental behaviors, with unmarried individuals being more proactive. Party membership correlates with greater concern for pollution.
2 The impact of environment literacy on rural residents' health level is complex. Positive attitudes towards environmental protection are significantly linked to better health status, and increased awareness and pollution perception contribute to improved health.
3 Among control variables, age, marital status, gender, and party membership significantly affect health. These findings inform more scientific and effective approaches to improving rural health.

This study presents a novel vantage point regarding the investigation of environmental literacy and the state of health. Moreover, it evaluates, to a certain extent, the efficacy of policies associated with ecological civilization. This indirect inquiry sheds light on the local residents' endorsement of environmental policies and support for related actions, while simultaneously providing a direct glimpse into the environmental consciousness, attitudes, behaviors, and perceptions of pollution held by the aforementioned rural dwellers. Drawing upon the empirical findings, the following recommendations are proffered: It is necessary for relevant governmental bodies and organizations to fortify environmental conservation education and technical training initiatives targeted at rural regions. Additionally, incentivizing eco-friendly behaviors and fostering the enhancement of educational attainment among residents of the mountainous rural areas should be prioritized. The

media and propaganda departments should actively engage in diverse environmental conservation training programs and dissemination of information, emphasizing the criticality of cultivating environmental literacy for the betterment of the rural ecosystem and individual well-being. Within this context, rural party members and officials ought to assume a pioneering and leading role to elevate rural residents' cognizance of environmental protection and encourage a heightened focus on personal health status.

## 5 Limitations

This study is not without certain limitations that warrant acknowledgement and further exploration. Firstly, it is necessary to recognize that the research was conducted exclusively within a representative mountainous region of Sichuan. Although data acquisition was carried out via random sampling to mitigate sampling bias, there may still exist isolated cases that deviate from the characteristics elucidated in this paper. Secondly, this paper also identifies avenues for potential advancement in future studies. At the same time, due to the reality that the existing rural residents in mountainous areas inevitably have a large proportion of older adults people and low education level, the different results of the research conclusions are also more targeted. For instance, the selection of indicators for control variables remains incomplete, and the inclusion of factors related to natural disasters could enhance the comprehensiveness of the assessment system. In addition, the analysis of the causal mechanisms between control variables and rural environmental results can be fortified. Thirdly, it is essential to recognize that the health status of rural inhabitants is a dynamically developing indicator. In future studies, there is potential for tracking data over subsequent years to facilitate a more comprehensive analysis of the long-term dynamics and impact mechanisms, thereby yielding more valuable insights for enhancing the health status and environmental consciousness of rural inhabitants. Moreover, given the perennial concern surrounding health-related matters, this paper substantiates a certain correlation between attitudes towards environmental protection, environmental preservation behaviors, and health degree. Consequently, further investigations should seek to address pertinent questions such as the effective methods to enhance environmental preservation behaviors and health degree among rural residents in mountainous regions, as well as the development of robust evaluation frameworks for assessing their health degree.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The studies involving humans were approved by the institutional and national research committee and the 1964 Helsinki declaration. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

## Author contributions

YS: Data curation, Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft. YY: Data curation, Methodology, Writing – review & editing. SL: Funding acquisition, Supervision, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# The impact of intrauterine adhesions on endometrial receptivity in patients undergoing *in vitro* fertilization-embryo transfer

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**Objective:** To clarify whether intrauterine adhesions (IUAs) affect endometrial receptivity (ER) on the day of ovulation and to compare patients with mild and moderate-severe adhesions.

**Methods:** This prospective cohort study included 592 infertile women with IUAs who underwent frozen-thawed embryo transfer (FET). Patients were divided into groups with or without IUAs; and pregnant and nonpregnant populations based on whether a clinical pregnancy was achieved. The ultrasound ER parameters on the ovulation day were compared. Patients with IUAs were then divided into mild or moderate-severe IUA subgroups according to IUA degree.

**Results:** The proportions of patients with Type B plus Type C endometrial morphology (94% vs. 75%, P<0.001), an endometrial thickness≥8mm (97% vs. 81%, P<0.001), an endometrial volume≥2ml (94% vs. 67%, P<0.001), a frequency of endometrial peristalsis≥2 times/min (84% vs. 53%, P<0.001), low subendometrial volume (11.54 ± 2.94 vs. 9.57 ± 2.35, P<0.001) and subendometrial vascularization flow index (VFI) values (2.70 ± 3.10 vs. 2.23 ± 2.23, P=0.033) and a low live birth rate (65% vs. 56%, P=0.039) were significantly higher in the group without IUAs than in the group with IUAs. The group with moderate-severe IUAs had lower proportion of patients with an endometrial thickness≥8mm (73% vs. 89%, P=0.008) and an endometrial volume ≥2ml (58% vs. 78%, P=0.005), a lower frequency of endometrial peristalsis≥2 times/min (42% vs. 65%, P=0.003), and low subendometrial volume (9.22 ± 2.29 vs. 9.97 ± 2.36, P=0.023) and subendometrial flow index (FI) (31.48 ± 3.64 vs. 33.43 ± 4.17, P=0.002) values than the group with mild IUAs; a high antral follicle count (AFC), basal follicle-stimulating hormone (FSH), and anti-Müllerian hormone (AMH) levels and an endometrial thickness≥8mm were independent predictors of clinical pregnancy.

**Conclusion:** IUAs can affect ER on the ovulation day and the live birth rate during natural cycles. Moderate-severe IUAs have a greater impact on ER than mild adhesions do; however, if these adhesions are treated properly, they do not have adverse effects on the clinical pregnancy rate. A high AFC, basal FSH and AMH levels and an endometrial thickness ≥8 mm were found to be independent predictors of clinical pregnancy.

KEYWORDS

**endometrial receptivity, intrauterine adhesion, frozen-thawed embryo transfer, natural cycle, ovulation day, transvaginal sonography**

# Introduction

Intrauterine adhesions (IUAs) are considered one of the main reproductive system diseases in women worldwide and are characterized by endometrial fibrosis with partial to complete obliteration of the uterine cavity and/or cervical canal (1, 2). The reported prevalence of IUAs varies between 0.3% and 21.5% (3). Any event that causes damage to the endometrium may lead to the development of IUAs, resulting in menstrual disturbances, infertility and (recurrent) pregnancy loss (4).

Transvaginal sonography (TVS) has been adopted as a routine method to assess endometrial receptivity (ER) (5). Hysteroscopic adhesiolysis is the standard treatment for removing IUAs, restoring the uterine architecture and facilitating communication among the uterine cavity, cervical canal and fallopian tubes to allow both normal menstrual flow and adequate sperm transportation (6). While B Pecorino et al. (7) reported that endometrial scratching in the patients with repeated implantation failure had no significant improvement in implantation and clinical pregnancy rates.

The relationship between IUAs and reproductive performance has been frequently described in the literature; IUAs, especially moderate and severe IUAs, may strongly impact fertility, predisposing individuals to pregnancy disorders and obstetric complications in subsequent pregnancies (8–10). Is this because IUAs have a negative impact on ER? The aim of the present study was to clarify whether IUAs affect ER on the day of ovulation and to further compare patients with mild and moderate–severe adhesions. To our knowledge, this is the first prospective study comparing ER on the day of ovulation during natural cycles between patients with and without a history of adhesions.

# Materials and methods

## Study design and participants

This prospective cohort study was conducted at the Reproductive and Genetic Hospital of CITIC-Xiangya from March 2019 to September 2023, and 592 infertile women (417 with a history of IUAs and 175 without no IUAs) who underwent frozen-thawed embryo transfer (FET) were included. Written informed consent was obtained from all participants. The institutional review board approved this study (date of approval: 11 September 2019; reference number: LL-SC-2019-023; Changsha, China).

The inclusion criteria for patients with a history of IUAs were as follows (1): underwent FET after adhesiolysis (2); underwent a natural FET cycle (3); aged 20–35 years (4); had a body mass index of 18–24 kg/m$^2$; and (5) had at least 1 high-quality embryo. The exclusion criteria were as follows (1): endometriosis, adenomyosis, or adenomyoma (2); endometritis (3); congenital uterine malformations (4); untreated hydrosalpinx; or (5) uterine cavity fluid (diameter ≥2 mm) caused by caesarean section diverticulum.

The inclusion criteria for the control group of patients with no IUAs were as follows (1): underwent FET (2); underwent a natural FET cycle (3); age 20-35 years (4); body mass index 18-24 kg/m2 (5) had at least 1 high-quality embryo. The exclusion criteria were (1): endometriosis, adenomyosis, or adenomyoma (2); intrauterine adhesion or endometritis (3);congenital uterine malformations (4); untreated hydrosalpinx; or (5) uterine cavity fluid caused by cesarean section diverticulum and diameter ≥2 mm.

## Adhesiolysis

IUAs were graded on the basis of the American Fertility Society classification (11): mild IUA was indicated by 1-4, moderate was indicated by 5-8, and severe was indicated by 9-12. All the included patients underwent adhesiolysis in our center before the *in vitro* fertilization (IVF) procedure, which was performed by an experienced surgeon under general anesthesia. The adhesions were dissected by using bipolar energy (Olympus) and/or hysteroscopic scissors (Karl Storz, Tuttlingen, Germany) until the uterine cavity was achieved to restore uterine morphology. Then, based on patients' uterine width, a heart-shaped intrauterine balloon (Cook Medical, Bloomington, IN, USA) or Foley catheter was inserted into the uterine cavity, and all patients received crosslinked hyaluronan gel (MateRegen; BioRegen Biomedical, Changzhou, China) to prevent adhesion reformation.

## *In vitro* fertilization procedure

Depending on the cause of infertility, IVF or intracytoplasmic sperm injection (ICSI) was applied for fertilization. In this study, all patients underwent FET during natural cycles. Thawed cleavage-stage embryos and blastocysts were transferred on the 3rd day and the 5th day after ovulation. Embryo morphology was scored on the basis of the Alpha Scientists in Reproductive Medicine and European Society of Human Reproduction and Embryology (ESHRE) Special Interest Group of Embryology (ASEBIR) consensus (12). A maximum of two embryos were transferred.

## Ultrasound evaluation

### Ultrasound diagnosis of IUAs

GE VOLUSON E8 ultrasound instrument (GE VOLUSON, E8, General Electric Tech Co., Ltd., New York, USA) equipped with a 5–9 MHz transvaginal three-dimensional (3D) probe was used for the preoperative evaluation of uterine cavity. 2D ultrasound was used routinely as a first-line diagnostic tool for the assessment of the endometrial integrity to look for disruptions of the endometrial–myometrial junction. Adhesions are seen as bands of myometrial tissue traversing the endometrial cavity and adjoining the opposing uterine walls. For suspected patients, 3D ultrasound was used to further clarify adhesions. During 3D TVS, the morphological characteristics suggesting IUA were classified into six categories: marginal irregularity, thinning (< 2 mm), defect, obliteration, fibrosis, or calcification (13, 14).

## Assessment of ER

TVS was performed on the day of ovulation for all included patients to evaluate ER. Ultrasound ER parameters, including endometrial thickness, morphology, volume, movement and blood perfusion, were assessed by the same senior ultrasonic doctor (Dr. Li) using the same ultrasound machine.

The maximum diameter of the endometrium was measured in the longitudinal plane. The Gonen classification criteria (15) were adopted to classify endometrial morphology: Type A: the increase in reflectivity leads to a completely homogeneous, hyperechoic endometrium, with the central echo line not visible; Type B: the endometrium has the same reflectivity compared to the surrounding myometrium, and the central echo line is not obvious or missing; and Type C: a "triple-line" endometrium is present, consisting of a prominent outer and central hyperechogenic line and inner hypoechogenic or black regions.

The movement of the endometrium was observed and recorded within 3 minutes and was divided into 5 types according to Ljland et al. (16) (1): positive wave: the peristaltic wave from the cervix to fundus (2); negative wave: the peristaltic wave from the fundus to the cervix (3); static wave: the endometrium is in a static state (4); bidirectional wave: the endometrium of both the uterine fundus and cervix contract simultaneously; and (5) random wave: irregular motion types with an uncertain direction or multiple starting points.

Endometrial blood perfusion was evaluated based on the Applebaum classification standard (17): I: vessels penetrate the outer hypoechoic area around the endometrium but do not enter the outer edge of the hyperechoic area; II: vessels penetrate the outer edge of the endometrium with high echogenicity but do not enter the internal area of low echogenicity; and III: vessels enter the hypoechogenic inner area of the endometrium.

The ultrasound machine was switched to 3D mode with power Doppler. Virtual organ computer-aided analysis (VOCAL) software was used to outline the endometrium, and the endometrial volume, endometrial vascularization index (VI), vascularization flow index (VFI) and flow index (FI) were obtained (18, 19).

## Outcome measures

Serum human chorionic gonadotropin (HCG) levels were measured 14 days (12 days after blastocyst transfer), and TVS was performed 4 weeks after transfer. The primary outcome was live birth, defined as the complete expulsion or extraction of a product of fertilization after 20 completed weeks of gestation that, after separation from the woman, breathed or showed other evidence of life, irrespective of whether the umbilical cord had been cut or the placenta was attached. The secondary outcome was clinical pregnancy, which was confirmed if a gestational sac was observed, and a viable pregnancy was diagnosed when fetal cardiac activity was detected (20).

The included patients were divided into groups according to the presence or absence of IUAs: the group with IUAs and the group without IUAs. All enrolled patients were further divided into two groups according to whether a clinical pregnancy was achieved: the pregnant group and the nonpregnant group. Patients with IUAs were then divided into mild or moderate-severe IUA subgroups according to the degree of IUAs. The ER parameters on the day of ovulation were compared between these groups.

## Statistical analysis

The distribution of patient demographics was analyzed via the Kolmogorov–Smirnov test. Continuous variables are expressed as means ± standard deviations (SDs). Categorical variables are described as frequencies and percentages. The Mann–Whitney U test or Student's t test was used to assess continuous variables, and the chi-square test or Fisher's exact test was used to assess differences in categorical variables between the pregnant group and the nonpregnant group. Univariate and multivariate logistic regression analyses were used to identify independent predictors of clinical pregnancy; odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. All the statistical analyses were performed via R software (version 4.3.2), and a two-sided p value $< 0.05$ was considered statistically significant.

# Results

## Characteristics and ultrasound parameters on the day of ovulation for patients without IUAs

During the study period, there were a total of 191 patients with a history of IUAs, after excluding 10 patients who were dropped and 6 patients of lost to follow up, 175 patients with previous IUAs (93 patients had moderate–severe IUAs, and 82 patients had mild IUAs) were included; A total of 447 patients who did not have a history of IUAs were enrolled, after excluding 19 patients who were dropped and 11 patients of lost to follow up, 417 patients were ultimately included as the control group. The overall clinical pregnancy rates were 69.9% (414/592) and 72.2% (301/417) for patients without IUAs, 64.6% (113/175) for patients with a history of IUAs [63.4% (59/93) for moderate-severe IUAs, 65.9% (54/82) for mild IUAs]. The basic, clinical and endometrial ultrasound characteristics of the overall group, pregnant group and nonpregnant group of patients without or with IUAs are displayed in Table 1.

Patients in the nonpregnant group had greater numbers of oocytes retrieved (1.21 ± 0.43 vs. 1.11 ± 0.35, P=0.011), higher basal follicle-stimulating hormone (FSH, 6.19 ± 2.94 vs. 5.42 ± 2.01, P=0.001) levels and lower antral follicle counts (AFCs, 15.03 ± 11.85 vs. 19.32 ± 15.29, P=0.024) than did those in the pregnant group. There were no significant differences in any endometrial or subendometrial ultrasound indicators on the day of ovulation between the nonpregnant group and the pregnant group.

## Characteristics and ultrasound parameters on the day of ovulation for patients with IUAs

For patients with IUAs, the nonpregnant group had a lower AFC (15.60 ± 17.07 vs. 21.74 ± 12.21, P<0.001), lower proportions of patients with Type B plus Type C endometrial morphology (66% (41/62) vs. 81% (91/113), P=0.034), an endometrial thickness ≥8 mm (68% (42/62) vs. 88% (99/113), P=0.001) and an endometrial volume ≥2 ml (55% (34/62) vs. 74% (84/113), P=0.008), and lower endometrial FI (27.92 ± 6.63 vs. 29.33 ± 5.75, P=0.041), subendometrial volume (9.06 ± 2.36 vs. 9.86 ± 2.30, P=0.046), subendometrial VI (5.32 ± 5.22 vs. 7.52 ± 6.95, P=0.031), and subendometrial VFI (1.71 ± 1.72 vs. 2.52 ± 2.42, P=0.023) values than those in the pregnant group. No significant differences in other ultrasound indicators were found.

## Characteristics and ultrasound parameters on the day of ovulation between patients with and without IUAs

Patients without IUAs generally had a longer duration of infertility (3.59 ± 2.35 years vs. 2.91 ± 1.88 years, P<0.001) and higher basal luteinizing hormone (LH) levels (5.34 ± 10.96 vs. 5.05 ± 6.02, P=0.014) than those with IUAs did; other basic characteristics, such as female age, BMI, number of oocyte retrieval cycles, AFC, FSH level, and anti-Müllerian hormone (AMH) level, were not significantly different between the two groups (P>0.05). In the comparison of the ER ultrasound parameters between the two groups, the proportions of patients with Type B plus Type C endometrial morphology (94% (392/417) vs. 75% (132/175), P<0.001), an endometrial thickness ≥8 mm (97% (405/417) vs. 81% (141/175), P<0.001), an endometrial volume ≥2 ml (94% (391/417) vs. 67% (118/175), P<0.001), a frequency of endometrial peristalsis ≥2 times/min (84% (349/417) vs. 53% (92/175), P<0.001), low subendometrial volume (11.54 ± 2.94 vs. 9.57 ± 2.35, P<0.001) and subendometrial VFI (2.70 ± 3.10 vs. 2.23 ± 2.23, P=0.033) values on the day of ovulation and a low live birth rate (65% (271/417) vs. 56% (98/175), P=0.039) were significantly higher in the group without IUAs than that in the group with IUAs.

For nonpregnant patients, the group without IUAs had a longer duration of infertility, a greater proportion of patients with Type B plus Type C endometrial morphology, greater proportions of patients with an endometrial thickness ≥8 mm, an endometrial volume ≥2 ml, a frequency of endometrial peristalsis ≥2 times/min, a subendometrial volume, a greater proportion of patients with type III endometrial blood flow, and greater subendometrial VFI and VI values than did the group with IUAs.

Compared with patients with IUAs, patients without IUAs also had a longer duration of infertility, a greater BMI, a greater basal LH level, a greater likelihood of having Type B plus Type C endometrial morphology, an endometrial thickness ≥8 mm, and an endometrial volume ≥2 ml, a greater frequency of endometrial peristalsis ≥2 times/min, a greater subendometrial volume, and a lower AFC.

## Characteristics and ultrasound parameters on the day of ovulation between patients with moderate-severe IUAs and patients with mild IUAs

There were no significant differences in any basic characteristics between patients with moderate-severe IUAs and patients with mild IUAs, but patients with moderate-severe IUAs had a lower likelihood of having an endometrial thickness ≥8 mm (73% (68/93) vs. 89% (73/82), P=0.008) and an endometrial volume ≥2 ml (58% (54/93) vs. 78% (64/82), P=0.005), a lower frequency of endometrial peristalsis ≥2 times/min (42% (39/93) vs. 65% (53/82), P=0.003), and lower subendometrial volume (9.22 ± 2.29 vs. 9.97 ± 2.36, P=0.023) and a subendometrial FI (31.48 ± 3.64 vs. 33.43 ± 4.17, P=0.002) values than did patients with mild IUAs (Table 2).

## Univariate and multivariate logistic regression analyses

Univariate and multivariate logistic regression analyses were used to obtain the ORs and 95% CIs of the independent risk factors contributing to clinical pregnancy. The results revealed that a high

TABLE 1 Comparisons of characteristics and ultrasound parameters on the day of ovulation.

| Variable | Non-IUAs | | | IUAs | | | p value | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 1 vs. 4 | 2 vs. 3 | 5 vs. 6 | 2 vs. 5 | 3 vs. 6 |
| | Overall | Nonpregnant group | Pregnant group | Overall | Nonpregnant group | Pregnant group | | | | | |
| Cycles | 417 | 116 | 301 | 175 | 62 | 113 | | | | | |
| **Baseline characteristics** | | | | | | | | | | | |
| Age (years) | 30.22 ± 3.17 | 30.19 ± 3.34 | 30.23 ± 3.12 | 30.34 ± 2.84 | 30.56 ± 2.72 | 30.22 ± 2.90 | 0.911 | 0.814 | 0.447 | 0.641 | 0.786 |
| Duration of infertility (years) | 3.59 ± 2.35 | 3.93 ± 2.91 | 3.47 ± 2.11 | 2.91 ± 1.88 | 2.84 ± 1.88 | 2.94 ± 1.89 | <0.001 | 0.368 | 0.693 | 0.012 | 0.017 |
| BMI (kg/m2) | 21.20 ± 1.71 | 21.00 ± 1.74 | 21.28 ± 1.69 | 20.94 ± 1.67 | 20.99 ± 1.49 | 20.91 ± 1.77 | 0.067 | 0.082 | 0.605 | 0.903 | 0.034 |
| Number of oocyte retrieval cycles | 1.14 ± 0.37 | 1.21 ± 0.43 | 1.11 ± 0.35 | 1.17 ± 0.42 | 1.18 ± 0.43 | 1.16 ± 0.41 | 0.555 | 0.011 | 0.737 | 0.535 | 0.301 |
| AFC | 18.18 ± 14.57 | 15.03 ± 11.85 | 19.32 ± 15.29 | 19.57 ± 14.38 | 15.60 ± 17.07 | 21.74 ± 12.21 | 0.190 | 0.024 | <0.001 | 0.476 | 0.022 |
| FSH | 5.62 ± 2.31 | 6.19 ± 2.94 | 5.42 ± 2.01 | 5.88 ± 2.03 | 6.08 ± 2.00 | 5.77 ± 2.05 | 0.098 | 0.001 | 0.327 | 0.935 | 0.075 |
| LH | 5.34 ± 10.96 | 6.25 ± 11.54 | 5.01 ± 10.75 | 5.05 ± 6.02 | 5.53 ± 7.26 | 4.79 ± 5.24 | 0.014 | 0.107 | 0.782 | 0.401 | 0.023 |
| AMH | 4.92 ± 3.78 | 4.88 ± 3.39 | 4.94 ± 3.92 | 5.14 ± 3.63 | 4.91 ± 3.97 | 5.26 ± 3.44 | 0.354 | 0.701 | 0.152 | 0.534 | 0.102 |
| **Ultrasound indicators on the day of ovulation** | | | | | | | | | | | |
| Endometrial morphology classification | | | | | | | <0.001 | 0.66 | 0.034 | <0.001 | <0.001 |
| Type A | 25 (6.0%) | 6 (5.2%) | 19 (6.3%) | 43 (25%) | 21 (34%) | 22 (19%) | | | | | |
| Type B+Type C | 392 (94%) | 110 (95%) | 282 (94%) | 132 (75%) | 41 (66%) | 91 (81%) | | | | | |
| Endometrial blood flow classification | | | | | | | 0.070 | 0.780 | 0.197 | 0.026 | 0.702 |
| I | 37 (8.9%) | 10 (8.6%) | 27 (9.0%) | 23 (13%) | 11 (18%) | 12 (11%) | | | | | |
| II | 330 (79%) | 90 (78%) | 240 (80%) | 140 (80%) | 49 (79%) | 91 (81%) | | | | | |
| III | 50 (12%) | 16 (14%) | 34 (11%) | 12 (6.9%) | 2 (3.2%) | 10 (8.8%) | | | | | |
| Endometrial thickness (mm) | 11.21 ± 2.08 | 11.29 ± 2.23 | 11.18 ± 2.02 | 9.53 ± 1.93 | 9.57 ± 2.41 | 9.50 ± 1.62 | <0.001 | 0.613 | 0.767 | <0.001 | <0.001 |
| <8 | 12 (2.9%) | 4 (3.4%) | 8 (2.7%) | 34 (19%) | 20 (32%) | 14 (12%) | | | | | |
| ≥8 | 405 (97%) | 112 (97%) | 293 (97%) | 141 (81%) | 42 (68%) | 99 (88%) | <0.001 | 0.745 | 0.001 | <0.001 | <0.001 |

*(Continued)*





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TABLE 1 Continued

| Variable | Non-IUAs | | | IUAs | | | p value | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | | | | | |
| | Overall | Nonpregnant group | Pregnant group | Overall | Nonpregnant group | Pregnant group | 1 vs. 4 | 2 vs. 3 | 5 vs. 6 | 2 vs. 5 | 3 vs. 6 |
| Ultrasound indicators on the day of ovulation | | | | | | | | | | | |
| Endometrial volume (ml) | 4.21 ± 1.72 | 4.11 ± 1.81 | 4.24 ± 1.69 | 2.89 ± 1.45 | 2.63 ± 1.41 | 3.03 ± 1.46 | <0.001 | 0.415 | 0.049 | <0.001 | <0.001 |
| <2 | 26 (6.2%) | 9 (7.8%) | 17 (5.6%) | 57 (33%) | 28 (45%) | 29 (26%) | | | | | |
| ≥2 | 391 (94%) | 107 (92%) | 284 (94%) | 118 (67%) | 34 (55%) | 84 (74%) | <0.001 | 0.424 | 0.008 | <0.001 | <0.001 |
| Frequency of endometrial peristalsis (time/min) | 2.85 ± 1.39 | 2.87 ± 1.48 | 2.84 ± 1.36 | 1.79 ± 1.42 | 1.57 ± 1.34 | 1.91 ± 1.46 | <0.001 | 0.767 | 0.127 | <0.001 | <0.001 |
| <2 | 67 (16%) | 21 (18%) | 46 (15%) | 83 (47%) | 35 (56%) | 48 (42%) | | | | | |
| ≥2 | 349 (84%) | 95 (82%) | 254 (85%) | 92 (53%) | 27 (44%) | 65 (58%) | <0.001 | 0.491 | 0.077 | <0.001 | <0.001 |
| Endometrial VI | 2.13 ± 2.96 | 2.19 ± 2.67 | 2.10 ± 3.06 | 1.95 ± 2.79 | 1.85 ± 2.28 | 2.00 ± 3.05 | 0.228 | 0.458 | 0.349 | 0.126 | 0.696 |
| Endometrial FI | 28.04 ± 6.71 | 28.86 ± 6.33 | 27.72 ± 6.83 | 28.83 ± 6.10 | 27.92 ± 6.63 | 29.33 ± 5.75 | 0.509 | 0.426 | 0.041 | 0.224 | 0.078 |
| Endometrial VFI | 0.81 ± 2.10 | 0.79 ± 1.19 | 0.81 ± 2.37 | 0.69 ± 1.19 | 0.69 ± 1.31 | 0.68 ± 1.12 | 0.460 | 0.296 | 0.251 | 0.122 | 0.855 |
| Subendometrial volume (ml) | 11.54 ± 2.94 | 11.30 ± 2.82 | 11.62 ± 2.99 | 9.57 ± 2.35 | 9.06 ± 2.36 | 9.86 ± 2.30 | <0.001 | 0.205 | 0.046 | <0.001 | <0.001 |
| Subendometrial VI | 7.47 ± 6.60 | 7.47 ± 5.79 | 7.48 ± 6.90 | 6.74 ± 6.46 | 5.32 ± 5.22 | 7.52 ± 6.95 | 0.056 | 0.55 | 0.031 | 0.005 | 0.759 |
| Subendometrial FI | 32.36 ± 4.76 | 32.84 ± 5.10 | 32.17 ± 4.61 | 32.39 ± 4.01 | 31.62 ± 4.17 | 32.81 ± 3.87 | 0.937 | 0.282 | 0.053 | 0.103 | 0.176 |
| Subendometrial VFI | 2.70 ± 3.10 | 2.57 ± 2.06 | 2.75 ± 3.42 | 2.23 ± 2.23 | 1.71 ± 1.72 | 2.52 ± 2.42 | 0.033 | 0.483 | 0.023 | 0.002 | 0.688 |
| Clinical pregnancy | | | | | | | 0.065 | | | | |
| No | 116 (28%) | | | 62 (35%) | | | | | | | |
| Yes | 301 (72%) | | | 113 (65%) | | | | | | | |
| Live birth | | | | | | | 0.039 | | | | |
| No | 146 (35%) | | | 77 (44%) | | | | | | | |
| Yes | 271 (65%) | | | 98 (56%) | | | | | | | |





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TABLE 2 Comparisons of characteristics and ultrasound parameters on the day of ovulation between the mild- and moderate-severe IUA groups.

| Variable | Overall | Moderate-to-severe IUAs | Mild IUAs | p value |
|---|---|---|---|---|
| Cycles | 175 | 93 | 82 | |
| **Baseline characteristics** | | | | |
| Age (years) | 30.34 ± 2.84 | 30.24 ± 2.89 | 30.47 ± 2.79 | 0.743 |
| Duration of infertility (years) | 2.91 ± 1.88 | 2.81 ± 1.72 | 3.01 ± 2.05 | 0.739 |
| BMI (kg/m2) | 20.94 ± 1.67 | 20.72 ± 1.52 | 21.20 ± 1.80 | 0.07 |
| Number of oocyte retrieval cycles | 1.17 ± 0.42 | 1.18 ± 0.44 | 1.15 ± 0.39 | 0.606 |
| AFC | 19.57 ± 14.38 | 17.62 ± 13.86 | 21.77 ± 14.73 | 0.057 |
| FSH | 5.88 ± 2.03 | 5.80 ± 2.13 | 5.98 ± 1.93 | 0.266 |
| LH | 5.05 ± 6.02 | 5.40 ± 7.42 | 4.66 ± 3.87 | 0.518 |
| AMH | 5.14 ± 3.63 | 4.82 ± 3.16 | 5.49 ± 4.08 | 0.446 |
| **Ultrasound indicators on the day of ovulation** | | | | |
| Endometrial morphology classification | | | | 0.958 |
| Type A | 43 (25%) | 23 (25%) | 20 (24%) | |
| Type B+Type C | 132 (75%) | 70 (75%) | 62 (76%) | |
| Endometrial blood flow classification | | | | 0.864 |
| I | 23 (13%) | 13 (14%) | 10 (12%) | |
| II | 140 (80%) | 73 (78%) | 67 (82%) | |
| III | 12 (6.9%) | 7 (7.5%) | 5 (6.1%) | |
| Endometrial thickness (mm) | 9.53 ± 1.93 | 9.07 ± 1.69 | 10.05 ± 2.07 | <0.001 |
| <8 | 34 (19%) | 25 (27%) | 9 (11%) | 0.008 |
| ≥8 | 141 (81%) | 68 (73%) | 73 (89%) | |
| Endometrial volume (ml) | 2.89 ± 1.45 | 2.59 ± 1.29 | 3.23 ± 1.56 | 0.002 |
| <2 | 57 (33%) | 39 (42%) | 18 (22%) | 0.005 |
| ≥2 | 118 (67%) | 54 (58%) | 64 (78%) | |
| Frequency of endometrial peristalsis (time/min) | 1.79 ± 1.42 | 1.43 ± 1.26 | 2.20 ± 1.49 | <0.001 |
| <2 | 83 (47%) | 54 (58%) | 29 (35%) | 0.003 |
| ≥2 | 92 (53%) | 39 (42%) | 53 (65%) | |
| Endometrial VI | 1.95 ± 2.79 | 2.13 ± 3.14 | 1.73 ± 2.34 | 0.311 |
| Endometrial FI | 28.83 ± 6.10 | 28.15 ± 6.37 | 29.60 ± 5.72 | 0.367 |
| Endometrial VFI | 0.69 ± 1.19 | 0.72 ± 1.29 | 0.65 ± 1.06 | 0.537 |
| Subendometrial volume (ml) | 9.57 ± 2.35 | 9.22 ± 2.29 | 9.97 ± 2.36 | 0.023 |
| Subendometrial VI | 6.74 ± 6.46 | 6.69 ± 6.19 | 6.79 ± 6.79 | 0.98 |
| Subendometrial FI | 32.39 ± 4.01 | 31.48 ± 3.64 | 33.43 ± 4.17 | 0.002 |
| Subendometrial VFI | 2.23 ± 2.23 | 2.14 ± 2.02 | 2.33 ± 2.45 | 0.842 |
| Clinical pregnancy | | | | 0.739 |
| No | 62 (35%) | 34 (37%) | 28 (34%) | |
| Yes | 113 (65%) | 59 (63%) | 54 (66%) | |

*(Continued)*

TABLE 2 Continued

| Variable | Overall | Moderate-to-severe IUAs | Mild IUAs | p value |
|---|---|---|---|---|
| Ultrasound indicators on the day of ovulation | | | | |
| Live birth | | | | 0.742 |
| No | 77 (44%) | 42 (45%) | 35 (43%) | |
| Yes | 98 (56%) | 51 (55%) | 47 (57%) | |

AFC, basal FSH level, and AMH level and an endometrial thickness ≥8 mm were independent predictors of clinical pregnancy after FET. Protective factors for clinical pregnancy after FET cycles include a greater AFC (aOR, 1.04; 95% CI, 1.02–1.06; P<0.001) and an endometrial thickness ≥8 mm on the day of ovulation (aOR, 2.31; 95% CI, 1.09–4.93; P=0.029); risk factors for clinical pregnancy after FET cycles include a greater basal FSH level (aOR, 0.90; 95% CI, 0.81–0.99; P=0.038) and a greater AMH level (aOR, 0.94; 95% CI, 0.88–1.00; P=0.044); and other endometrial and subendometrial ultrasound indicators on the day of ovulation and the presence of uterine adhesions do not affect the clinical pregnancy rate (Table 3).

## Discussion

In this study, we compared ER on the day of ovulation between patients with and without a history of IUAs and reported that the live birth rate was significantly higher in patients without IUAs than in patients with IUAs. Compared with patients without IUAs, patients with IUAs (regardless of pregnancy status) had a thinner endometrium, a lower likelihood of having Type B+C endometrial morphology, a smaller volume, a lower frequency of endometrial peristalsis, a smaller subendometrial volume, and less subendometrial blood perfusion. However, there was no significant difference in the live birth rate between patients with mild and moderate–severe adhesions. Compared with patients with moderate–severe adhesions, those with mild adhesions had greater endometrial thickness and volume, peristaltic frequency, subendometrial volume and subendometrial blood perfusion. A high AFC and an endometrial thickness ≥8 mm are protective factors for clinical pregnancy, while high levels of FSH and AMH are risk factors for clinical pregnancy.

IUA development is multifactorial with multiple predisposing and causal factors, which often cause subfertility or infertility (21). Currently, hysteroscopic adhesiolysis is widely considered the gold standard for the diagnosis and treatment of IUAs (22). TVS is a first-line diagnostic method for IUAs and a noninvasive modality for analyzing ER (23).

In this study, IUAs affected the endometrial thickness, volume, morphology, subendometrial blood perfusion and endometrial peristalsis. Some previous studies have suggested that the "triple line sign" on the day of ovulation is related to clinical pregnancy and that adhesion disrupts the morphology of the endometrium (24, 25); thus, the proportion of patients with the triple line sign also decreases significantly (Types B+C). The live birth rate of patients with IUAs was lower, which was consistent with the results of previous studies (26, 27), but there was no significant difference in the clinical pregnancy rates, indicating that IUAs may have little effect on embryo implantation but may impair the ability to tolerate pregnancy in the long term.

Notably, there was no significant difference in the ultrasound parameters of patients without IUAs, regardless of pregnancy status. Among patients with IUAs, pregnant patients had significantly better endometrial ultrasound indicators (thickness, volume, blood perfusion) than nonpregnant patients did. However, there was no significant difference in peristaltic waves between pregnant and nonpregnant patients with adhesions, which is likely due to adhesion affecting the flexibility of endometrial peristalsis. However, we observed that there was no significant difference in endometrial blood perfusion between patients with adhesions and those without adhesions. This may be because, in this study, relatively few patients had moderate–severe adhesions, and most of these patients had moderate adhesions; thus, there was no significant difference in blood perfusion between patients with mild and moderate–severe adhesions.

Studies generally suggest that moderate to severe IUAs have a greater impact on pregnancy outcomes (27, 28). Although the ER indicators of moderate–severe adhesions were better than those of mild adhesions in this study, there was no significant difference in the live birth rate or clinical pregnancy rate between these two groups. This may be related to the high proportion of moderate adhesions, or it may indicate that patients with adhesions can achieve satisfactory pregnancy outcomes with proper treatment before transfer.

When ovarian reserve function and endometrial development are good, the probability of clinical pregnancy is relatively high. Similarly, decreased ovarian function (elevated FSH) is a risk factor for clinical pregnancy, which is consistent with previous research results (29, 30). Notably, the results of this study indicate that a high AMH level is a risk factor for clinical pregnancy.

The present work has several significant strengths. First, this was the first prospective study comparing ER on the day of ovulation during natural cycles between patients with and without a history of IUAs. Second, the degree of influence of mild and moderate–severe adhesions was also analyzed. However, several limitations remain. First, this study analyzed only the ER status of the endometrium on the day of ovulation in natural FET cycles, and the impact of adhesions on other populations and nonovulation days remains to be verified. Second, among patients

TABLE 3 Univariate and multivariate logistic regression analyses of risk factors contributing to clinical pregnancy.

| Characteristic | Univariate Logistic Regression | | | | | Multivariate Logistic Regression | | | |
|---|---|---|---|---|---|---|---|---|---|
| | N | Event N | OR | 95% CI | p value | Event N | OR | 95% CI | p value |
| Age (years) | 584 | 413 | 0.99 | 0.93, 1.05 | 0.710 | 388 | 1.04 | 0.97, 1.12 | 0.220 |
| Duration of infertility (years) | 572 | 402 | 0.96 | 0.89, 1.04 | 0.330 | 388 | 0.92 | 0.84, 1.00 | 0.053 |
| BMI | 584 | 413 | 1.07 | 0.96, 1.19 | 0.240 | 388 | 1.06 | 0.95, 1.19 | 0.320 |
| Cycles | 585 | 414 | 0.63 | 0.41, 0.98 | 0.040 | 388 | 0.69 | 0.42, 1.15 | 0.150 |
| AFC | 585 | 414 | 1.03 | 1.01, 1.04 | <0.001 | 388 | 1.04 | 1.02, 1.06 | <0.001 |
| FSH | 574 | 407 | 0.88 | 0.80, 0.95 | 0.004 | 388 | 0.90 | 0.81, 0.99 | 0.038 |
| LH | 575 | 407 | 0.99 | 0.97, 1.01 | 0.260 | 388 | 1.00 | 0.98, 1.02 | 0.730 |
| AMH | 572 | 405 | 1.01 | 0.96, 1.06 | 0.700 | 388 | 0.94 | 0.88, 1.00 | 0.044 |
| Endometrial morphology classification | 592 | 414 | | | | 388 | | | |
| Type A | | | — | — | | | — | — | |
| Type B+Type C | | | 1.63 | 0.96, 2.73 | 0.067 | | 1.27 | 0.66, 2.42 | 0.460 |
| Endometrial blood flow classification | 592 | 414 | | | | 388 | | | |
| I | | | — | — | | | — | — | |
| II | | | 1.28 | 0.72, 2.24 | 0.390 | | 1.03 | 0.51, 2.02 | 0.940 |
| III | | | 1.32 | 0.61, 2.84 | 0.480 | | 0.98 | 0.37, 2.58 | 0.970 |
| Endometrial thickness (mm) | 592 | 414 | | | | 388 | | | |
| <8 | | | — | — | | | — | — | |
| ≥8 | | | 2.78 | 1.51, 5.13 | <0.001 | | 2.31 | 1.09, 4.93 | 0.029 |
| Endometrial volume (ml) | 592 | 414 | | | | 388 | | | |
| <2 | | | — | — | | | — | — | |
| ≥2 | | | 2.10 | 1.30, 3.37 | 0.002 | | 1.25 | 0.62, 2.51 | 0.530 |
| Frequency of endometrial peristalsis (times/min) | 591 | 413 | | | | 388 | | | |
| <2 | | | — | — | | | — | — | |
| ≥2 | | | 1.56 | 1.05, 2.30 | 0.026 | | 1.08 | 0.66, 1.76 | 0.760 |
| VI | 588 | 410 | 1.00 | 0.94, 1.07 | >0.990 | 388 | 0.97 | 0.87, 1.08 | 0.540 |
| FI | 588 | 410 | 0.99 | 0.96, 1.02 | 0.530 | 388 | 0.99 | 0.95, 1.03 | 0.730 |
| VFI | 588 | 410 | 1.01 | 0.92, 1.13 | 0.920 | 388 | 0.99 | 0.86, 1.21 | 0.900 |
| Subendometrial volume (ml) | 592 | 414 | 1.08 | 1.01, 1.15 | 0.019 | 388 | 1.02 | 0.93, 1.11 | 0.700 |
| Subendometrial VI | 588 | 410 | 1.02 | 0.99, 1.05 | 0.200 | 388 | 1.01 | 0.88, 1.09 | 0.790 |
| Subendometrial FI | 588 | 410 | 1.00 | 0.96, 1.04 | 0.870 | 388 | 0.97 | 0.91, 1.02 | 0.240 |
| Subendometrial VFI | 588 | 410 | 1.06 | 0.99, 1.15 | 0.110 | 388 | 1.10 | 0.95, 1.64 | 0.380 |
| group | 592 | 414 | | | | 388 | | | |
| Non-IUAs | | | — | — | | | — | — | |
| IUAs | | | 0.70 | 0.48, 1.03 | 0.066 | | 0.89 | 0.55, 1.44 | 0.630 |

OR, odds Ratio; CI, confidence Interval; BMI, body mass index; AFC, antral follicle count; FSH, follicle-stimulating hormone; LH, luteinizing hormone; AMH, anti-Müllerian hormone; VI, vascularization index; FI, flow index; VFI, vascularization-flow index; IUA, intrauterine adhesion.

with moderate-severe IUAs, the proportion of those with severe adhesions is relatively small, which may have a certain impact on the observation results. Third, no comparison of ER was made preadhesiolysis and postadhesiolysis.

In conclusion, IUAs can affect ER on the day of ovulation and the live birth rate during natural cycles. Moderate–severe IUAs have a greater impact on ER than mild IUAs do; however, if these adhesions are treated properly, they do not have adverse effects on

the clinical pregnancy rate. A high AFC and an endometrial thickness ≥8 mm are protective factors for clinical pregnancy, whereas high FSH and AMH levels are risk factors for clinical pregnancy. However, the conclusions and ideas of such reviews need to be validated in other populations, as well as in studies with larger sample sizes and longer follow-up periods.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

## Ethics statement

The studies involving humans were approved by The Ethics Committee of the Reproductive and Genetic Hospital of CITIC-Xiangya. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

## Author contributions

YO: Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing. YP: Formal analysis, Methodology, Writing – original draft, Writing – review & editing. MZ: Data curation, Writing – review & editing. YM: Data curation, Writing – review & editing. FG: Data curation, Funding acquisition, Writing – review & editing. YL: Data curation, Writing – review & editing. HC: Data curation, Writing – review & editing. XL: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Sleep quality during pregnancy following assisted reproductive technology and natural conceiving: a prospective birth cohort study

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**Purpose:** To investigate the association of sleep quality during pregnancy on *in vitro* fertilization/intra-cytoplasmic sperm injection (IVF/ICSI) and natural conceiving (NC), as well as anxiety, depression, and perceived stress.

**Methods:** This prospective cohort study includes 500 infertile pregnant women undergoing IVF/ICSI and 678 NC women in a Sichuan birth cohort. Data on sleep, anxiety, depression, and stress was collected in the first trimester (T1), second trimester (T2), and third trimester (T3) using integrated questionnaires. Sleep quality is quantified by the Pittsburgh Sleep Quality Index (PSQI) with a cut-point of 5 indicating poor sleep. The Self-rating Anxiety Scale (SAS), the Center for Epidemiologic Study of Depression scale (CES-D), and the Perceived Stress scale (PSS) were used for assessing anxiety, depression, and perceived stress symptoms. Additionally, the matched husbands are surveyed. Multivariable logistic regression models with adjustments for influencing factors were used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for the associations of sleep quality.

**Results:** In the IVF/ICSI group, 61.1%, 55.5%, and 66.5% of participants in T1, T2, and T3 reported poorer sleep quality compared to the NC group, which had 43.2%, 37.4%, and 46.4% throughout the same trimesters. Additionally, the IVF/ICSI group exhibited higher levels of negative psychological factors as measured by the CES-D and PSS during T1 and T2, showing statistical significance in T1 ($P = 0.008$, $P < 0.001$) and T2 ($P = 0.038$, $P < 0.001$), except at T3 ($P = 0.107$, $P = 0.253$). In addition to psychological factors and IVF/ICSI treatment, poor sleep quality was also associated with advanced age. However, there was no significant difference in sleep quality between the husbands of the IVF/ICSI and NC groups.

**Conclusion:** The study reveals that women receiving IVF/ICSI treatment are at a higher risk of experiencing sleep disturbances throughout their pregnancy compared with women with natural conception. While partners typically do not report major sleep problems, they do exhibit increased anxiety levels. These findings underscore the necessity for screening and addressing sleep issues in women pregnant through assisted IVF/ICSI treatment, to promote their well-being during this critical period.

KEYWORDS

**sleep quality, anxiety, depression, perceived stress, assisted reproductive technology, natural conceiving, pregnancy**

## Introduction

Sleep is a vital physiological process essential for maintaining both physical and mental health, with individuals spending approximately one-third of their lifespan asleep. During pregnancy, ensuring adequate and restorative sleep is crucial not only for the mother's well-being but also for the optimal development of the fetus. The National Sleep Foundation recommends that adults should aim for a sleep duration of seven to nine hours per night (1). A comprehensive review by Maniaci et al. highlights that conditions, like obstructive sleep apnea during pregnancy can affect maternal and fetal health, emphasizing the broader implications of sleep disturbances for pregnant women (2). These issues could negatively impact both maternal and fetal outcomes, potentially leading to complications such as preeclampsia, hypertension, gestational diabetes, cesarean deliveries, and excessive weight gain during pregnancy. However, specific guidelines for sleep duration, quality, and insomnia during pregnancy remained insufficient. Furthermore, sleep quality and disturbances are often linked to significant determinants of women's health, particularly during critical life stages such as menstruation, pregnancy, and menopause. For instance, sleep disturbances are often associated with higher levels of depression and stress, as well as an increased risk of cardiovascular disease, hypertension, and diabetes (3). Furthermore, poor sleep quality and the prevalence of depression tend to be escalated throughout pregnancy (4). These issues could adversely affect both maternal and fetal outcomes, potentially leading to complications, including preeclampsia, hypertension, gestational diabetes, cesarean deliveries, and excessive weight gain during pregnancy (3, 5–7).

Animal studies have demonstrated that persistently disrupted light/dark cycles could significantly impair the preovulatory LH surge, leading to reduced fertility. In contrast, the reproductive effects of a single-phase shift on female mice could be minimized (8). Furthermore, sleep deprivation had been shown to diminish oocyte output, as transient activation of wake-promoting dopaminergic neurons negatively impacts reproductive performance (9). Sleep disorders were notably prevalent undergoing assisted reproductive technology (ART) treatments, and affected 57% of women undergoing *in vitro* fertilization/intra-cytoplasmic sperm injection (IVF/ICSI) before treatment, 43% during the stimulation phase, and 29% following embryo transfer (10). Philipsen et al. found that mean PSQI global scores before treatment were 8.1, with 91% of participants having PSQI scores > 5, indicating poor sleep quality (11). Research indicated that insufficient nocturnal sleep—specifically, less than seven hours per night with disrupted sleep patterns, is associated with reduced oocyte and embryo production. Conversely, excessively prolonged nocturnal sleep might decrease the likelihood of achieving a successful pregnancy. Additionally, the relationship between nocturnal sleep duration and ART outcomes is influenced by factors such as subjective sleep quality and maternal age (12).

Strong correlations have been established between sleep quality and both physical and mental well-being (13). The interplay between negative emotions and sleep difficulties can create a harmful cycle. Given that the high prevalence of sleep disorders among expectant mothers and their significant association with psychological symptoms, it is crucial to prioritize screening and counseling for psychological disorders in pregnant women to enhance sleep quality (4). Previous research also indicated that there are small but significant associations between stress and distress, which can further diminish the likelihood of successful pregnancies through ART (14). Moreover, the rates of anxiety and depression fluctuate depending on the stage of IVF/ICSI treatment. While many studies had examined women's psychological health before and after IVF (15–17), few had focused on sleep quality and the psychological factors influencing sleep among IVF/ICSI patients during pregnancy. As a result, the relationship between sleep during pregnancy and positive psychological aspects remained poorly understood.

The existing body of research has largely focused on assessing sleep quality in individuals undergoing ART treatments. However, there is a notable gap in studies that investigate the impact of sleep quality among IVF/ICSI patients during pregnancy, particularly in comparison to women, who conceive naturally. In this study, we aim to gather data on sleep characteristics among women undergoing IVF/

ICSI and those achieving natural conception. We hypothesize that both negative and positive psychological factors play a significant role in influencing sleep quality.

# Materials and methods

## Participants and study design

In this prospective cohort study, we recruited a convenience sample of 557 females who underwent ART treatment and 691 females who were natural conception and delivered at West China Second University Hospital of Sichuan University between January 2016 and July 2018. All couples included in the study were not involved in any type of prospective interventional trials. All protocols were approved by the Medical Science Ethics Board of West China Second University Hospital of Sichuan University.

## Data collection

First, each participant completed a questionnaire on demographic information and at least once of questionnaire. In this prospectively longitudinal study, data was collected in the first trimester (T1, before 12 weeks of gestation), second trimester (T2, between 13-28 weeks of gestation), and third trimester (T3, after 28 weeks of gestation) using integrated questionnaires. All the subjects were fully informed of the procedures, and their written informed consent and approval were obtained. Husbands for the matched couple were questioned once during pregnancy.

Due to incomplete questionnaire data, 57 participants from the Assisted Reproductive Technology (ART) group and 13 from the naturally conceived (NC) group were excluded from the study as they did not respond to any questionnaires. As a result, the final analysis included 500 individuals from the IVF/ICSI group and 678 from the NC group. Simultaneously, the study encompassed 498 partners of women who conceived through IVF/ICSI and 621 partners of women who conceived naturally. A detailed inclusion and exclusion flow chart was shown in Figure 1.

## Pittsburgh sleep quality index

Sleep quality during pregnancy was assessed by the Pittsburgh Sleep Quality Index (PSQI), a self-reported 19-item questionnaire (18, 19). The PSQI global score, which consists of seven component scores: subjective sleep quality, subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medication, and daytime dysfunction, was used to evaluate sleep quality. Each component score was assigned a value of 0-3 points. The PSQI global score varied from 0 to 21, with higher scores suggesting worse sleep quality. In this study, sleep quality was classified into good (score ≤ 5) and poor (score> 5) according to the global PSQI scores (19). Among the poor quality, women with scores from 6 to 10 were considered fairly good, from 11 to 15 as fairly bad, and above 15 as bad sleep.

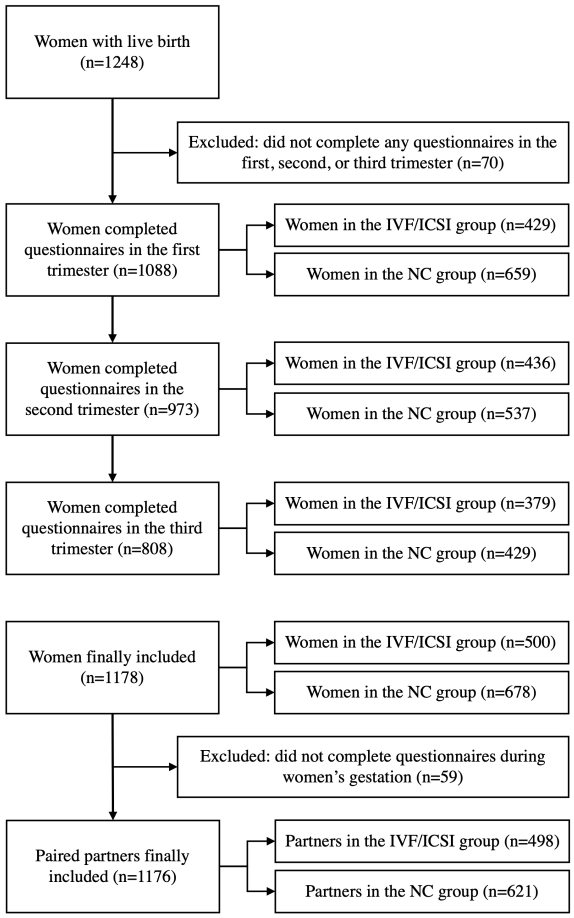


FIGURE 1
Participants flow chart.

## Self-rating anxiety scale

Anxiety was measured with the Zung's Self-Rating Anxiety Scale (SAS) (20). The scale includes 20 items that cover 4 groups of manifestations: cognitive, autonomic, motor, and central nervous system symptoms. Each item is scored on a Likert-type scale of 1-4 (1 = none or a little of the time, 2 = some of the time, 3= good part of the time, 4 = most or all of the time). Anxiety standard scores ≥ 50 were considered to be at risk for clinical anxiety. Among the anxiety group, women with scores from 50 to 60 were considered as mild anxiety, from 60 to 70 as middle anxiety, and above 70 as severe anxiety (21).

## Center for epidemiologic study of depression scale

Depression was assessed using the Center for Epidemiologic Study of Depression Scale (CES-D). The CES-D consists of 20 items which are rated using a 4-point ordered response set to indicate how frequently symptoms were experienced during the previous week (0 = rarely or none of the time, 1= some or a little of the time, 2 = occasionally or a moderate amount of the time, 3 = most or all of the

time). Total score of CES-D was generated by summing their item responses and ranging from 0 to 60 (higher scores indicating more depressive symptoms). Participants with CES-D scores ≥16 were considered at risk for clinical depression (22).

## Perceived stress scale

Perceived stress was assessed with the Perceived Stress Scale (PSS-10) (23), which consists of 10 items purported to measure the degree of nonspecific appraised stress in the past month on a 5-point Likert scale (0 = never, 1 = almost never, 2= sometimes, 3 = fairly often, 4 = very often). The total PSS score ranged from 0 to 40, The stress level increases depending on the increase in score.

## Statistical analysis

SPSS 25.0 (IBM, Armonk, NY, USA) was used to analyze the study data. Baseline characteristics were summarized as mean (standard deviation, SD) for continuous variables, and frequencies (percentages) for categorical variables. Differences in these characteristics between the three nighttime sleep duration groups were compared by analysis of variance (for continuous variables) and *Chi*-square tests (for categorical variables). Nominal variables were tested either with the *Chi*-square test or Fisher's exact test. Odds ratios (ORs) and their 95% confidence intervals (CIs) were estimated through multivariable logistic regression models to evaluate the influencing factors of the sleep quality of participants. The P-value of $< 0.05$ was considered statistically significant.

# Results

## Baseline characteristics and obstetric characteristics

A total of 1,178 cases of women were included in the final analysis of this study, with 500 participants in the IVF/ICSI group, with 429, 436, and 379 women in T1, T2 and T3 of pregnancy. The average( ± SD) age and pre-BMI were 32.24 ± 3.28 and 21.19 ± 2.47. Most participants were of Han ethnicity (97%). In the NC group, 678 women were included, with 659, 537, and 429 women in T1, T2 and T3 of pregnancy. The average( ± SD) age and pre-BMI were 30.67 ± 3.41 and 20.76 ± 2.78. Most participants were of Han ethnicity (99.26%). The basic characteristics of the patients are presented in Table 1. The rate of twin pregnancies varied significantly between the groups, with 20.6% of the IVF/ICSI-treated women and 0.88% of the NC women having twins. Furthermore, we observed that the incidence of preterm birth was 15.8% in the IVF/ICSI group compared to 4.9% in the naturally conceiving group. Meanwhile, the prevalence of intrahepatic cholestasis of pregnancy, gestational hypertension, and hypothyroidism was notably higher among women undergoing IVF/ICSI compared to those in the NC group. Additionally, the occurrence of low-birth-weight infants was significantly higher among the IVF/ICSI group than in those conceived naturally (Table 1).

## Sleep quality, anxiety, depression, and perceived stress in different trimesters in pregnant women

In T1, 429 women from the IVF/ICSI group, and 659 women from the NC group completed the questionnaire. The median global PSQI scores were 7 for the IVF/ICSI group and 5 for the NC group ($P< 0.001$). During T2, 436 women in the IVF/ICSI group participated in the survey, with537 women in the NC group. The median PSQI scores for this trimester were 6 points for the IVF/ICSI group and 5 points for the NC group ($p < 0.001$). In T3, 379 women from the IVF/ICSI group answered the questionnaire, compared to 429 women from the NC group. The PSQI scores were presented as a median of 7 points in the IVF/ICSI group and 5 points in the NC group ($p < 0.001$). Overall, when compared to the NC group during pregnancy, women undergoing ART exhibited significantly poorer sleep quality.

Pregnant women undergoing IVF/ICSI showed similar median scores on SAS throughout their pregnancy compared to those who conceived naturally (Table 2). Specifically, the scores were 39.79 for the IVF/ICSI group versus 39.91 for the NC group in T1 ($P = 0.827$), 38.52 versus 38.77 points in T2 ($P = 0.874$), and 40.01 versus 40.07 points in T3 ($P = 0.689$).

In contrast, the median scores for CES-D and PSS in the IVF/ICSI group were significantly lower than those in the NC group during T1 and T2. Specifically, the CES-D scores were 13.74 for the IVF/ICSI group compared to 15.11 for the NC group in T1 ($P = 0.008$) and 11.88 versus 13.1 in T2 ($P = 0.038$). For the PSS, the scores were 12.28 for the IVF/ICSI group versus 14.02 in T1 ($P < 0.0001$) and 11.51 compared to 12.56 in T2 ($P < 0.001$). However, in T3, the median scores of CES-D and PSS for both groups were similar across groups: CES-D was 12.63 versus 13.77 ($P = 0.107$) and PSS was 11.93 versus 12.29 ($P = 0.253$) (Table 2).

## The relationship between sleep quality and trimesters

In the IVF/ICSI group, 38.90% (167 out of 429) of women reported experiencing good sleep quality during T1, which declined to 33.5% (127 out of 379) in T3. Both of these figures were lower than the 44.5% (194 out of 436) who reported good sleep quality in T2. Similarly, in the NC group, the second trimester also showed the highest proportion of reported good sleep quality. Throughout their pregnancies, only a small number of women in the NC group reported poor sleep quality. In contrast, 17.7% (67 out of 379) of women in the IVF/ICSI group reported poor sleep quality in the third trimester, a significant increase from the 9.1% (39 out of 429) reported in T1 and 8.5% (37 out of 436) in T2. Overall, the second trimester was associated with the highest T1quality of sleep for both groups throughout the entire pregnancy (Figure 2).

We further studied the relationship between demographics and fertility characteristics, psychological characteristics, and sleep quality. A logistic stepwise regression model was

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TABLE 1 Demographic characteristic of obstetrics outcome between the IVF/ICSI and NC group.

| Characteristic | IVF/ICSI group (n=500) | NC group (n=678) | P value |
|---|---|---|---|
| Female age | 32.24 ± 3.28 | 30.67 ± 3.41 | <0.001 |
| Male age | 34.10 ± 4.18 | 32.21 ± 4.55 | <0.001 |
| Pre-BMI, kg/m2 | 21.19 ± 2.47 | 20.76 ± 2.78 | 0.006 |
| BMI at delivery | 26.29 ± 2.78 | 26.68 ± 11.43 | 0.456 |
| Weight gain during gestation | 13.13 ± 4.66 | 13.56 ± 4.39 | 0.107 |
| Education level | | | 0.000 |
| Middle school or less | 4.60% (23/500) | 5.16% (35/678) | |
| High school | 7.00% (35/500) | 2.95% (20/678) | |
| Graduate or higher | 88.40% (442/500) | 95.42% (647/678) | |
| Ethics | | | 0.003 |
| Han | 97% (485/500) | 99.26% (673/678) | |
| Minority | 3% (15/500) | 7.37% (5/678) | |
| Preconception Disease History | | | |
| PCOS | | | |
| Yes | 4.2% (21/500) | 1.90% (13/678) | 0.023 |
| No | 5.8% (479/500) | 98.10% (7/678) | |
| EMS | | | |
| Yes | 2.8% (14/500) | 1.00% (7/678) | 0.027 |
| No | 97.2% (479/500) | 99.00% (/678) | |
| Obstetrical outcomes | | | |
| Gestational Hypertension | 9.40% (47/500) | 3.98% (27/678) | <0.001 |
| Gestational Diabetes Mellitus | 18.80% (94/500) | 16.52% (112/678) | 0.301 |
| Intrahepatic Cholestasis of Pregnancy | 7.00% (35/500) | 3.10% (21/678) | 0.002 |
| Placental Abruption | 1.40% (7/500) | 0.88% (6/678) | 0.287 |
| Placental Previa | 4.00% (20/500) | 1.33% (9/678) | 0.237 |
| Oligohydramnios | 3.00% (15/500) | 2.51% (17/678) | 0.365 |
| Intrauterine growth restriction | 0.60% (3/500) | 2.65% (18/678) | 0.505 |
| Macrosomia | 2.00% (10/500) | 2.65% (18/678) | 0.301 |
| Low birth weight infants | 4.00% (20/500) | 1.33% (9/678) | 0.003 |
| Multiple pregnancy | 20.60% (103/500) | 0.88% (6/678) | <0.001 |
| Preterm birth | 15.8% (79/500) | 4.9% (33/678) | <0.001 |

constructed for sleep quality based on female age, pre-BMI, ethnicity, educational level, conception methods (IVF/ICSI or NC group), number of pregnancies, prior diseases (polycystic ovary syndrome and endometriosis), anxiety degree, depressive symptoms, and PPS. We found that female age, conception methods, anxiety degree, depressive symptoms, and PPS affect the sleep quality of pregnant women during the whole gestation (Table 3).

## Sleep quality, anxiety, depression, and perceived stress in paired partners

In the study, 498 matched partners from the IVF/ICSI group and 621 partners from the NC group were included. The male partners in the IVF/ICSI group exhibited slightly higher SAS scores compared to those in the NC group (P<0.001), indicating a potential increase in anxiety levels. However, there was no

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TABLE 2 Sleep quality, anxiety, depression, and stress in different trimesters between the IVF/ICSI and NC group.

| Psychological Characteristics | T1 | | P value | T2 | | P value | T3 | | P value |
|---|---|---|---|---|---|---|---|---|---|
| | IVF/ ICSI group (n=429) | NC group (n=659) | | IVF/ ICSI group (n=436) | NC group (n=537) | | IVF/ ICSI group (n=379) | NC group (n=429) | |
| **PSQI** | 7 (4, 10) | 5 (4, 7) | <0.001 | 6 (4, 10) | 5 (3, 7) | <0.001 | 7 (5, 13) | 5 (4, 7) | <0.001 |
| **Sleep quality** | | | <0.001 | | | <0.001 | | | <0.001 |
| Good | 38.93% (167/429) | 56.75% (374/659) | | 44.50% (194/436) | 62.57% (336/537) | | 33.51% (127/379) | 53.61% (230/429) | |
| Fairly good | 38.69% (166/429) | 38.39% (253/659) | | 33.03% (144/436) | 33.89% (182/537) | | 34.04% (129/379) | 40.09% (172/429) | |
| Fairly bad | 13.29% (57/429) | 4.70% (31/659) | | 13.99% (61/436) | 3.17% (17/537) | | 14.78% (56/379) | 6.29% (27/429) | |
| Bad | 9.09% (39/429) | 0.15% (1/659) | | 8.49% (37/436) | 0.37% (2/537) | | 17.68% (67/379) | 0.00% (0/429) | |
| **SAS** | 40 (35, 45) | 40 (35, 45) | 0.827 | 38 (33, 42) | 38 (33, 43) | 0.874 | 40 (35, 44.5) | 40 (33, 45) | 0.689 |
| **Anxiety degree** | | | 0.347 | | | 0.865 | | | 0.024 |
| Normal | 89.98% (386/429) | 89.38% (589/659) | | 92.20% (402/436) | 91.43% (491/537) | | 88.65% (336/379) | 87.41% (375/429) | |
| Mild anxiety | 9.56% (41/429) | 9.26% (61/659) | | 6.65% (29/436) | 7.08% (38/537) | | 11.08% (42/379) | 10.02% (43/429) | |
| Middle anxiety | 0.47% (2/429) | 1.37% (9/659) | | 1.15% (5/436) | 1.49% (8/537) | | 0.26% (1/379) | 2.56% (11/429) | |
| **CES-D** | 13 (8, 19) | 15 (9, 20) | 0.008 | 11 (6, 17) | 12 (6, 19) | 0.038 | 12 (6, 18) | 13 (6, 20) | 0.107 |
| **Depressive symptoms** | | | 0.005 | | | 0.003 | | | 0.002 |
| No | 62.94% (270/429) | 54.78% (361/659) | | 71.10% (310/436) | 62.38% (335/537) | | 69.39% (263/379) | 59.67% (256/429) | |
| Yes | 37.06% (159/429) | 45.22% (298/659) | | 28.90% (126/436) | 37.62% (202/537) | | 30.61% (116/379) | 40.33% (173/429) | |
| **PSS** | 13 (9, 16) | 14 (11, 18) | <0.001 | 11 (8, 15) | 13 (9, 17) | <0.001 | 12 (8, 16) | 12 (9, 16) | 0.253 |

T1, the first trimester; T2, the second trimester; T3, the third trimester; PSQI, Pittsburgh Sleep Quality Index; SAS, Self-Rating Anxiety Scale; CES-D, Center for Epidemiologic Study of Depression Scale; PSS, Perceived Stress Scale.

significant differences in sleep quality, depression levels, or stress perception of the male partners in the IVF/ICSI and NC groups (Table 4).

## Discussion

In this prospective cohort study, our findings reveal that women in the IVF/ICSI group consistently report significantly poorer sleep quality throughout all three trimesters compared to naturally conceiving women. To our knowledge, this study is the first to provide a comprehensive assessment of sleep quality, anxiety, depression, and perceived stress across different stages of pregnancy in both IVF/ICSI and naturally conceiving individuals. In the stratified analysis, we discovered that the association of sleep quality during the gestation during gestation was specifically influenced by maternal age, conception methods, anxiety status, depressive symptoms, and perceived stress. To the best of our knowledge, this is the first prospective study to evaluate sleep quality differences between the IVF/ICSI and NC groups during pregnancy.

The study revealed that a troubling trend in sleep quality among women undergoing IVF/ICSI during pregnancy. Only 38.93% reported good sleep quality in T1, decreasing to 33.51% in T3, though there was a slight improvement to 44.5% in T2. These findings are significant, as they aligned with previous research

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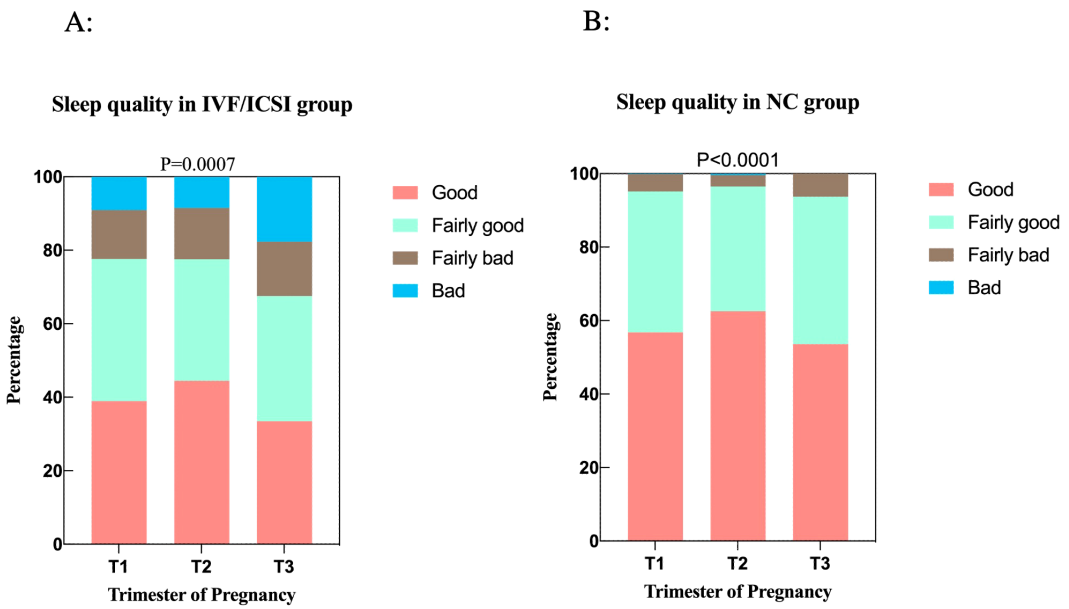


FIGURE 2
The relationship between sleep quality and trimesters. **(A)** Sleep quality in the IVF/ICSI group among three trimesters. **(B)** Sleep quality in the NC group among three trimesters.

TABLE 3 Logistic regression analysis of sleep quality during pregnancy.

| Characteristics | T1 | | | T2 | | | T3 | | |
|---|---|---|---|---|---|---|---|---|---|
| | OR | 95%CI | P Value | OR | 95%CI | P Value | OR | 95%CI | P Value |
| Age (year) | 0.954 | 0.917-0.991 | 0.016 | 0.939 | 0.901-0.979 | 0.003 | 0.912 | 0.869-0.957 | <0.001 |
| Pre-BMI (kg/m2) | 0.997 | 0.949-1.047 | 0.905 | 1.008 | 0.955-1.063 | 0.775 | 1.016 | 0.956-1.079 | 0.615 |
| Ethnicity | 0.827 | 0.929-2.289 | 0.715 | 0.227 | 0.062-0.836 | 0.026 | 0.431 | 0.128-1.448 | 0.173 |
| Educational Level | 0.844 | 0.592-1.205 | 0.351 | 0.931 | 0.652-1.329 | 0.693 | 0.743 | 0.499-1.106 | 0.143 |
| Conception method | | | | | | | | | |
| Natural conceiving | 1 | | | 1 | | | 1 | | |
| IVF/ICSI | 2.345 | 1.745-3.152 | <0.001 | 2.451 | 1.809-3.321 | <0.001 | 2.605 | 1.851-3.667 | <0.001 |
| Multiple Pregnancy | 0.918 | 0.566-2.489 | 0.729 | 0.631 | 0.391-1.016 | 0.058 | 0.85 | 0.49-1.474 | 0.563 |
| Prior PCOS | 1.16 | 0.54-2.492 | 0.704 | 1.694 | 0.782-3.672 | 0.181 | 0.822 | 0.3-2.255 | 0.703 |
| Prior EMS | 0.809 | 0.273-2.392 | 0.701 | 0.662 | 0.252-1.734 | 0.401 | 0.479 | 0.169-1.36 | 0.167 |
| Anxiety degree | | | | | | | | | |
| Normal | 1 | | | 1 | | | 1 | | |
| Mild Anxiety | 0.456 | 0.272-0.763 | 0.003 | 0.631 | 0.352-1.133 | 0.123 | 0.655 | 0.355-1.208 | 0.175 |
| Middle anxiety | 0.193 | 0.0023-1.584 | 0.126 | 0.125 | 0.016-0.999 | 0.05 | 0 | 0 | 0.999 |
| Depressive Symptoms | | | | | | | | | |
| Yes | 1 | | | 1 | | | 1 | | |
| No | 1.891 | 1.402-2.55 | <0.001 | 1.884 | 1.336-2.656 | <0.001 | 1.352 | 0.899-2.033 | 0.148 |
| PSS | 0.957 | 0.929-0.985 | 0.003 | 0.95 | 0.921-0.98 | 0.001 | 0.903 | 0.872-0.936 | <0.001 |

T1, the first trimester; T2, the second trimester; T3, the third trimester; BMI, Body Mass Index; PSS, Perceived Stress Scale; PCOS, Polycystic Ovary Syndrome; EMS, Endometriosis; IVF/ICSI, *In vitro* Fertilization/Intracytoplasmic Sperm Injection.

TABLE 4 Sleep quality, anxiety, depression, and stress in paired partner.

| Psychological Characteristics | IVF/ICSI group (n=498) | NC group (n=621) | P value |
|---|---|---|---|
| **PSQI** | 4 (2,6) | 4 (2,6) | 0.349 |
| **SAS** | 36 (31,40) | 33 (30,38) | <0.001 |
| **CES-D** | 10 (6,10) | 10 (5,16) | 0.786 |
| **PSS** | 14 (11,18) | 14 (11,18) | 0.709 |

PSQI, Pittsburgh Sleep Quality Index; SAS, Self-Rating Anxiety Scale; CES-D, Center for Epidemiologic Study of Depression Scale; PSS, Perceived Stress Scale.

showing that pregnancy often leads to sleep disturbances, especially for those with infertility histories. Multivariate logistic regression analysis showed that age, conception methods, depression, anxiety, and perceived stress significantly impacted sleep quality. Although age was found to be significantly associated with reduced sleep quality, the group of women receiving IVF/ICSI treatment is significantly older than the natural conception group, which obviously impacts the finding of the difference between the two groups. Previous studies also have shown that sleep quality in pregnant women declines with increasing age (24). Furthermore, a meta-analysis of quantitative sleep parameters has demonstrated that sleep quality naturally diminishes as individuals age (25). Pregnant women aged 30 and older are more likely to experience stress and depressive symptoms during pregnancy, which may also elevate the risk of postpartum depression in this age group (26).

Sleep disorders are prevalent during pregnancy. Previous studies demonstrated that pregnant women experience variable levels of sleep quality deficits across all trimesters (24). PSQI scores exhibited an upward trend as pregnancy progressed, with the sleep quality of pregnant women being particularly susceptible to disruptions in the late stages of pregnancy (4). Contributing factors include emotional stress from the IVF process, physical discomfort as pregnancy progresses, and hormonal changes affecting sleep (27). Notably, the observed sleep decline in the NC group suggests that sleep issues during pregnancy are influenced by physiological and psychological demands rather than just conception methods. This study is consistent with previous findings, which suggest that around 46% of pregnant people experience sleep difficulties, with a notable drop in sleep quality occurring during the third trimester (28). This highlights the need for further investigation into sleep patterns among pregnant women, taking into account their medical histories and current health. During pregnancy, the significance of sleep for maintaining physical health was highlighted linking compromised sleep quality to negative pregnancy outcomes. The decline in sleep quality may impact maternal and fetal health, increasing risks of gestational complications, postpartum depression, and impaired maternal-fetal attachment. Studies have identified associations between sleep-disordered breathing and conditions such as gestational diabetes and hypertension (3, 29, 30). Additionally, insomnia and obstructive sleep apnea have been correlated with the increased risk of preterm birth (29).

Anxiety, depression, and stress associated with infertility treatment are essential issues. Our results reveal that while both groups exhibited similar median anxiety scores as measured by the SAS, pregnant women in the IVF/ICSI group reported significantly lower median scores on both the CES-D and PSS during T1 and T2. The retained comparability of anxiety scores across both groups throughout the pregnancy, with no statistically significant differences, suggested that the process of conception-whether through IVF/ICSI or natural means- does not lead to significant differing levels of anxiety in pregnant women. This could imply that the act of pregnancy itself, along with the accompanying physiological and emotional changes, may predispose all pregnant individuals to experience a standard level of anxiety regardless of their conception method. Significant differences in CES-D and PSS scores during the first two trimesters indicate that the IVF/ICSI group experiences lower depressive symptoms and perceived stress early in pregnancy. This may stem from a sense of accomplishment and relief from overcoming infertility. However, in the third trimester, CES-D and PSS scores equalize between groups, likely due to increased discomfortness and anxiety about childbirth affecting all pregnant women. This aligned with literature indicating that the third trimester often brings heightened physical discomfort and concerns about maternal health, impacting mental health. These findings underscored the need for individualized care approaches, suggesting that early emotional support and psychological interventions for the IVF/ICSI group could enhance their positive mental state. A study involving 842 patients undergoing IVF treatment showed that 39.4% of patients felt anxious, and 28.5% had depressive symptoms (31). Even though it is not entirely clear to what extent mental disorders affect fertility and to what extent infertility affects mental health (32), targeted interventions addressing psychological factors are crucial as pregnancies progress and challenges intensify.

The analysis of partners of pregnant women in the IVF/ICSI and NC groups revealed important insights into the emotional landscape surrounding ART and natural conception. With 498 partners from the IVF/ICSI group and 621 from the NC group, the study robustly compared mental health indicators, particularly anxiety levels measured by SAS scores. Partners in the IVF/ICSI group showed slightly higher anxiety scores, suggesting that the stress of infertility treatments affects their mental well-being. The emotional toll of IVF/ICSI, marked by uncertainty and financial strain, likely contributed to this heightened anxiety, aligning with literature on the psychological burden faced by couples undergoing fertility treatments. Interestingly, despite the increased anxiety, no significant differences in sleep quality, depression, or perceived stress were found between the two groups. This might suggest that while anxiety is a concern for IVF/ICSI partners, it does not lead to poorer sleep or higher depression levels compared to NC partners. The lack of sleep quality differences may reflect a broader trend where partners face similar sleep challenges during pregnancy, regardless of the conception method. These findings highlighted the need to consider partners' mental health, especially for those undergoing IVF/ICSI. Future research should explore factors contributing to partner anxiety and potential interventions.

One limitation of this study is the absence of data on participants' prior history of sleep, depression, and anxiety. This information could have provided a more comprehensive understanding of the psychological factors influencing sleep quality during pregnancy, particularly in women undergoing IVF/ICSI treatment. The lack of this data may limit our ability to fully assess the impact of pre-existing mental health conditions on the observed outcomes. Another notable limitation of this study is the reliance on self-reported questionnaires to assess sleep quality among participants. While these instruments provide valuable insights, they are subjective and may be influenced by various biases, including recall bias and social desirability bias. Previous research mainly focused on specific aspects of psychological distress without considering the interplay of multiple influencing factors. A more reliable insights into this association was highlighted. For sleep issues during pregnancy, there are pharmacological options, but there's a lack of human data on their safety during pregnancy and lactation, raising concerns about potential birth defects and neonatal withdrawal (33). Future studies should utilize longitudinal designs to track mental health changes throughout pregnancy and identify key factors. This knowledge is vital for healthcare providers to establish support systems that help pregnant women maintain their psychological well-being during this critical period.

## Conclusions

Achieving a pregnancy and establishing a family life holds significant value for individuals undergoing IVF/ICSI as it greatly influences their overall life happiness. Consequently, even slight increases in the likelihood of achieving a pregnancy may warrant the implementation of various measures aimed at achieving success. Our study indicates that a notable concern regarding sleep quality during pregnancy among women in both IVF/ICSI and NC groups, calling for immediate attention from health professionals to address this crucial aspect of maternal health. Moreover, our current understanding of the potential impact of sleep, perceived stress, and distress on the progression from early pregnancy stage to live births remains incomplete. While IVF/ICSI partners exhibit higher anxiety, the absence of significant differences in other mental health aspects underscores the importance of understanding psychological dynamics and addressing the mental health needs of both partners to foster a supportive pregnancy environment.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

## Ethics statement

The studies involving humans were approved by the Medical Science Ethics Board of West China Second University Hospital of Sichuan University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

## Author contributions

YX: Formal Analysis, Investigation, Writing – original draft, Data curation, Methodology, Conceptualization. RP: Data curation, Investigation, Writing – review & editing. LX: Investigation, Formal Analysis, Validation, Writing – review & editing. SL: Funding acquisition, Supervision, Writing – review & editing. XL: Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Associations of maternal PM2.5 exposure with preterm birth and miscarriage in women undergoing *in vitro* fertilization: a retrospective cohort study

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**Background:** Excessive exposure to PM2.5 can be detrimental to reproductive health. The objective of this study was to investigate the potential associations between ambient PM2.5 exposure during different periods and negative pregnancy outcomes, such as miscarriage and preterm birth, in patients who underwent assisted reproductive technology (ART).

**Methods:** This retrospective cohort study examined the outcomes of 2,839 infertile women aged ≤ 45 years who underwent their first fresh or frozen-thawed embryo transfer at the Shanghai First Maternity and Infant Hospital between April 2016 and December 2019. Satellite data were used to determine the daily average levels of PM2.5, and exposure was categorized as excessive if it exceeded the WHO's interim target 2 level of 50 μg/m$^3$. The analysis was conducted separately for seven different periods. Our study used multinomial logistic regression models to explore the potential associations between PM2.5 exposure and adverse pregnancy outcomes. Sensitivity analysis was conducted by excluding women who underwent blastocyst transfer.

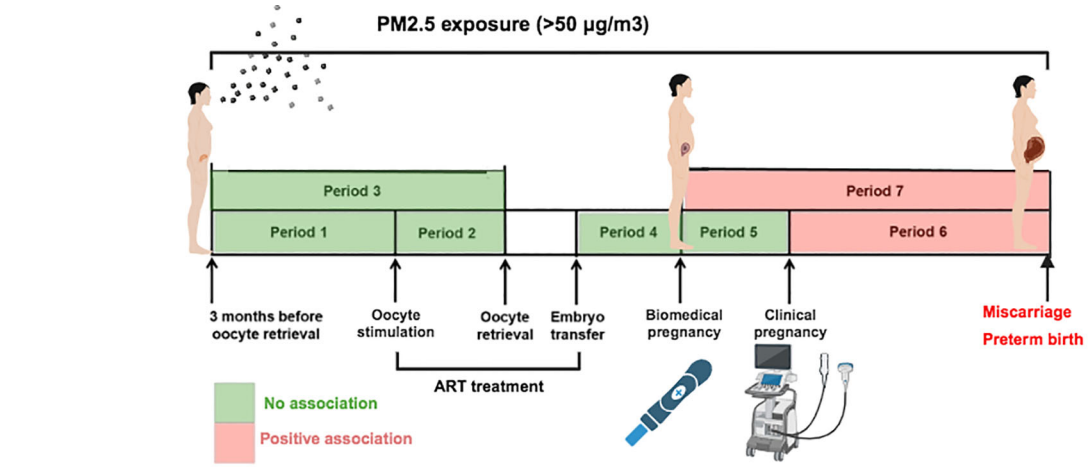
**Results:** Daily PM2.5 exposure exceeding the threshold (50 μg/m$^3$) was associated with an increased risk of miscarriage during the period after confirmation of clinical pregnancy or biochemical pregnancy, with adjusted odds ratios (AORs) of 2.22 (95% CI 1.75-2.81) and 2.23 (95% CI 1.68-2.96), respectively. Moreover, for each increase of 10 μg/m$^3$ above the threshold for PM2.5, there was a 46% elevated risk of preterm birth (AOR = 1.46, 95% CI 1.09-1.94) during the period after the confirmation of clinical pregnancy and a 61% elevated risk of preterm birth (AOR = 1.61, 95% CI 1.16-2.23) during the period after the confirmation of biochemical pregnancy. Our stratified analyses revealed

that women with an endometrial thickness <11 mm or who underwent frozen embryo transfer were more vulnerable to PM2.5 exposure, leading to higher rates of preterm birth.

**Conclusion:** Excessive PM2.5 exposure after biochemical pregnancy or clinical pregnancy was associated with increased risks of preterm birth and miscarriage among women who underwent ART.

KEYWORDS

PM2.5 exposure, preterm birth, miscarriage, particulate matter, *in vitro* fertilization




GRAPHICAL ABSTRACT

## Introduction

Ambient fine particulate matter with an aerodynamic diameter ≤ 2.5 μm (PM2.5) poses a health threat to populations worldwide and has detrimental effects on multiple organs and systems, including the reproductive system (1, 2). In addition, physiological changes during pregnancy make pregnant women particularly susceptible to the negative health impacts of PM2.5 (3). Previous research has indicated a link between PM2.5 and potential risks to fecundability and fertility (4, 5), as well as unfavorable pregnancy outcomes such as miscarriage (6), fetal growth restriction (7), low birth weight, and preterm birth (3, 8, 9). However, cohort studies regarding PM2.5 exposure and pregnancy outcomes were mainly conducted in women who conceived naturally (2, 7, 8). However, there is a gap in knowledge concerning the potential link between PM2.5 exposure and adverse pregnancy outcomes among women undergoing ART treatment.

The estimated rate of infertility among couples of reproductive age in China is as high as 25%, which exceeds the global average of 15% (10). An increasing number of infertile couples are undergoing ART treatments to conceive and have children, with in vitro fertilization (IVF) being the most commonly utilized method (11). Previous studies have demonstrated that women who conceive through IVF may be more vulnerable to ambient air pollution, including PM2.5 exposure, than women who conceive naturally (12), with a decreased probability of oocyte yield, clinical pregnancy and live birth among women with high ambient air pollution during pregnancy (11, 13–21). Limited research has been

**Abbreviations:** PM2.5, aerodynamic diameters ≤ 2.5 μm; ART, assisted reproductive technology; IVF, *in vitro* fertilization; hCG, human chorionic gonadotropin; SOP, standard operating procedure; COS, controlled ovarian stimulation; ICSI, intracytoplasmic sperm injection; BMI, body mass index; MAIAC, Multi-Angle Implementation of Atmospheric Correction; AOD, aerosol optical depth; MERRA-2, Modern-Era Retrospective Analysis for Research and Applications; AQGs, Air Quality Guidelines; AOR, adjusted odds ratio; 95% CI, 95% confidence interval; SD, standard deviation; IQR, interquartile range.

conducted on the potential link between PM2.5 exposure and adverse pregnancy outcomes, including preterm birth and pregnancy loss, in women undergoing ART.

Preterm birth and pregnancy loss, including miscarriage and biochemical pregnancy loss, are major adverse pregnancy outcomes of IVF treatment for patients who have a positive human chorionic gonadotropin (hCG) test (22). Nevertheless, there is a lack of research on the relationship between exposure to PM2.5 and preterm birth and/or pregnancy loss in women undergoing IVF, and the findings are inconclusive (12, 19, 23). In a prospective study of women in the United States, chronic daily PM2.5 exposure (mean concentration of 8.8 $\mu g/m^3$) from the date of the first positive hCG test until the date of pregnancy loss or live birth was not significantly associated with pregnancy loss (19). The lack of a significant association might be due to the lower concentration of PM2.5 exposure, which was within the normal level of the WHO's 24-hour air quality guidelines (PM2.5 < 15 $\mu g/m^3$) (19, 24). In contrast, another prospective cohort study in China showed a significant increase in the likelihood of biochemical pregnancy loss for every 10 $\mu g/m^3$ increase in PM2.5 exposure from the time of hCG testing to 30 days after embryo transfer. The average daily PM2.5 exposure of patients in this study was 45.4 $\mu g/m^3$ (25). The available evidence on the link between preterm birth and excessive PM2.5 exposure is scarce. A retrospective study in Hangzhou of China, revealed that daily PM2.5 exposure at a mean level of 36.0 $\mu g/m^3$ was significantly associated with an increased risk of preterm birth in all periods of pregnancy (from 85 days before oocyte retrieval to delivery outcome) among the ART population (23). However, a national cohort study in China reported that an increased risk of preterm birth in newborns conceived by ART was only linked to excessive PM2.5 exposure (median 55.0 $\mu g/m^3$) during the third trimester (12).

Therefore, the current understanding of the association between PM2.5 exposure and adverse pregnancy outcomes, such as preterm birth and pregnancy loss, in women undergoing IVF is incomplete and uncertain. Moreover, there is insufficient evidence to determine which stage of pregnancy after IVF treatment is most susceptible to adverse pregnancy outcomes resulting from high levels of PM2.5 exposure. Our study aimed to investigate the effects of PM2.5 exposure on adverse pregnancy outcomes (i.e., pregnancy loss and preterm birth) between different pregnancy periods among women who underwent ART.

# Methods

## Study design and subject recruitment

This was a retrospective cohort study. The subjects of this study were women residing in Shanghai who were transferred their first fresh or frozen embryos at the Centre for Assisted Reproduction of Shanghai First Maternity and Infant Hospital from April 2016 to December 2019. The following patients who underwent ART at our center were not included in this study: (1) were aged more than 45 years, (2) used donor semen, (3) underwent preimplantation genetic testing, (4) exhibited oocyte maturation *in vitro*, (5) had an endometrial thinness <8 mm with fresh embryo transfer, and (6) had an endometrial thinness <7 mm with frozen embryo transfer. Figure 1 shows the flowchart outlining the recruitment and follow-up of the study participants. This study received approval from the Research Ethics Committee of Shanghai First Maternity and Infant Hospital (KS22298).

## ART procedures

The center followed a standard operating procedure (SOP) to administer controlled ovarian stimulation (COS) treatment to all participants. The details of the COS protocol were described previously (21). ART procedures, including semen preparation, conventional IVF or intracytoplasmic sperm injection (ICSI) processes, embryo culture, and assessment, were performed as described previously (26). According to the Center's SOP, women who underwent fresh embryo transfer received one or two high-quality embryos three or five days after oocyte retrieval with the guidance of transabdominal ultrasound. Luteal phase support was initiated on the day of oocyte retrieval (27). The procedures for endometrial preparation and luteal phase support in frozen-thawed embryo transfer have been previously described (21). Women with a positive hCG test continued to receive luteal-phase support until ten weeks of pregnancy.

## Outcome definition and covariate data collection

The presence of biochemical pregnancy was determined by a serum hCG level exceeding 10 mIU/ml two weeks after embryo transfer (16). The term biochemical pregnancy loss refers to an early pregnancy loss that does not develop into a clinical pregnancy (28). All pregnant women were followed up until delivery or miscarriage. Clinical pregnancy was indicated upon the identification of a gestational sac through ultrasound four weeks after embryo transfer. Miscarriage was defined as the spontaneous loss of an intrauterine pregnancy before the 20th week of gestation (29). Live birth was defined as the delivery of one or more living infants with a gestational age of 20 weeks or more or a birth weight exceeding 1,000 g (30). Delivery between 20 and 37 weeks of gestation was classified as preterm birth, while delivery at 37 weeks of gestation or later was considered full-term birth (31).

As previously described, we retrieved covariate data from medical records at the Centre, which included information on the age of the females, age of the males, body mass index (BMI), address, level of education, employment status, duration and causes of infertility, COS method, duration of ovarian stimulation, dosage of gonadotropin stimulation used, progesterone level on the trigger day, total number of oocytes retrieved, method used for fertilization, thickness of the endometrial lining on the day the embryo was transferred, year and season of embryo transfer, number of transferred embryos and whether the transferred embryos were fresh or frozen (21).

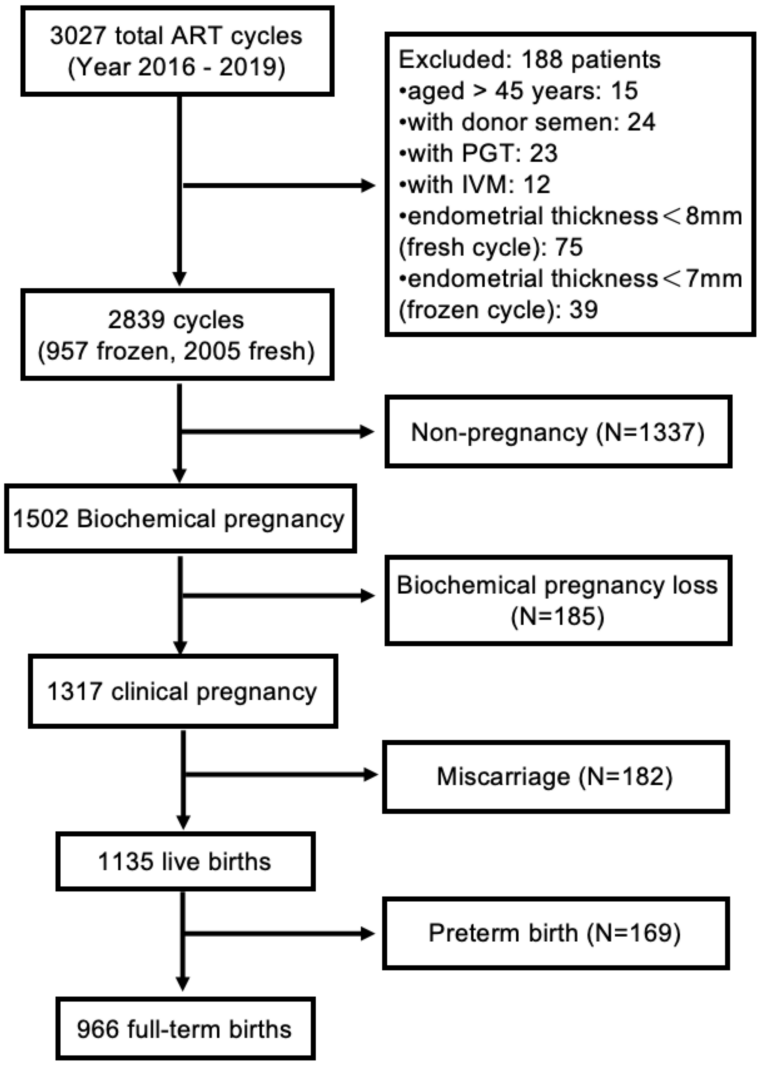



FIGURE 1
Subject recruitment and follow-up.

## Definition of periods of IVF and pregnancy

As shown in Figure 2, we used 7 periods of IVF treatment and pregnancy to study the effects of daily exposure to PM2.5 on the outcomes of IVF therapy. The periods were as follows: period 1 included the time between the 3 months prior to oocyte retrieval and gonadotropin-induced ovarian stimulation; period 2 included the time between gonadotropin-induced ovarian stimulation and oocyte retrieval; period 3 included the total 3 months preceding oocyte retrieval; period 4 included the time from embryo transfer to the time of serum hCG testing; period 5 followed from the hCG serum test to the time of ultrasound testing to confirm intrauterine pregnancy; period 6 included the time of ultrasound testing to the time of delivery or miscarriage; and finally, period 7 included the

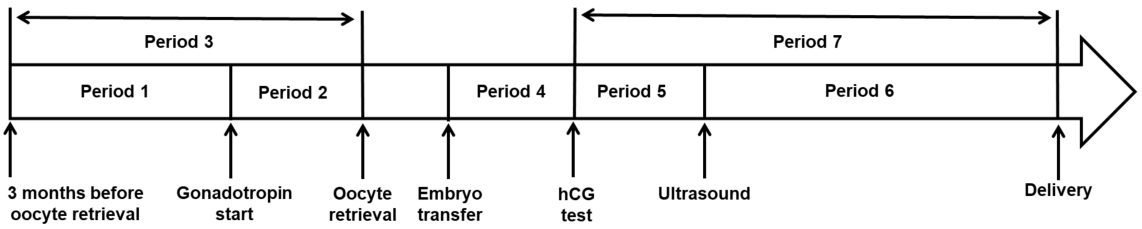



FIGURE 2
Period definition of IVF stages and pregnancy.

whole period between the hCG serum testing stage and the time of delivery or miscarriage.

## Assessment of ambient PM2.5 exposure

Following an established method (31, 32), we used a satellite-based model with a spatial resolution of 1x1 km to estimate the daily concentrations of PM2.5 during each of the seven periods studied. This was done utilizing a random forest algorithm where the PM2.5 measurements of >1,500 ground-based monitoring sites between 2013 and 2019 were used as the dependent variable, and the multiangle implementation of atmospheric correction (MAIAC) aerosol optical depth (AOD) retrieval data were used as the independent variables. We also utilized additional predictors, including population density, land use data, meteorological variables, and the Modern-Era Retrospective Analysis for Research and Applications (MERRA-2) PM2.5 products. In terms of missing AOD data, we approached this issue through a gap-filling method where estimated PM2.5 data were generated by combining findings from models with and without AOD data. Each participant had their daily mean PM2.5 concentration assigned by connecting the 1x1 km grid data to their residential address geocodes (32).

## Statistical analysis

One-way ANOVA was utilized to assess variations among continuous variables that followed a normal distribution, while the Kruskal–Wallis test was used for continuous variables with a skewed distribution. The chi-square test and Fisher's exact test were applied to assess the categorical variables between different IVF/ICSI outcomes or time periods.

The average daily PM2.5 concentration among our participants across the study period was 36.9 μg/m$^3$, which is close to the current suggested Air Quality Guidelines (AQGs) 24-hour interim target 3 (i.e., 37.5 μg/m$^3$) (33)). We also plotted a series of model deviances for the association between the average PM2.5 concentration and IVF outcomes and found that the minimum deviance values were greater than 40 μg/m$^3$ (Supplementary Figure 1). Therefore, we used the 24-hour interim target 2 (50 μg/m$^3$) (34) as a threshold for excessive PM2.5 exposure (24).

Multinomial logistic regression analysis was conducted to determine the adjusted odds ratios (AORs) and their corresponding 95% confidence intervals (95% CIs) for the associations between PM2.5 exposure and pregnancy outcomes among women receiving infertility treatments. PM2.5 was included in the models as a continuous variable representing concentrations exceeding 50 μg/m$^3$, and the AORs represented the change in odds of adverse outcomes per 10 μg/m$^3$ increase in PM2.5 above this threshold. To address potential confounding factors, we adjusted for both known and potential variables in our multinomial regression models, as these variables have been identified as potential risk factors for IVF pregnancy outcomes (35, 36). The previous study have suggested that clinical pregnancy and live birth rates declined with each millimeter below 8 mm in fresh IVF-ET and below 7 mm in frozen-ET (37). To validate the estimates, we performed sensitivity analyses by excluding women who underwent blastocyst transfer. R statistical software version 3.6.3, with the 'nnet' package used in multinomial logistic regression, was used for conducting the statistical analyses. A two-tailed $p$ value of less than 0.05 was considered to indicate statistical significance.

# Results

A total of 2,839 women whose mean age was 32.7 ± 3.9 years were included in the final data analysis. The majority (69.1%) of participants were treated with fresh embryo transfer, while 30.9% received frozen embryo transfer. Table 1 displays the demographic and clinical characteristics of the participants using descriptive statistics. According to pregnancy outcomes, participants were classified into five groups: full-term birth (34.0% of cases), preterm birth (5.9% of cases), miscarriage (6.4% of cases), biochemical pregnancy loss (6.5% of cases), and nonpregnancy (47.0% of cases).

The daily PM2.5 concentrations of the participants during different periods are shown in Table 2. There were significant differences in the average daily PM2.5 concentration among women with distinct pregnancy outcomes during Period 4 (from embryo transfer to the serum hCG test), Period 6 (after confirmation of clinical pregnancy), and Period 7 (after confirmation of biochemical pregnancy). The results displayed in Table 3 demonstrate a significant correlation between each 10 μg/m$^3$ increase in PM2.5 above the threshold of 50 μg/m$^3$ and increased risk of miscarriage (AOR = 2.22; 95% CI: 1.75-2.81 for Period 6; AOR = 2.23; 95% CI: 1.68-2.96 for Period 7), using full-term birth as the reference group (Table 3). In addition, excessive PM2.5 pollution above the 50 μg/m$^3$ threshold was associated with a 46% increase in the risk of preterm birth (AOR = 1.46, 95% CI: 1.09-1.94) during period 6 and a 61% increase in the risk of preterm birth (AOR = 1.61, 95% CI: 1.16-2.23) during period 7.

We examined whether the estimated associations between PM2.5 and preterm birth and miscarriage differed among subgroups in Period 7. As shown in Figure 3, the estimated association with preterm birth varied by endometrial thickness and type of embryo transfer. Women with an endometrial thickness <11 mm or frozen embryo transfer were more susceptible to the adverse effects of a 10 μg/m$^3$ increase in PM2.5 above the threshold on preterm birth rates; however, there was no significant variation in the association between excessive PM2.5 exposure and miscarriage among these subgroups for Period 7. The association remained almost unchanged in the sensitivity analyses (Supplementary Table 1).

# Discussion

The findings of our study suggest a significant association between maternal exposure to high levels of ambient PM2.5 (above 50 μg/m$^3$) after confirmation of biochemical pregnancy or

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TABLE 1 Demographic characteristics of couples who underwent IVF with different outcomes.

| Variables | All cases (N=2839) | Full-term birth (N=966) | Preterm birth (N=169) | Miscarriage (N=182) | Biochemical pregnancy loss (N=185) | Non-pregnancy (N=1337) | *p*-value |
|---|---|---|---|---|---|---|---|
| **Female age (years)** | 32.7 ± 3.9 | 31.9 ± 3.3 | 31.7 ± 3.1 | 33.4 ± 4.4 | 32.4 ± 3.9 | 33.3 ± 4.1 | **< 0.001** |
| **Male age (years)** | 34.7 ± 5.2 | 34.1 ± 4.7 | 33.4 ± 4.2 | 35.5 ± 6.0 | 34.6 ± 4.8 | 35.3 ± 5.5 | **< 0.001** |
| **Female BMI (kg/m$^2$)** | | | | | | | 0.445 |
| Underweight | 283 (10.2) | 111 (11.7) | 16 (9.9) | 13 (7.3) | 13 (7.1) | 130 (9.9) | |
| Normal weight | 1594 (57.3) | 544 (57.4) | 88 (54.3) | 102 (57.3) | 105 (57.4) | 755 (57.7) | |
| Overweight/Obesity | 903 (32.5) | 293 (30.9) | 58 (35.8) | 63 (35.4) | 65 (35.5) | 424 (32.4) | |
| **Education level of female** | | | | | | | 0.156 |
| High school or below | 299 (10.5) | 80 (8.3) | 18 (10.7) | 16 (8.8) | 26 (14.1) | 159 (11.9) | |
| Tertiary education | 2138 (75.4) | 745 (77.2) | 127 (75.1) | 138 (75.8) | 138 (74.6) | 990 (74.2) | |
| Postgraduate education | 398 (14.1) | 140 (14.5) | 24 (14.2) | 28 (15.4) | 21 (11.4) | 185 (13.9) | |
| **Type of infertility** | | | | | | | 0.077 |
| Primary infertility | 1821 (64.2) | 648 (67.2) | 116 (68.6) | 111 (61.7) | 112 (60.9) | 834 (62.4) | |
| Secondary infertility | 1014 (35.8) | 317 (32.8) | 53 (31.4) | 69 (38.3) | 72 (39.1) | 503 (37.6) | |
| **Factors of infertility** | | | | | | | 0.168 |
| Male | 656 (23.2) | 230 (23.9) | 44 (26.2) | 37 (20.6) | 46 (25.1) | 299 (22.4) | |
| Female | 1627 (57.5) | 535 (55.6) | 89 (53.0) | 119 (66.1) | 109 (59.7) | 775 (58.1) | |
| Both | 311 (11.0) | 103 (10.7) | 23 (13.7) | 12 (6.7) | 19 (10.4) | 154 (11.5) | |
| Unknown | 234 (8.3) | 95 (9.9) | 12 (7.1) | 12 (6.7) | 9 (4.9) | 106 (7.9) | |
| **Duration of infertility (years)** | 3.1 ± 2.2 | 3.0 ± 2.1 | 3.0 ± 2.2 | 3.1 ± 2.6 | 3.0 ± 2.1 | 3.2 ± 2.3 | 0.325 |
| **Gonadotropin doses (IU)** | 1800 (900) | 1800 (825) | 1875 (900) | 1800 (816) | 1931 (1050) | 1875 (975) | 0.214 |
| **Duration of stimulation (days)** | 10 (4) | 10 (3) | 10 (3) | 10 (4) | 10 (4) | 10 (3) | **0.002** |
| **Stimulation protocol** | | | | | | | **< 0.001** |
| GnRH agonist | 1483 (52.2) | 553 (57.2) | 113 (66.9) | 76 (41.8) | 97 (52.4) | 644 (48.2) | |
| GnRH antagonist | 710 (25.0) | 225 (23.3) | 29 (17.2) | 45 (24.7) | 45 (24.3) | 366 (27.4) | |
| Other protocols | 646 (22.8) | 188 (18.5) | 27 (15.9) | 61 (33.5) | 43 (23.2) | 327 (24.5) | |
| **Number of retrieved oocytes** | 10.6 ± 6.4 | 11.1 ± 6.1 | 11.2 ± 5.9 | 10.7 ± 7.6 | 10.6 ± 6.5 | 10.1 ± 6.4 | **0.003** |
| **Fertilization method** | | | | | | | 0.216 |
| IVF | 1939 (68.3) | 670 (69.4) | 120 (71.0) | 131 (72.0) | 115 (62.2) | 903 (67.5) | |
| ICSI | 900 (31.7) | 296 (30.6) | 49 (29.0) | 51 (28.0) | 70 (37.8) | 434 (32.5) | |
| **Rate of fertilization (%)** | 73.3 ± 22.2 | 73.8 ± 21.7 | 75.4 ± 21.3 | 74.5 ± 21.7 | 76.7 ± 18.8 | 72.1 ± 23.1 | **0.021** |
| **Rate of good embryos (%)** | 26.6 ± 22.2 | 29.3 ± 21.5 | 31.5 ± 23.4 | 27.8 ± 21.5 | 29.5 ± 21.4 | 23.5 ± 22.4 | **< 0.001** |
| **Endometrial thickness (mm)** | 11.0 ± 2.2 | 11.1 ± 2.1 | 10.9 ± 1.9 | 10.7 ± 2.1 | 11.3 ± 2.4 | 10.9 ± 2.2 | **0.010** |
| **Type of embryo transfer** | | | | | | | **0.008** |
| Fresh | 1961 (69.1) | 691 (71.5) | 128 (75.7) | 111 (61.0) | 120 (64.9) | 911 (68.1) | |
| Frozen | 878 (30.9) | 275 (28.5) | 41 (24.3) | 71 (39.0) | 65 (35.1) | 426 (31.9) | |

*(Continued)*

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TABLE 1 Continued

| Variables | All cases (N=2839) | Full-term birth (N=966) | Preterm birth (N=169) | Miscarriage (N=182) | Biochemical pregnancy loss (N=185) | Non-pregnancy (N=1337) | *p*-value |
|---|---|---|---|---|---|---|---|
| **Year of embryo transfer** | | | | | | | **< 0.001** |
| 2016 | 403 (14.2) | 130 (13.5) | 22 (13.0) | 29 (15.9) | 25 (13.5) | 197 (14.7) | |
| 2017 | 726 (25.6) | 279 (28.9) | 57 (33.7) | 35 (19.2) | 60 (32.4) | 295 (22.1) | |
| 2018 | 811 (28.5) | 295 (30.5) | 72 (42.6) | 46 (25.3) | 47 (25.4) | 351 (26.3) | |
| 2019 | 899 (31.7) | 262 (27.1) | 18 (10.7) | 72 (39.6) | 53 (28.6) | 494 (36.9) | |
| **Number of transferred embryos** | | | | | | | **< 0.001** |
| One | 1192 (42.0) | 335 (34.7) | 18 (10.7) | 73 (40.1) | 72 (38.9) | 694 (51.9) | |
| Two | 1647 (58.0) | 631 (65.3) | 151 (89.3) | 109 (59.9) | 113 (61.1) | 643 (48.1) | |
| **Stage of transferred embryos** | | | | | | | 0.082 |
| Blastocyst | 135 (4.8) | 38 (3.9) | 3 (1.8) | 12 (6.6) | 8 (4.3) | 74 (5.5) | |
| Cleavage | 2704 (95.2) | 928 (96.1) | 166 (98.2) | 170 (93.4) | 177 (95.7) | 1263 (94.5) | |

SD, standard deviation; BMI, body mass index; IQR, interquartile range.
Bold values are statistically significant.

clinical pregnancy and increased risks of miscarriage and preterm birth in women who underwent ART treatment. Moreover, a significant association between PM2.5 exposure and miscarriage was observed in all subgroups, covering diverse endometrial thickness ranges and types of embryo transfer, whereas the detrimental effects on preterm birth were more pronounced among women with endometrial thickness <11 mm or who underwent frozen embryo transfer.

The relationships between exposure to PM2.5 and adverse pregnancy outcomes, such as preterm birth and pregnancy loss, among women undergoing IVF treatment are still not fully understood. The existing research presents varying findings, with some studies suggesting a potential association between PM2.5 exposure and adverse outcomes, while others do not find significant correlations (12, 19, 23, 25). Factors such as the concentration and duration of PM2.5 exposure, as well as geographical location, may influence the outcomes observed. Studies conducted in areas with higher levels of PM2.5 pollution tend to show a stronger association with adverse pregnancy outcomes (13, 23, 25).

Only a few population-based studies have examined the effects of maternal exposure to PM2.5 on miscarriage (38). A retrospective cohort study conducted in a naturally conceived population demonstrated that miscarriage was associated with maternal acute exposure to ambient PM2.5 during the four weeks after conception (38). Furthermore, a significant relationship was observed between PM2.5 and fetal death caused by miscarriage (39). However, the effect of PM2.5 exposure in the ART population on the incidence of miscarriage is unclear. We found that ambient PM2.5 exposure after the confirmation of biochemical or clinical pregnancy was associated with a greater risk of miscarriage in infertile women undergoing IVF cycles. In

TABLE 2 Description of the PM2.5 concentration in different periods among IVF patients.

| | All cases (N=2839) | Full-term birth (N=966) | Preterm birth (N=169) | Miscarriage (N=182) | Biochemical pregnancy loss (N=185) | Non-pregnancy (N=1337) | *p*-value |
|---|---|---|---|---|---|---|---|
| **Period 1** | 37.0 ± 9.4 | 37.2 ± 9.3 | 37.5 ± 9.0 | 37.1 ± 9.6 | 36.5 ± 9.4 | 36.8 ± 9.5 | 0.741 |
| **Period 2** | 37.0 ± 12.9 | 37.0 ± 12.3 | 37.0 ± 13.2 | 37.3 ± 13.1 | 37.6 ± 12.7 | 36.9 ± 13.2 | 0.965 |
| **Period 3** | 37.0 ± 8.9 | 37.2 ± 8.7 | 37.4 ± 8.5 | 37.1 ± 9.1 | 36.6 ± 8.8 | 36.9 ± 9.1 | 0.846 |
| **Period 4** | 36.9 ± 13.0 | 38.0 ± 13.0 | 36.4 ± 13.5 | 35.7 ± 13.9 | 37.5 ± 13.9 | 36.2 ± 12.7 | **0.011** |
| **Period 5** | 37.6 ± 16.1 | 38.1 ± 16.2 | 37.1 ± 15.1 | 36.1 ± 15.1 | 37.1 ± 17.2 | — | 0.366 |
| **Period 6** | 36.6 ± 6.6 | 36.7 ± 4.9 | 37.6 ± 6.0 | 34.7 ± 12.4 | — | — | **0.015** |
| **Period 7** | 36.6 ± 6.2 | 36.8 ± 4.7 | 37.6 ± 5.8 | 34.8 ± 11.2 | — | — | **0.010** |

Bold values are statistically significant.

TABLE 3 Associations of daily PM2.5 above the WHO interim target 2 in different periods with IVF outcomes among participants in Shanghai.

| | Preterm birth (N=169) | Miscarriage (N=182) | Biochemical pregnancy loss (N=185) | Non-pregnancy (N=1337) |
|---|---|---|---|---|
| | AOR (95% CI) [a]* | AOR (95% CI) [a]* | AOR (95% CI) [a]* | AOR (95% CI) [a]* |
| **Period 1** | 0.93 (0.83-1.04) | 0.97 (0.87-1.09) | 0.97 (0.87-1.09) | 0.97 (0.91-1.03) |
| **Period 2** | 1.04 (0.96-1.12) | 1.04 (0.97-1.12) | 1.02 (0.95-1.10) | 1.03 (0.99-1.07) |
| **Period 3** | 1.00 (0.88-1.12) | 0.97 (0.85-1.10) | 1.01 (0.89-1.13) | 1.00 (0.93-1.07) |
| **Period 4** | 0.94 (0.87-1.02) | 0.97 (0.90-1.05) | 0.99 (0.92-1.06) | 0.97 (0.93-1.00) |
| **Period 5** | 0.99 (0.93-1.06) | 0.97 (0.90-1.04) | 1.03 (0.97-1.09) | — |
| **Period 6** | **1.46 (1.09-1.94)** | **2.22 (1.75-2.81)** | — | — |
| **Period 7** | **1.61 (1.16-2.23)** | **2.23 (1.68-2.96)** | — | — |

AOR, adjusted odds ratio; 95% CI, 95% confidence interval.
*Full-term birth was used as the reference level, and the AOR is for each 10 μg/m³ increment of ambient PM2.5.
[a]Adjusted for the age of the male and female, type of infertility, factors of infertility, duration of stimulation, stimulation protocol, number of retrieved oocytes, rate of good embryos, thickness of the endometrium, type of embryo transfer, year of embryo transfer, number of transferred embryos, stage of transferred embryos, and rate of fertilization.
Bold values are statistically significant.

contrast, two multicenter retrospective cohort studies in the USA and China suggested that PM2.5 was not associated with a decreased risk of live birth or increased risk of pregnancy loss in women who underwent ART (18, 20). The discrepancy in the literature may be due to differences in study design (exposure at patients' address or IVF unit), air pollution monitoring mode (monitoring station or different validated models), different IVF cycle protocols among the centers, PM2.5 concentrations and organic composition (high or low in different regions), and demographic characteristics of the study population.

The global number of preterm births associated with PM2.5 was estimated to be approximately 2.7 million in 2010 (40). Preterm birth is a major contributor to perinatal and early neonatal mortality and has been linked to adverse long-term health consequences, including cognitive, immunological, neurodevelopmental, and cardiovascular diseases (3). With the development of infertility treatment, the goal is to help infertile couples obtain healthy babies, not just live births. Therefore, it is crucial to investigate and understand the effects of PM2.5 on preterm birth among the population undergoing IVF treatment. A study in the USA reported that a 10% decrease in PM2.5 levels nationwide in 2008 contributed to a reduction of 5,016 preterm births, potentially reducing government expenditures by hundreds of millions of dollars annually, and that when additional health expenditures for preterm birth offspring in later years are taken into account, the savings in health finances could exceed $1 billion (41). Furthermore, a national cohort study in mainland China demonstrated that women who underwent IVF treatment had a greater risk of preterm birth due to PM2.5 exposure during the third trimester than women who conceived naturally (12). Although the local government has implemented stringent policies to reduce PM2.5 levels, exposure to unhealthy levels of PM2.5 is common for Shanghai residents (42). In this study, we found that a high level of PM2.5 exposure above the WHO interim target 2 (50 μg/m$^3$) during pregnancy was significantly related to increased odds of preterm birth

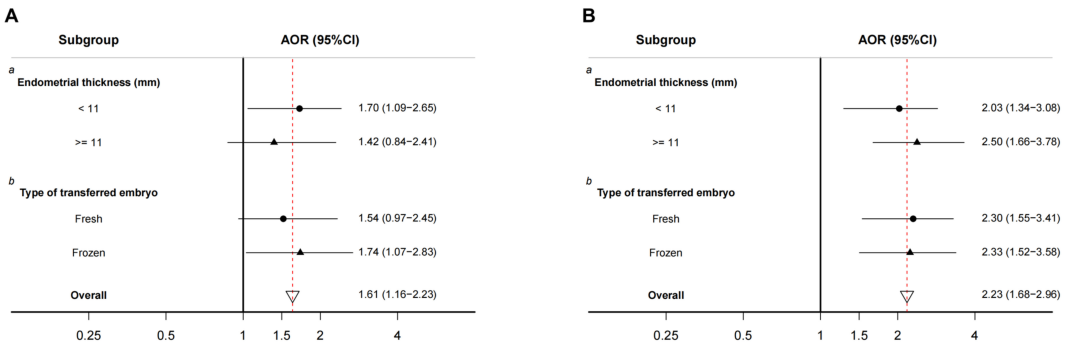


FIGURE 3
Stratified analyses of associations between daily PM2.5 above the WHO interim target 2 in period 7, **(A)** preterm birth and **(B)** miscarriage. AOR, adjusted odds ratio; 95% CI, 95% confidence interval. The full-term birth group was used as a reference. AOR for each 10 μg/m$^3$ increment of ambient PM2.5. [a] Adjusted for the age of male and female, type of infertility, factors of infertility, duration of stimulation, stimulation protocol, number of retrieved oocytes, rate of good embryos, type of embryo transfer, year of embryo transfer, number of transferred embryos, stage of transferred embryos, and rate of fertilization. [b] Adjusted for the age of the male and female, type of infertility, factors of infertility, duration of stimulation, stimulation protocol, number of retrieved oocytes, rate of good embryos, thickness of the endometrium, year of embryo transfer, number of transferred embryos, stage of transferred embryos, and rate of fertilization.

following the IVF cycle. However, Shi et al. reported a significant link between PM2.5 exposure and a greater rate of preterm birth within 85 days before oocyte retrieval, from the onset of gonadotropin administration until oocyte retrieval, throughout the first trimester of pregnancy, and throughout the entire IVF pregnancy (23). A potential explanation for this might be that the percentage of two embryos transferred in their study was as high as 84.5% (58% in our study), and the preterm birth rate in their study was much greater than that in our study (9.25% *versus* 5.95%). Previous research has shown an association between embryo transfer and an increased risk of preterm birth (43, 44). Consequently, transferring multiple embryos as an independent risk factor for preterm birth may influence the outcomes of PM2.5 exposure.

Stratified analyses provide additional evidence that excessive PM2.5 exposure over the threshold (i.e., 50 $\mu g/m^3$) may act together with other biological factors (e.g., endometrial thickness) and clinical treatment (e.g., the type of embryo transfer) to induce systemic inflammation and affect pregnancy duration (45, 46). A thin endometrium is associated with poor vascularization and reduced blood supply to the placenta, resulting in increased oxidative stress and inflammatory reactions (47). As a result, this condition can potentially compromise the process of placentation and hinder fetal growth, thereby contributing to adverse maternal and perinatal outcomes (48). Our observations indicated that subjects who had an endometrial thickness <11 mm rather than an endometrial thickness ≥ 11 mm had a greater risk of preterm birth when exposed to PM2.5 after biochemical pregnancy. In addition, our findings indicate that the adverse effects of transferring frozen embryos might contribute to an increased susceptibility to preterm birth when exposed to high levels of PM2.5. Retrospective studies suggested that frozen embryo transfer significantly increased the chance of preterm birth and abnormal placentation even after adjusting for BMI, maternal age and other confounders (49, 50). The combination of frozen embryo transfer and exposure to high levels of PM2.5 may have a synergistic effect, further increasing vulnerability to preterm birth. The underlying mechanisms of this interaction are not fully understood but may involve the compromised uterine environment and altered immune responses associated with frozen embryo transfer (51). Therefore, women who bear these risk factors may be a specific target group for future interventions and preventive measures.

Evidence from animal models suggests that mitochondrial dysfunction, oxidative stress, inflammation, and epigenetic alterations could be the underlying mechanisms of the reproductive toxicity of PM2.5 (52–54). High doses of PM2.5 induce the apoptosis of ovarian granulosa cells and oocytes and disrupt embryo development and impair placentation (54). PM2.5 was also associated with more severe lipid peroxidation and inadequate antioxidant capacity in women who suffer a miscarriage than in the normal pregnant population (55). Exposure to PM2.5 might interfere with the transport of oxygen and nutrients and/or accumulation in the placenta to directly cause placental inflammation and senescence, which can lead to preterm birth and miscarriage (56). Further investigation is needed to understand the mechanisms underlying the observed associations between PM2.5 exposure and miscarriage and preterm birth.

There are several strengths in our study. To our knowledge, this is one of the few studies to examine the relationship between PM2.5 exposure and adverse pregnancy outcomes in ART population. Our findings suggest that PM2.5 exposure is associated with a considerable burden of miscarriage and preterm birth among women undergoing infertility treatment. By avoiding outdoor activities in areas with air pollution, pregnant women who undergo IVF treatment may reduce their exposure to PM2.5 particles and mitigate the associated risks to their pregnancy. In addition, previous cohort studies were mainly conducted in women who conceived naturally (2, 7, 8), for whom it is challenging to identify the specific susceptible exposure. Instead, the stage of pregnancy during ART treatment is clearly recorded (e.g., dates of gonadotropin stimulation, embryo transfer, hCG test, and ultrasound examinations). This study used a characterized model with well-defined exposure timelines to study potential susceptible windows, which provides important insights for further study. Moreover, the satellite-based PM2.5 exposure assessment in our study could provide more detailed and additional useful information than the ground-based exposure assessment (57).

There are a few limitations of our study. First, this study analyzed only embryo transfer cycles, excluding cycles that were terminated or without viable embryos, potentially causing selection bias. The explanation for this is that this particular population exhibits the most serious consequences of IVF treatment, making it difficult to determine the impact of PM2.5 pollution on these occurrences. However, this selection bias may cause the adverse effects of PM2.5 to decrease to null values. Second, our data were obtained from medical records as a retrospective study, containing only limited information on individual covariates; thus, residual confounding effects could not be completely ruled out. Third, the absence of details regarding the utilization of fresh air filtration could result in misclassification of the exposure. Hence, it is necessary to conduct multicenter prospective studies to validate the results of this study.

## Conclusions

This study demonstrated that women who received ART and who were exposed to PM 2.5 after pregnancy had a greater risk of preterm birth and miscarriage. Women with an endometrial thickness <11 mm or who underwent frozen embryo transfer were more susceptible to increased risks of preterm birth associated with PM2.5. Additionally, an association between PM2.5 exposure and miscarriage after pregnancy was found in all subgroups, encompassing a wide range of endometrial thickness levels and various types of embryo transfer. Nevertheless, the underlying mechanism of the adverse effects of PM2.5 on pregnancy outcomes among women undergoing ART needs further investigation.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

## Ethics statement

This study received approval from the Research Ethics Committee of Shanghai First Maternity and Infant Hospital (KS22298). Written informed consent was waived due to the nature of retrospective, and anonymous clinical data was used and analyzed.

## Author contributions

MC: Conceptualization, Data curation, Funding acquisition, Writing – review & editing. QC: Data curation, Writing – original draft, Writing – review & editing. GL: Conceptualization, Data curation, Formal analysis, Writing – review & editing. CS: Data curation, Writing – review & editing. CL: Methodology, Writing – review & editing. XM: Methodology, Writing – review & editing. WL: Writing – review & editing. AQ: Writing – review & editing. OB: Writing – review & editing. HK: Methodology, Writing – review & editing. FW: Writing – review & editing. LT: Conceptualization, Supervision, Writing – review & editing. XT: Project administration, Resources, Supervision, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2025.1460976/full#supplementary-material

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# Higher Life's Crucial 9 protects against infertility among U.S. women aged 18–45 years

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**Objective:** Infertility is not only a reproductive issue but is also closely linked to cardiovascular health and other factors. Life's Crucial 9 (LC9) is a set of lifestyle guidelines aimed at improving cardiovascular health, yet its potential association with infertility remains underexplored. This study aims to investigate the relationship between LC9 and infertility, providing new insights and strategies for the prevention and management of infertility.

**Methods:** This study utilized data from the National Health and Nutrition Examination Survey (NHANES) from 2013 to 2018. Multivariate logistic regression and weighted quantile sum (WQS) regressions were employed to investigate the association between LC9 and infertility. Restricted cubic spline (RCS) analysis explored the linear or non-linear relationship between LC9 and infertility. Interaction analyses were conducted on subgroups to validate the findings.

**Results:** There was a significant negative association between LC9 and infertility. After adjusting for covariates, for every 10-point increase in LC9, there was a 35% decrease in the prevalence of infertility ($P < 0.001$). This negative correlation persisted when LC9 was divided into quartiles. Moreover, as LC9 increased, there was a trend towards lower infertility prevalence ($P$ *for trend* $< 0.001$). WQS analyses showed consistent associations (OR=0.27, 95%CI: 0.14, 0.53), with sleep health score, psychological health score, and Body mass index score as significant factors. The dose-response curve indicated a linear association between LC9 and infertility, with higher LC9 associated with lower infertility risk.

**Conclusion:** The results of this study show a strong negative correlation between LC9 and the prevalence of infertility. Clinically, these findings offer hope for infertility patients, suggesting that adherence to a higher LC9 score significantly reduces the risk of infertility. This will provide a new avenue for infertility prevention and management, offering hope and potential relief to infertile patients.

KEYWORDS

**infertility, Life's Crucial 9, association, National Health and Nutrition Examination Survey, cardiovascular health**

# 1 Introduction

Infertility is defined as the inability to conceive after at least 12 months of unprotected intercourse. Approximately 8%-12% of couples of reproductive age worldwide suffer from infertility (1). Although male infertility accounts for more than half of these cases, the burden of infertility is still predominantly borne by women (2). Infertility is a condition characterized by reproductive dysfunction resulting from various etiologies and is a significant reproductive health issue for couples of childbearing age. The causes of infertility can be attributed to organic factors (such as pelvic diseases) or iatrogenic factors (such as postoperative adhesions), but lifestyle and psychological stress may also play a role (3). The risk factors and mechanisms of infertility remain subjects of ongoing research. Several studies have indicated that infertility is associated with sleep (3), diet (4), appropriate exercise (5), diabetes (6), obesity (7), dyslipidemia (8), cardiovascular diseases (9), and smoking (10).

In 2010, the American Heart Association (AHA) defined cardiovascular health (CVH) and quantified it using seven metrics known as Life's Simple 7, which includes diet, physical activity, smoking status, BMI, total cholesterol, blood pressure, and blood glucose. In 2022, the AHA added sleep as a new component of CVH, introducing Life's Essential 8 (LE8) to further encourage personal and community health and to provide a more comprehensive reflection of cardiovascular health (11). On February 27, 2024, an article published in Circulation highlighted that individual mental health is a crucial component of enhancing cardiovascular health (12). Therefore, it is proposed to expand Life's Essential 8 (LE8) by adding a mental health metric, creating what can be termed Life's Crucial 9 (LC9). LC9 encompasses four health behaviors (diet, physical activity, smoking cessation, sleep) and five health factors (healthy weight, normal cholesterol levels, blood glucose, blood pressure, and mental health). Compared to LE8, LC9 redefines cardiovascular health by incorporating the new mental health metric, making it more aligned with clinical practice. Consequently, LC9 can better represent an individual's cardiovascular health status and more accurately reflect the underlying pathophysiological changes within the body.

To date, numerous studies have explored the impact of the four health above behaviors and five health factors of CVH on infertility. However, the relationship between LC9 (the composite metric) and infertility has not yet been investigated. Previous research has indicated that infertility is associated with lower CVH scores in middle-aged women, focusing solely on diet, sleep, physical activity, smoking, BMI, total cholesterol, blood pressure, and blood glucose (13), without considering the impact of mental health on infertility. Nevertheless, mental health is very prevalent in the occurrence and development of infertility (14–19), and various psychological interventions are currently employed to address infertility (20).

With the ongoing changes in global lifestyles and environmental factors, the incidence of infertility has shown a marked upward trend. Given its complex etiology and uncertain treatment options, identifying modifiable factors associated with infertility has become a critical focus of current research, aiming to intervene and prevent its occurrence. Therefore, this study utilized data from the 2013-2018 cycles of the National Health and Nutrition Examination Survey (NHANES) in the United States. This database, with its comprehensive individual health indicators and lifestyle data, provides a broad population sample and a solid foundation for investigating infertility-related factors and their impact on cardiovascular health. For the first time, this study systematically analyzed the relationship between various independent LC9 factors and infertility, evaluating their potential roles in preventing infertility. This research not only aims to uncover further details about the complex causes of infertility but also provides important evidence-based support for clinical practice, advancing the development and implementation of public health strategies.

# 2 Methods

## 2.1 Study population

The National Health and Nutrition Examination Survey (NHANES) is a nationally representative cross-sectional survey conducted through home interviews and mobile examination centers, aimed at assessing the health and nutritional status of the U.S. population (21). This survey utilized data from 29,400 participants over three cycles of NHANES, spanning from 2013 to 2018. After excluding individuals <18 or >45 years old (n = 21,186), males (n = 3,891), and participants with missing or incomplete LC9 and infertility data (n = 1,975), a total of 2,348 participants were included in the final analysis. Figure 1 displays a flowchart of the entire selection process. NHANES is approved by the Research Ethics Review Board of the National Center for Health Statistics, and all participants provided informed consent (22). The data used in this study are de-identified and publicly available (https://www.cdc.gov/nchs/nhanes/index.htm).

## 2.2 Measurement

### 2.2.1 Assessment of Life's Crucial 9

LC9 includes nine metrics: four health behaviors (healthy diet, physical activity, smoking cessation, and healthy sleep) and five health factors (weight management, cholesterol control, blood glucose management, blood pressure management, and mental health). Detailed instructions on calculating each participant's LC9 score using the NHANES database can be found in Supplementary Table S1. In summary, each of the nine LC9 metrics has a score ranging from 0 to 100. The overall LC9 score is determined by averaging the scores of the nine individual metrics. A healthy diet is assessed using the Healthy Eating Index 2015 (HEI-2015) (23, 24). The components and scoring criteria of HEI-2015 are detailed in Supplementary Table S2. Sleep health, smoking status, physical activity, and mental health are derived from standardized questionnaires, while BMI, blood pressure, blood glucose, and cholesterol are obtained by trained professionals from the NHANES database (https://www.cdc.gov/nchs/nhanes/index.htm).

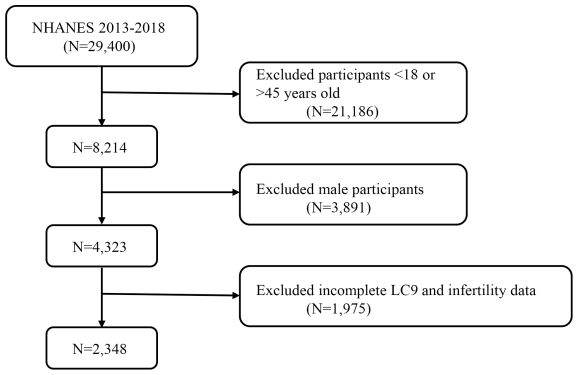


FIGURE 1
A flow diagram of eligible participant selection in the National Health and Nutrition Examination Survey.

### 2.2.2 Diagnosis of infertility

As in previous studies (25, 26), infertility is defined as a decrease in the ability of an individual or their partner to conceive, evidenced by the inability to achieve pregnancy after one year or more of regular, unprotected intercourse. In this study, the assessment of infertility was derived from the NHANES Reproductive Health Questionnaire (RHQ074). Specifically, participants were asked the following question: "Have you ever tried to become pregnant for a year or longer without becoming pregnant?" Those who answered "yes" were classified as infertile (https://wwwn.cdc.gov/Nchs/Nhanes/2013-2014/RHQ_H.htm#RHQ074).

## 2.3 Covariables

According to previous studies (25, 26), the covariates in this research include age, race, marital status, education level, family poverty-to-income ratio (PIR), smoking, alcohol consumption, Physical activity, hypertension, diabetes, and hypercholesterolemia. For detailed information on these covariates, please refer to Supplementary Table S3.

## 2.4 Statistical analyses

In this study, all data were statistically analyzed using R (version 4.3.1). The data were weighted, with continuous variables presented as mean ± standard deviation, and p-values calculated using weighted linear regression models. Percentages for categorical variables (weighted N, %) and their p-values were calculated using weighted chi-square tests. The association between LC9 and infertility was analyzed using multivariable logistic regression models, where LC9 was categorized into quartiles. Trend tests and p-values for linear trends were calculated to determine the consistency of the relationship. Three models were constructed in this study: (1) an unadjusted crude model; (2) a model adjusted for age, race, education level, marital status, and family poverty-to-income ratio (PIR); and (3) a model further adjusted for smoking, alcohol consumption, Physical activity, hypertension, diabetes, and hypercholesterolemia. A smooth curve fitting was applied to further explore the potential linear relationship between LC9 and infertility. Additionally, odds ratios (ORs) were calculated for every 10-point increase in LC9, with subgroup analyses conducted based on age, race, marital status, education level, PIR, smoking, physical activity, alcohol consumption, hypertension, diabetes, and hypercholesterolemia.

In addition, we applied the WQS to explore the overall effect of each LC9 metric on infertility. LC9 metric with a WQS weighting (ranging from 0 to 1 and summing to 1) higher than 0.111 (the mean of the 9 LC9 metric) were identified as major contributors. The significance was determined by p-values below 0.05.

# 3 Results

## 3.1 Characteristics of the participants

This study included 2,348 women aged 18 to 45, representing approximately 36.26 million reproductive-aged women in the United States. The prevalence of infertility was 13% (equivalent to 4.66 million), with an average (SD) CVH score of 70.74 (14.23). Table 1 (weighted) indicates that compared to women without infertility, those with infertility had lower CVH scores [no infertility: 76.62 (13.29); infertility: 70.74 (14.23)]. Statistically significant differences (all $p<0.05$) were observed between infertility and non-infertility groups in terms of age, cohabitation status, smoking, alcohol consumption, hypertension, diabetes, and hyperlipidemia. Compared to women without infertility, those with infertility tended to be older (35-45 years), married, Caucasian, and heavy drinkers. The unweighted baseline characteristics are detailed in Supplementary Table S4.

## 3.2 Association between LC9 and infertility

As shown in Table 2, three different models were used to assess the association between LC9 and its nine sub-scores with infertility. In Model 3, after full adjustment for covariates, each 10-point increase in LC9 was associated with a 35% reduction in the odds of infertility [odds ratio: 0.65 (95% confidence interval: 0.54, 0.80)]. As the LC9 quartiles increased (with Q1 as the reference), the incidence of infertility progressively decreased, with the corresponding results being: Q2: [odds ratio: 0.53 (95% confidence interval: 0.32, 0.87)], Q3: [odds ratio: 0.33 (95% confidence interval: 0.17, 0.62)], and Q4: [odds ratio: 0.27 (95% confidence interval: 0.15, 0.50)]. Additionally, a statistically significant trend was observed with lower infertility incidence as LC9 increased (P for trend < 0.001). In all models, except for the physical activity score, other LC9 sub-scores maintained a significant negative association with infertility. Furthermore, consistent results were obtained in the unweighted analysis (Supplementary Table S5). Figure 2 further illustrates the significant negative correlation between LC9 and the prevalence of infertility (P for overall < 0.001; P for non-linear = 0.468).

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TABLE 1 Baseline characteristics of all participants were stratified by infertility, weighted.

| Characteristic | Overall, N = 36,266,192 (100%) | Non-infertility, N = 31,597,899 (87%) | Infertility, N = 4,668,293 (13%) | P Value |
|---|---|---|---|---|
| **No. of participants in the sample** | 2,348 | 2,065 | 283 | - |
| **Age (%)** | | | | **<0.001** |
| *18-25* | 8,475,204 (23%) | 7,935,345 (25%) | 539,859 (12%) | |
| *26-34* | 11,943,370 (33%) | 10,579,120 (33%) | 1,364,250 (29%) | |
| *35-45* | 15,847,619 (44%) | 13,083,435 (41%) | 2,764,184 (59%) | |
| **Race (%)** | | | | 0.153 |
| *Non-Hispanic White* | 20,817,235 (57%) | 17,847,849 (56%) | 2,969,386 (64%) | |
| *Other* | 6,503,832 (18%) | 5,865,240 (19%) | 638,592 (14%) | |
| *Non-Hispanic Black* | 4,655,868 (13%) | 4,104,606 (13%) | 551,263 (12%) | |
| *Mexican American* | 4,289,256 (12%) | 3,780,205 (12%) | 509,052 (11%) | |
| **Married/live with partner (%)** | | | | **<0.001** |
| *No* | 14,416,365 (40%) | 13,408,319 (42%) | 1,008,046 (22%) | |
| *Yes* | 21,849,827 (60%) | 18,189,580 (58%) | 3,660,247 (78%) | |
| **Education level (%)** | | | | 0.971 |
| *Below high school* | 3,785,024 (10%) | 3,300,393 (10%) | 484,631 (10%) | |
| *High School or above* | 32,481,168 (90%) | 28,297,507 (90%) | 4,183,662 (90%) | |
| **PIR (%)** | | | | 0.260 |
| *Not Poor* | 24,693,013 (72%) | 21,283,046 (72%) | 3,409,967 (75%) | |
| *poor* | 9,400,423 (28%) | 8,281,507 (28%) | 1,118,915 (25%) | |
| **Smoking (%)** | | | | **0.048** |
| *Never* | 24,972,694 (69%) | 22,049,273 (70%) | 2,923,421 (63%) | |
| *Former* | 4,638,919 (13%) | 3,997,170 (13%) | 641,749 (14%) | |
| *Current* | 6,654,580 (18%) | 5,551,457 (18%) | 1,103,123 (24%) | |
| **Physical activity (%)** | | | | 0.470 |
| *Inactive* | 4,284,392 (15%) | 3,706,519 (14%) | 577,874 (16%) | |
| *Active* | 25,233,812 (85%) | 22,243,259 (86%) | 2,990,553 (84%) | |
| **Drinking (%)** | | | | **0.047** |
| *former* | 1,741,402 (5.0%) | 1,350,142 (4.4%) | 391,260 (8.7%) | |
| *heavy* | 9,055,486 (26%) | 7,731,973 (25%) | 1,323,513 (29%) | |
| *mild* | 9,565,997 (27%) | 8,453,627 (28%) | 1,112,371 (25%) | |
| *moderate* | 10,241,369 (29%) | 9,041,706 (30%) | 1,199,663 (27%) | |
| *never* | 4,539,156 (13%) | 4,069,652 (13%) | 469,504 (10%) | |
| **Hypertension (%)** | | | | **0.001** |
| *No* | 30,640,616 (84%) | 27,036,788 (86%) | 3,603,829 (77%) | |
| *Yes* | 5,625,576 (16%) | 4,561,112 (14%) | 1,064,465 (23%) | |
| **Diabetes (%)** | | | | **0.007** |
| *No* | 34,396,392 (95%) | 30,134,993 (95%) | 4,261,399 (91%) | |
| *Yes* | 1,869,800 (5.2%) | 1,462,907 (4.6%) | 406,894 (8.7%) | |

*(Continued)*

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TABLE 1 Continued

| Characteristic | Overall, N = 36,266,192 (100%) | Non-infertility, N = 31,597,899 (87%) | Infertility, N = 4,668,293 (13%) | P Value |
|---|---|---|---|---|
| **High cholesterol (%)** | | | | **0.007** |
| *No* | 31,460,705 (87%) | 27,690,895 (88%) | 3,769,810 (81%) | |
| *Yes* | 4,805,488 (13%) | 3,907,004 (12%) | 898,484 (19%) | |
| **Mean LC9 score (mean (SD))** | 75.86 (13.56) | 76.62 (13.29) | 70.74 (14.23) | **<0.001** |
| **Mean HEI-2015 diet score (mean (SD))** | 37.78 (31.99) | 38.44 (32.15) | 33.35 (30.56) | **0.048** |
| **Mean physical activity score (mean (SD))** | 76.88 (39.10) | 77.54 (38.64) | 72.42 (41.93) | 0.148 |
| **Mean tobacco exposure score (mean (SD))** | 75.86 (38.95) | 76.62 (38.43) | 70.68 (42.01) | **0.039** |
| **Mean sleep health score (mean (SD))** | 84.63 (23.22) | 85.05 (22.95) | 81.76 (24.84) | 0.088 |
| **Mean psychological health score (mean (SD))** | 86.84 (25.63) | 87.50 (24.84) | 82.35 (30.11) | **0.037** |
| **Mean body mass index score (mean (SD))** | 59.59 (37.11) | 61.17 (36.58) | 48.84 (38.96) | **0.002** |
| **Mean blood lipid score (mean (SD))** | 80.25 (26.29) | 80.90 (25.88) | 75.87 (28.59) | **0.027** |
| **Mean blood glucose score (mean (SD))** | 93.80 (16.76) | 94.46 (15.93) | 89.30 (21.03) | **<0.001** |
| **Mean blood pressure score (mean (SD))** | 87.14 (22.34) | 87.89 (21.90) | 82.04 (24.57) | **0.002** |

Mean (SD) for continuous variables: the P value was calculated by the weighted linear regression model.
Percentages (weighted N, %) for categorical variables: the P value was calculated by the weighted chi-square test.
LC9, Life's Crucial 9; CVH, cardiovascular health; PIR, Ratio of family income to poverty; ORs, odds ratios; CI, confidence interval.
The bold values are less than 0.05.

## 3.3 Subgroup analysis

The results of the subgroup analysis are shown in Figure 3. In most subgroups, LC9 scores were negatively correlated with infertility ($P < 0.05$). There was a significant interaction between LC9 scores and age ($P < 0.05$). For every 10-point increase in LC9, the odds of infertility decreased by 69% [0.31 (0.17, 0.57)] in women aged 18-25, which was significantly higher than the reduction observed in women aged 26-34 [0.60 (0.45, 0.80)] and those aged 35-45 [0.74 (0.56, 0.96)]. Similarly, in the unweighted subgroup analysis shown in Figure 3B, there were significant interactions between LC9 scores and both age and blood pressure ($P < 0.05$).

## 3.4 WQS analyses

The WQS index from the WQS regression was negatively associated with the risk of infertility (OR 0.27,95% CI 0.14 to 0.53) (Supplementary Table S6). Figure 4 showed that all sub-scores were negatively associated with infertility, with sleep health (weight = 0.244) identified as the most important factor influencing the presence of infertility, followed by psychological health and Body mass index score (weights = 0.178 and 0.148).

# 4 Discussion

In this study, we found a significant negative correlation between CVH (quantified by LC9 scores) and the risk of infertility. Subgroup analysis further revealed a more pronounced interaction between LC9 scores and age in women younger than 35 years. This underscores the potential impact of cardiovascular health on the occurrence of infertility and highlights the importance of early monitoring and control of CVH, as quantified by LC9 scores, to reduce the incidence of infertility. In addition, the WQS regression results showed that sleep health (weight = 0.244) was considered the most important factor influencing the occurrence of infertility, followed by mental health and body mass index score (weights = 0.178 and 0.148).

To our knowledge, this is the first study to investigate the association between the new CVH metric (LC9) and the prevalence of infertility. Previous research has indicated that women with infertility have an increased risk of poorer CVH in middle age (quantified by LE8 scores) compared to women without infertility (13). This suggests a link between infertility and cardiovascular health, providing valuable insights for exploring CVH metrics and their relationship with infertility.

However, merely indicating the association between LE8 and infertility is insufficient. Infertility is a multifactorial condition closely related to various cardiovascular risk factors. For instance, a cross-sectional study involving 1,820 participants highlighted a strong association between sleep disorders and female infertility, potentially linked to the activation of the HPA axis (hypothalamic-pituitary-adrenal axis), which reduces endometrial receptivity (27). Additionally, a prospective study involving 385,292 participants demonstrated that healthy sleep patterns are associated with a reduced risk of cardiovascular diseases, coronary heart disease, and stroke (28). Another case-control study of 180 infertile women and 540 fertile women indicated that the psychological health of infertile women might be adversely affected (29). These

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TABLE 2 Adjusted odds ratios (ORs) of LC9 and infertility, weighted.

| LC9 components (per 10 scores) | Model 1 [OR (95% CI)] | *P* | Model 2 [OR (95% CI)] | *P* | Model 3 [OR (95% CI)] | *P* |
|---|---|---|---|---|---|---|
| **Total LC9 score** | 0.74 (0.65, 0.83) | **<0.001** | 0.72 (0.64, 0.82) | **<0.001** | 0.65 (0.54, 0.80) | **<0.001** |
| Quartile | | | | | | |
| Q1 | 1 (ref.) | | 1 (ref.) | | 1 (ref.) | |
| Q2 | 0.62 (0.43, 0.89) | **0.010** | 0.58 (0.39, 0.87) | **0.009** | 0.53 (0.32, 0.87) | **0.014** |
| Q3 | 0.46 (0.30, 0.72) | **<0.001** | 0.41 (0.26, 0.65) | **<0.001** | 0.33 (0.17, 0.62) | **0.001** |
| Q4 | 0.33 (0.20, 0.55) | **<0.001** | 0.31 (0.19, 0.53) | **<0.001** | 0.27 (0.15, 0.50) | **<0.001** |
| ***P for trend*** | <0.001 | | <0.001 | | <0.001 | |
| Subgroup LC9 scores | | | | | | |
| HEI diet score | 0.95 (0.90, 1.00) | **0.046** | 0.94 (0.89, 0.99) | **0.017** | 0.95 (0.90, 1.01) | 0.100 |
| Physical activity score | 0.97 (0.93, 1.01) | 0.140 | 0.98 (0.93, 1.02) | 0.130 | 1.11 (0.94, 1.32) | 0.200 |
| Tobacco exposure score | 0.96 (0.93, 0.99) | **0.028** | 0.96 (0.92, 0.99) | **0.024** | 0.98 (0.78, 1.32) | >0.9 |
| Sleep health score | 0.95 (0.90, 0.99) | **0.041** | 0.92 (0.88, 0.98) | **0.005** | 0.92 (0.85, 0.99) | **0.026** |
| Psychological health | 0.93 (0.89, 0.98) | **0.009** | 0.91 (0.86, 0.95) | **<0.001** | 0.92 (0.87, 0.97) | **0.006** |
| Body mass index score | 0.92 (0.87, 0.96) | **<0.001** | 0.92 (0.87, 0.97) | **0.003** | 0.92 (0.86, 0.98) | **0.010** |
| Blood lipid score | 0.93 (0.88, 0.99) | **0.014** | 0.96 (0.90, 1.02) | 0.200 | 0.98 (0.90, 1.05) | 0.500 |
| Blood glucose score | 0.86 (0.81, 0.92) | **<0.001** | 0.89 (0.83, 0.95) | **0.001** | 0.85 (0.74, 0.98) | **0.029** |
| Blood pressure score | 0.90 (0.85, 0.96) | **0.001** | 0.92 (0.86, 0.98) | **0.015** | 0.92 (0.82, 1.02) | 0.100 |

Model 1: no covariates were adjusted.
Model 2: age, education level, marital, PIR, and race were adjusted.
Model 3: age, education level, marital, PIR, race, smoking, drinking, Physical activity, hypertension, diabetes, and high cholesterol were adjusted.
LC9, Life's Crucial 9; PIR, Ratio of family income to poverty; ORs, odds ratios; CI, confidence interval.
The bold values are less than 0.05.

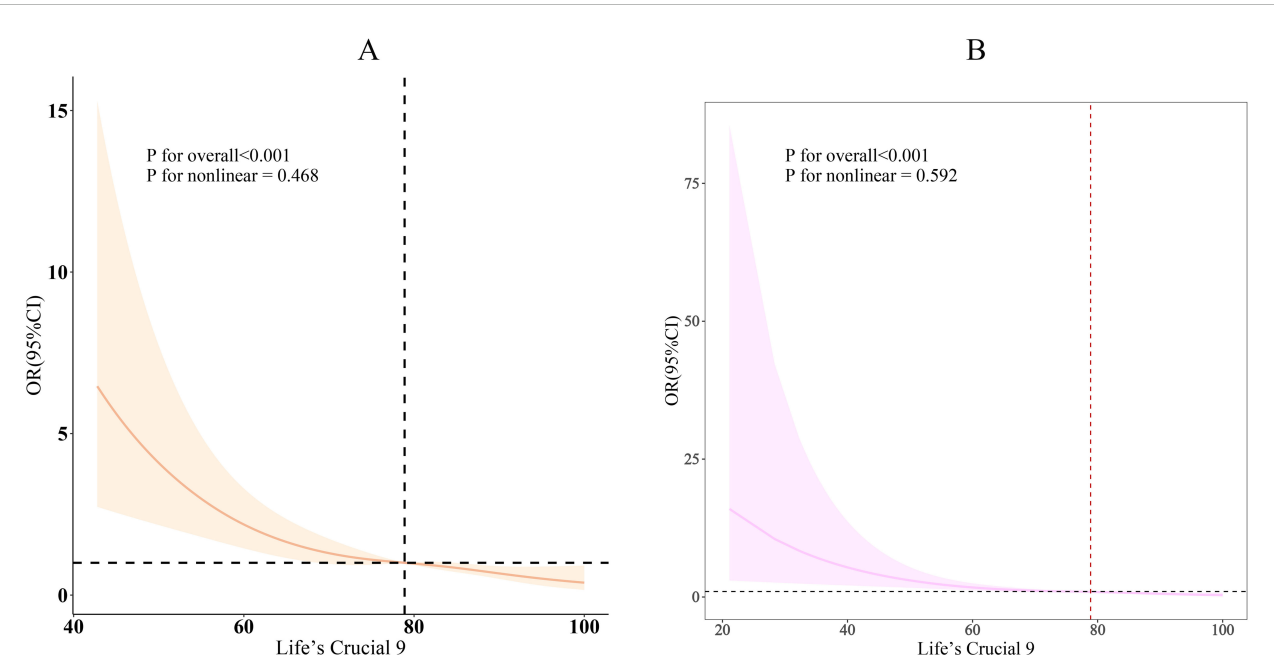


FIGURE 2
Dose-response relationships between LC9 and infertility. **(A)** weighted; **(B)** unweighted. OR (solid lines) and 95% confidence levels (shaded areas) were adjusted for age, education level, marital, PIR, race, smoking, drinking, Physical activity, hypertension, diabetes, and high cholesterol.

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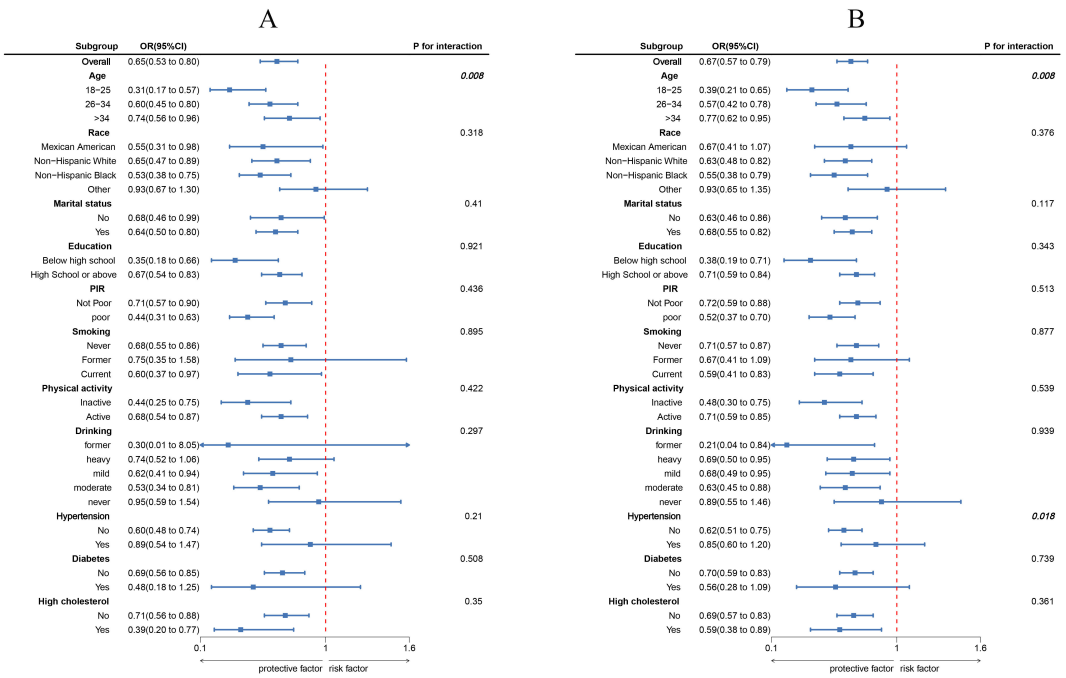


FIGURE 3
Subgroup analysis between LC9 and infertility. **(A)** weighted; **(B)** unweighted. ORs were calculated as per 10-unit increase in LC9. Analyses were adjusted for age, education level, marital, PIR, race, smoking, drinking, Physical activity, hypertension, diabetes, and high cholesterol.

shared risk factors may underlie both infertility and cardiovascular disease, with women experiencing infertility having a higher prevalence of cardiovascular risk factors even before conception (30). These pieces of evidence suggest that the CVH score assessed by LC9 could serve as an indicator of both cardiovascular health status and infertility incidence. Our study results demonstrate a negative linear relationship between CVH and infertility from various perspectives. For example, WQS results indicate that sleep

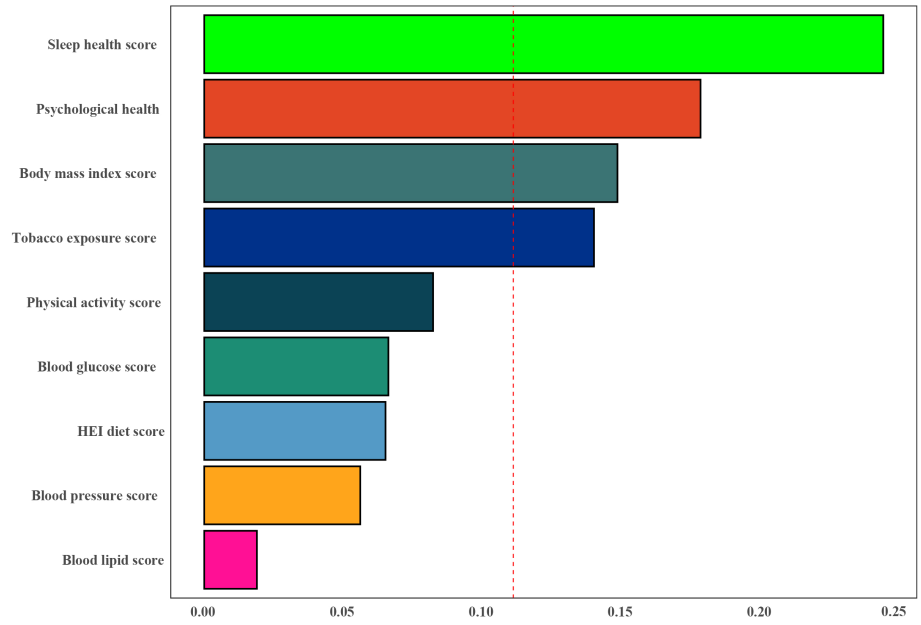


FIGURE 4
Weights represent the proportion of partial effect for each LC9 metric in the WQS regression. The model adjusted for age, education level, marital status, PIR, and race.

health, mental health, BMI, and tobacco exposure are more closely associated with infertility after adjusting for other confounding factors.

The mechanisms underlying the relationship between cardiovascular health and infertility are multifaceted and complex. Possible mechanisms include:(1) Insulin Resistance (IR): Insulin resistance is a pathogenic mechanism in various diseases. Research suggests that IR may affect oocyte quality by reducing mitochondrial function and exacerbating oxidative stress in follicles, thereby further impairing mitochondrial function and inducing the release of inflammatory factors (31). (2) Lipid Metabolism Disorders: Dyslipidemia, a major contributor to cardiovascular disease, is typically characterized by elevated circulating levels of low-density lipoprotein cholesterol (LDL-C) and/or triglycerides (TG), along with reduced levels of high-density lipoprotein cholesterol (HDL-C). Women with lipid metabolism disorders tend to exhibit deteriorated cumulus-oocyte complex (COC) morphology and a decreased number of cleavage-stage embryos. Elevated TC concentrations may adversely affect endometrial receptivity, leading to infertility (8). (3) Endothelial Dysfunction: It is well-known that the incidence of cardiovascular diseases is significantly lower in premenopausal women compared to men, largely due to the protective role of estrogen on cardiovascular health. Estrogen regulates lipoproteins and possesses anti-apoptotic properties by increasing the activation of the protective PI3K/Akt pathway and reducing inflammation and cytokine production. Endothelial cell damage is closely related to cardiovascular diseases, and estrogen also plays a role in vascular function regulation. Studies suggest that the decline in regulatory function is due to estrogen loss rather than aging (32). Endocrine abnormalities causing female infertility, such as polycystic ovary syndrome (PCOS), are associated with endocrine disorders like hyperandrogenism.

Studies have shown that sleep disorders, including disturbances in sleep duration and continuity, can interfere with reproductive capabilities or lead to the activation of the hypothalamic-pituitary-adrenal (HPA) axis, further disrupting reproductive functions. Sleep disorders may also affect reproductive hormones such as thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), and prolactin (PRL) (3). Age is negatively correlated with oocyte quality, potentially due to the accumulation of DNA damage in oocytes from environmental stress factors over time or the first-in-first-out principle, where higher-quality oocytes are ovulated earlier in life. Additionally, the shortening of oocyte telomeres with age contributes to the decline in oocyte and embryo viability (33). A meta-analysis indicated that psychological interventions could improve pregnancy rates (20). For example, a study by Rong Zhou and colleagues (34) demonstrated that cognitive behavioral therapy (CBT) and the integrative body–mind–spirit (BMS) approach play important roles in improving pregnancy rates, with BMS being associated with reduced anxiety. Additionally, a prospective, controlled, single-blind, randomized study by Alice D. Domar et al. (35), which included 184 women who had been attempting to conceive for 1 to 2 years, demonstrated that group psychological interventions appeared to increase pregnancy rates in women with infertility. Moreover, factors such as obesity, diet, smoking (33), and lipid abnormalities—components of LC9—affect these pathophysiological processes. Therefore, these studies collectively emphasize the importance of LC9 in infertility and provide further theoretical support for this research.

The results of this study provide important guidance for the management and prevention of infertility. This is the first time LC9 has been used to predict the risk of infertility, demonstrating its potential clinical value. By adjusting for other confounding factors, the results are more reliable, and the representative sample obtained through national sampling allows for extrapolation to a larger population. However, there are limitations: (1) This study is cross-sectional, which prevents establishing a causal relationship between LC9 and infertility (36). Large-scale cohort studies are needed to further validate the current findings. (2) Data collection primarily relied on questionnaires, which may introduce measurement errors. (3) Despite adjusting for many confounding factors, the limitations of the NHANES database prevented us from including certain potential confounders of infertility, such as male-only causes, uterine structural abnormalities, and other anatomical factors, which may have a weaker association with CVH. (4) This study did not take into account participants' job status or their exposure to other chemical and physical pollutants. For example, global warming and working in hot and humid environments may impact reproductive health in both men and women, potentially leading to infertility, which could affect the study's findings. Therefore, caution should be exercised when interpreting the results due to this limitation. In future studies, we plan to include these factors as confounding variables in the model to improve the accuracy of infertility research.

## 5 Conclusion

In conclusion, the findings of this study reveal a significant protective association between Life's Crucial 9 (LC9) and the incidence of infertility. This discovery not only provides scientific support for the application of LC9 in infertility risk assessment but also underscores its potential clinical significance in promoting reproductive health. By integrating LC9 into routine health management, healthcare professionals can more effectively identify and manage high-risk populations for infertility, thereby enhancing overall reproductive health. Furthermore, this study lays the groundwork for future research to validate the efficacy and mechanisms of LC9 in larger populations, advancing the development of infertility prevention strategies.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

## Author contributions

SH: Conceptualization, Data curation, Investigation, Software, Writing – original draft, Writing – review & editing. SD: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. SC: Formal Analysis, Methodology, Project administration, Supervision, Writing – review & editing. HG: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2025.1465022/full#supplementary-material

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# Association between the body roundness index and female infertility: a cross-sectional study from NHANES

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**Background:** Infertility is strongly associated with obesity. The body roundness index (BRI) is a more accurate assessment of visceral fat content than the body mass index (BMI). However, current evidence on the association between visceral fat accumulation and infertility remains insufficient and controversial. Therefore, we utilized the 2017-2020 National Health and Nutrition Examination Survey (NHANES) database to explore the correlation between BRI and infertility.

**Methods:** We used multiple logistic regression, smoothed curve fitting, subgroup analyses, and interaction tests to investigate the potential association between BRI and infertility. Additionally, we assessed the ability of BRI and BMI to predict infertility risk using receiver operating curve (ROC) analysis and calculate the area under the curve (AUC),sensitivity, and specificity.

**Results:** In the study, 1463 women aged 20 to 45 participated, and 172 of them were found to be infertile. After adjusting for all factors except body measurements, the findings indicated that for each one-unit increase in BRI, there was a 19% increase in the risk of infertility (OR = 1.19, 95% CI 1.05, 1.34). The analysis also revealed a positive nonlinear relationship between BRI and infertility. Furthermore, based on the ROC curves, it was observed that BRI was a more reliable predictor of infertility risk compared to BMI (BRI AUC = 0.5773, BMI AUC = 0.5681).

**Conclusion:** This study demonstrated a positive association between higher BRI values and infertility among adult women in the United States and showed a stronger association than BMI.

KEYWORDS

**NHANES, infertility, body roundness index (BRI), obesity, cross-sectional studies**

# Introduction

Infertility is a medical condition that prevents pregnancy after more than one year of regular sexual intercourse without any contraception (1). According to the World Health Organization report, infertility affects about 17.5% (2) of the population of reproductive age, causing a substantial psychological and financial burden on the patients (3–5).

With lifestyle changes, obesity has become one of the critical risk factors for infertility (6). Although widely used, traditional measurements such as BMI have gradually revealed their limitations in reflecting body fat distribution (7). In 2013, Thomas DM et al. (8) proposed the BRI as a new predictor of visceral adipose tissue and percentage of body fat, which estimates total body fat and body mass by considering the human body as an ellipsoid and combining height and waist circumference to calculate the percentage of total and localized fat, responding to the individual's body shape characteristics.

This study aims to investigate the association between BRI and infertility and whether it is superior to BMI. It will help healthcare professionals to better identify and manage women at high risk of infertility and provide a basis for future research on the relationship between visceral fat accumulation and women's reproductive health.

# Materials and methods

## Survey description

The National Health and Nutrition Examination Survey (NHANES) is a comprehensive national survey conducted by the Centers for Disease Control and Prevention (CDC) to evaluate the health and nutritional status of the U.S. population. Due to its use of probability sampling, NHANES data is considered highly credible and valuable for academic research. The 2017-2020 NHANES serves as the primary data source for this analysis. The National Center for Health Statistics (NCHS) Ethics Review Board has approved all study protocols for NHANES, and each participant has provided written informed consent. Detailed NHANES study designs and data are publicly available at http://www.cdc.gov/nchs/nhanes/.

## Study population

Initially, 15,560 participants from the 2017-2020 NHANES were enrolled. In order to select the most eligible subjects for the current study, the researchers further evaluated these individuals. First, they excluded 7,839 male subjects and female subjects younger than 20 years (n = 3,086) or older than 45 years (n = 2,796). Second, participants with missing data on self-reported infertility (n = 282) and body mass index (n = 55) were excluded. Participants who had both ovaries removed (n = 35), underwent hysterectomy (n = 54), or were pregnant at the time of examination (n = 68) were also excluded. Ultimately, 1,463 eligible women participated in the study (Figure 1).

## Exposure and ending definition

The exposure factor was BRI, which was calculated using the formula: $BRI = 364.2 - 365.5 \times \sqrt{1 - \frac{[WC(m)/2\pi]^2}{[0.5 \times Height(m)]^2}}$ (8, 9). The height and waist circumference data were obtained from the participants' physical examination documents. Body measurements were taken by professionally trained health technicians at the Mobile Health Screening Center and were recorded with the corresponding person to ensure accuracy. Participants were asked to remove their clothes and shoes before the measurements were taken. Height was measured while standing, and waist circumference was measured at the midpoint between the lower ribs and the upper part of the hips in a standing position.

The outcome indicator is self-reported female infertility, which was obtained from the Reproductive Health Questionnaire. The questionnaire was administered by professionally trained interviewers using the Ministry of Education's computer-assisted personal interview system. Participants were asked about their pregnancy history with the question, "Have you been trying to get pregnant for one year?" Women who answered "yes" were categorized as having infertility, and those who answered "no" were categorized as not having infertility (Questionnaire variable name: RHQ074).

## Covariates

Covariates in this study included age, race, education level, marital status, poverty-to-income ratio (PIR), smoking status, hypertension, diabetes mellitus, regular menstruation, sex hormone use, history of treatment for pelvic infection, waist circumference, and BMI. In our analyses, we used the questionnaire "Your doctor told you that you have diabetes" to determine if participants had diabetes. Those who answered "yes" were categorized as diabetic. Similar questionnaires were used to identify patients with hypertension and other conditions. Additionally, we used the questionnaire "Have you smoked at least 100 cigarettes in your life" to determine smoking status, categorizing those who answered "yes" as smokers. The above covariates were determined professionally after reviewing existing studies. All data collection and measurement procedures for these covariates are available on the NCHC website. Moreover, measurement procedures for these covariates are available on the NCHC website.

Questions about missing data, after data processing, the only variable with missing values was the poverty-to-income ratio (PIR), with a missing data percentage of 10.2% (168/1463). The missing values were estimated using the continuous iterative imputation method, which is based on the conditional distribution of other

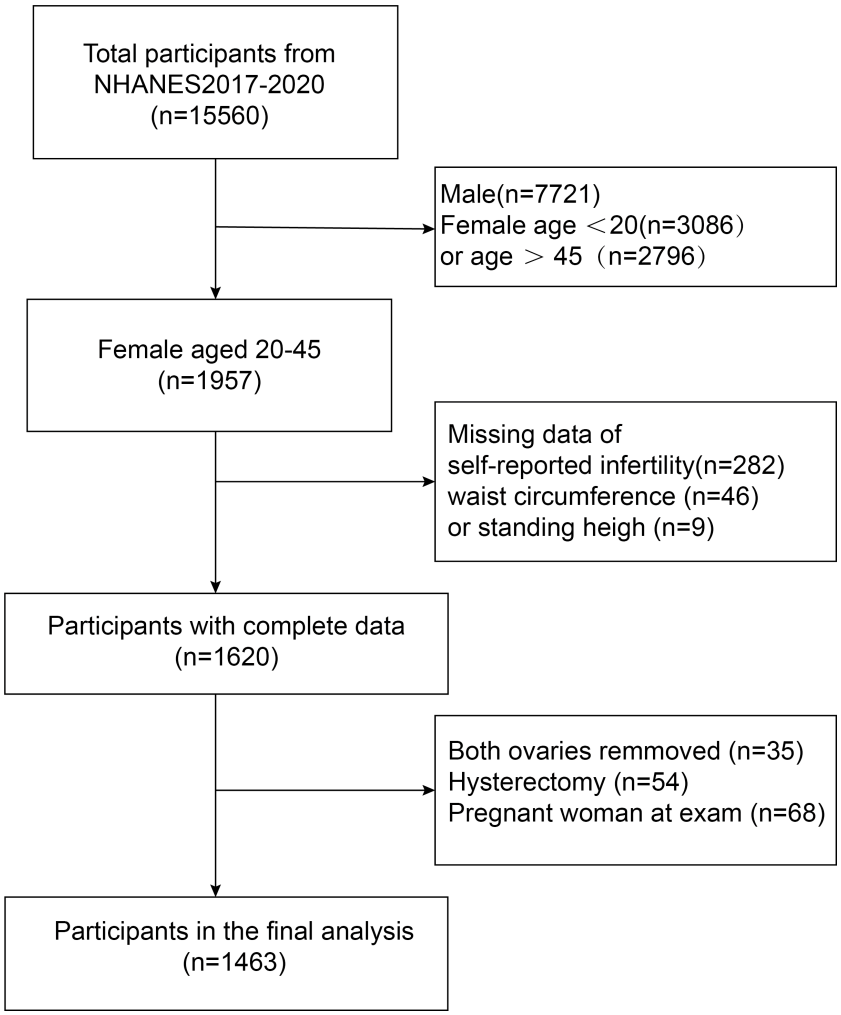


FIGURE 1
Flow chart for participants recruitment, NHANES 2017-2020.

variables. This approach improves the accuracy and reliability of subsequent analysis.

## Statistical analysis

Statistical analyses were performed using Empower Stats software (X&Y et al., USA) and the R package (version 3.4.3). $p < 0.05$ was considered statistically significant. Data were obtained from NHANES 2017-2020. The NHANES study employed a sophisticated multi-stage probability sampling design to collect data, which was taken into account in our analysis. To ensure representativeness of the sample, we performed weighted multivariate logistic regression analyses. Demographic and measurement indicators were descriptively analyzed for the study population. These indicators were categorized into two groups according to the presence or absence of infertility. Continuous variables were expressed as Mean ± Standard Error (Mean ± S.E.), and a t-test was used to compare groups. Categorical variables were expressed as frequencies (constitutive ratios) [n (%)], and comparisons between groups were made using the chi-square test. Multivariate regression models were used to examine the association between BRI and infertility. In model 1, the covariates were not adjusted. In model 2, adjustments were made for age and race. Model 3 was adjusted for the following factors: age, race, marital status, education level, household income, poverty rate, smoking at least 100 cigarettes, diabetes, hypertension, previous use of female hormones, previous treatment for pelvic infection, and regularity of menstruation in the past 12 months. Model 3 also used the RCS curve to assess the association between BRI and infertility. In addition, subgroup analyses and interaction tests were performed according to age, marital status, smoking status, diabetes, and

regularity of menstruation. We aim to identify possible impact modifiers and understand the differences in results among various groups and circumstances. The sensitivity of BRI and BMI in predicting infertility was assessed by examining the receiver operating characteristic curve (ROC) and calculating the area under the curve (AUC).

TABLE 1 Weighted demographic characteristics of selected participants from the NHANES 2017-2020.

| Variables | Non-infertility | Infertility | *P*-value |
|---|---|---|---|
| Age (years) | 31.74 ± 7.51 | 33.17 ± 6.45 | 0.0208 |
| Race/ethnicity(%) | | | 0.4653 |
| Mexican American | 11.85 | 12.02 | |
| Other Hispanic | 8.19 | 12.12 | |
| Non-Hispanic White | 55.24 | 54.58 | |
| Non-Hispanic Black | 13.95 | 13.10 | |
| Other Race | 10.77 | 8.17 | |
| Education level (%) | | | 0.1608 |
| Less than 9th grade | 2.56 | 2.00 | |
| 9-11th grade | 5.83 | 9.47 | |
| High school graduate/GED or equivalent | 22.34 | 25.10 | |
| Some college or AA degree | 30.83 | 33.24 | |
| College graduate or above | 38.43 | 30.19 | |
| Marital status(%) | | | <0.0001 |
| Living with Partner | 56.23 | 78.17 | |
| Live alone | 43.77 | 21.83 | |
| Ratio of family income to poverty | 2.82 ± 1.68 | 2.79 ± 1.57 | 0.8401 |
| Smoked≥100 cigarettes in life(%) | | | 0.0003 |
| Yes | 30.22 | 44.24 | |
| No | 69.78 | 55.76 | |
| Hypertension(%) | | | 0.8819 |
| Yes | 10.87 | 10.63 | |
| No | 88.98 | 89.37 | |
| Not recorded | 0.15 | | |
| Diabetes(%) | | | <0.0001 |
| Yes | 1.90 | 9.49 | |
| No | 96.77 | 88.73 | |
| Borderline | 1.16 | 1.78 | |
| Not recorded | 0.17 | | |
| Regular periods(%) | | | 0.0249 |
| Yes | 92.81 | 87.80 | |
| No | 7.19 | 12.20 | |
| Ever use female hormones(%) | | | |
| Yes | 4.68 | 4.03 | 0.8453 |
| No | 95.20 | 95.97 | |
| Not recorded | 0.12 | | |
| Ever treated for a pelvic infection(%) | | | 0.2143 |
| Yes | 4.24 | 7.13 | |
| No | 95.54 | 92.87 | |
| Not recorded | 0.22 | | |
| Waist Circumference (cm) | 94.47 ± 18.97 | 102.99 ± 20.49 | <0.0001 |
| Body Mass Index (kg/m2) | 29.32 ± 8.63 | 32.52 ± 9.40 | <0.0001 |
| Body Roundness Index/BRI | 5.41 ± 1.40 | 5.89 ± 1.43 | <0.0001 |

Data in the table: For continuous variables: survey-weighted mean (95% confidence interval), P-value was by survey-weighted linear regression (wtmecprp). For categorical variables: survey-weighted percentage (95% confidence interval), P-value was by survey-weighted Chi-square test (wtmecprp).

# Results

## Baseline characteristics of participants

In Table 1, we can see the characteristics of participants aged 20 to 45 years based on whether they had infertility. Out of 1463 participants, 172 (11.76%) were found to be infertile. The research showed that older women who lived with a partner who smoked at least 100 cigarettes, had diabetes mellitus, irregular menstruation, higher waist circumference, body mass index, and body roundness index reported a higher prevalence of infertility.

## Associations between BRI and risk of infertile

In Table 2, it is evident that there is a positive association between BRI and the prevalence of infertility. This positive correlation remained consistent in model 3 (OR 1.19; 95% CI 1.05-1.34), indicating that each unit increase in BRI raises the risk of infertility by 19%. For sensitivity analysis, we converted BRI from a continuous variable to a categorical variable (quartile). The likelihood of infertility in Q4 was 83% higher compared to Q1. However, the difference between Q2 and Q1 was not statistically significant (OR 1.17; 95% CI 0.68-2.02), nor was the difference between Q3 and Q1 (OR 1.52; 95% CI 0.89-2.59). Additionally, we used smoothed curve fitting based on model 3 to investigate the

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TABLE 2 Association between the body roundness index and female infertility.

| Exposure | OR (95%CI), *P*-value | | |
|---|---|---|---|
| | Model 1 | Model 2 | Model 3 |
| BRI | 1.19 (1.07, 1.33) 0.0010 | 1.18 (1.06, 1.32) 0.0036 | 1.19 (1.05, 1.34) 0.0070 |
| BRI Quartile | | | |
| Q1, [2.845, 4.534] | 1(Reference) | 1(Reference) | 1(Reference) |
| Q2, [4.539, 5.474] | 1.33 (0.81, 2.18) 0.2571 | 1.24 (0.75, 2.05) 0.3971 | 1.17 (0.68, 2.02) 0.5621 |
| Q3, [5.476, 6.581] | 1.48 (0.91, 2.40) 0.1148 | 1.38 (0.84, 2.27) 0.1989 | 1.52 (0.89, 2.59) 0.1276 |
| Q4, [6.585, 11.827] | 1.99 (1.25, 3.17) 0.0036 | 1.85 (1.14, 2.99) 0.0122 | 1.83 (1.08, 3.11) 0.0254 |

Model 1: no covariates were adjusted;
Model 2: age and race were adjusted;
Model 3: age, race, education level, marital status, ratio of family income to poverty, smoked≥100 cigarettes in life, hypertension, diabetes, regular periods, ever use female hormones, ever treated for a pelvic infection were adjusted;
BRI, body roundness index; OR, odds ratio; CI, confidence interval.

relationship between BRI and infertility, revealing a positive nonlinear relationship (Figure 2).

## Subgroup analyses

Subgroup analyses and interaction tests were performed using different variables to investigate the stability of the association between BRI and female infertility across subgroups. Subgroup analyses were stratified by age, marital status, diabetes, smoking status, and menstrual pattern. In all subgroups, BRI values were generally and consistently positively associated with the risk of infertility. The smoking group showed a significant interaction effect (P for interaction=0.0027), suggesting that smoking or not smoking may have a differential effect on the relationship between BRI and infertility (Figure 3).

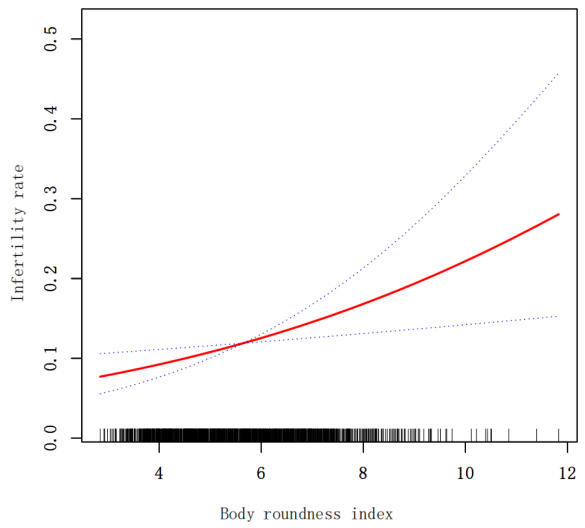


FIGURE 2
Association between BRI and infertility rate.

## Comparison of BRI and BMI in predicting infertility

In this study, we initially calculated the sensitivity and specificity at varying thresholds, subsequently employing the Youden index to assess the performance of BRI and BMI at distinct thresholds. Youden's index = sensitivity + specificity - 1.We identified the optimal cut-off point through the selection of a threshold that maximized the Youden index. This process ensured an optimal balance between positive and negative predictions. The results demonstrate that BRI exhibits a Youden index of 14.48%, an optimal threshold of 6.2945, an AUC of 0.5773, a sensitivity of 43.60%, and a specificity of 70.95%. The Youden index for BMI is 12.77%, with an optimal threshold of 3.345 and an AUC of 0.5681. The sensitivity is 41.28%, while the specificity is 71.49%. BRI demonstrated superior performance relative to BMI. Furthermore, a receiver operating characteristic (ROC) curve was plotted. The receiver operating characteristic (ROC) curve illustrates the correlation between sensitivity and the false positive rate (1-specificity) at varying thresholds. The optimal inflection point is defined as the point on the receiver operating characteristic (ROC) curve that is furthest from the diagonal line (Table 3, Figure 4).

# Discussion

In this cross-sectional study, which included 1,463 women of childbearing age attending NHANES in the United States from 2017-2020, of whom 172 reported infertility (prevalence 11.76%), we found a positive nonlinear association between BRI and infertility. After adjusting for all covariates except body measurements and converting the BRI to categorical variables by quartiles (Q1-Q4), the positive association remained, with a 19% increase in infertility risk for each unit increase in the BRI. Individuals with a high BRI score (Q4) had a higher probability of developing infertility compared to Q1. In subgroup analyses and interaction tests for age, marital status, diabetes mellitus, smoking

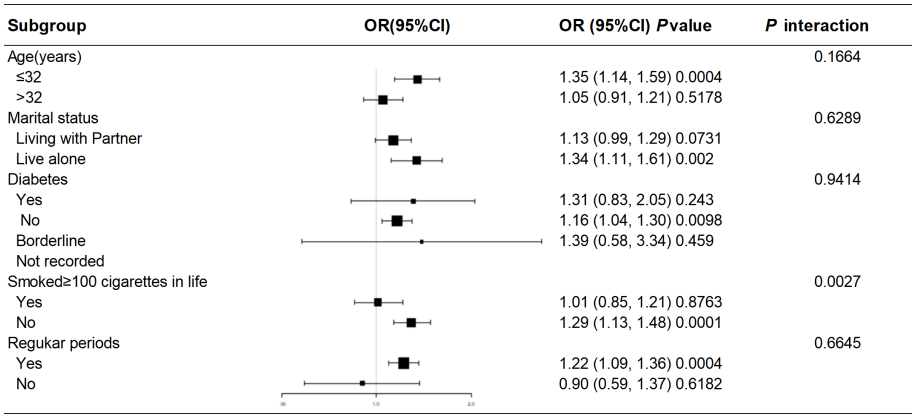


FIGURE 3
Subgroup analysis for the association between BRI and female infertility.

TABLE 3 Comparison of ROC curves for BRI and BMI in predicting infertility.

| Anthropometric Measures | Youden index | Best thresholds | Sensitivity | Specificity | AUC(95%CI) | *P*-value |
|---|---|---|---|---|---|---|
| BRI | 0.1448 | 6.2945 | 0.4360 | 0.7095 | 0.5773 (0.5321,0.6225) | 0.0010 |
| BMI | 0.1277 | 33.4500 | 0.4128 | 0.7149 | 0.5681 (0.5215,0.6147) | 0.0024 |

status, and menstrual regularity, the smoking group showed a significant interaction effect (P for interaction=0.0027), suggesting that smoking status may modulate the effect of BRI on infertility. To further explore the ability of the BRI to predict infertility, we performed a ROC analysis and compared the BRI with BMI in predicting infertility. The BRI was found to be superior to BMI in its ability to predict infertility.

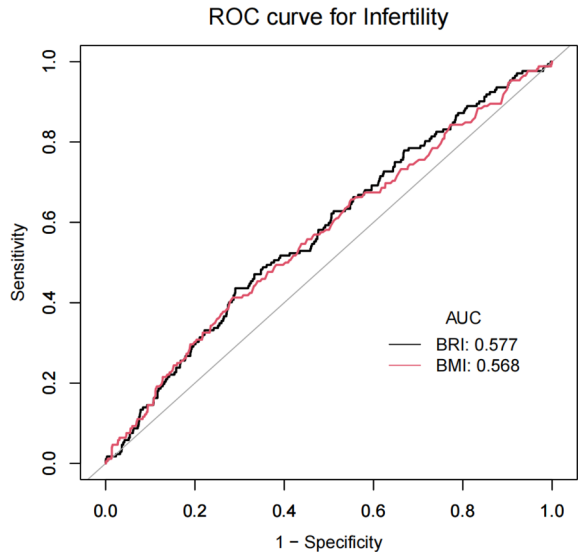


FIGURE 4
Comparison of ROC curves for BRI and BMI in predicting infertility.

In this study, BRI was used as a potential predictor of female infertility in the NHANES cohort. Although previous studies have identified a link between obesity and reproductive dysfunction (10, 11), our study highlights that BRI is more effective than BMI in capturing the effects of central obesity. This distinction is critical because central obesity is more closely associated with metabolic disorders (12), which can impair reproductive function (13). Prior research has indicated that smoking may potentially induce apoptosis of ovarian follicle cells in women, which could subsequently affect the anatomy and function of the uterus and fallopian tubes, among other reproductive organs. This may consequently lead to an increased risk of infertility (14–16). He et al. analyzed data from 3,665 female participants aged 18–45 in the NHANES (2013-2018) study and found that the risk of infertility in smokers was 41.8% higher than in never smokers. However, the results of the subgroup analysis demonstrated that the relationship between smoking status and infertility varied across different ethnic groups and age categories. In particular, the study revealed that the correlation between smoking and infertility was only significant for women aged 25–38 and Mexican Americans (17). The findings of our research demonstrate that BRI exerts a more pronounced effect on the likelihood of infertility in women who do not engage in smoking. The observed correlation between smoking and infertility suggests a possible mechanism by which the two may be associated, but the exact nature of this mechanism requires further investigation. The identification of interactions between smoking and BRI provides new insights into how lifestyle factors influence the relationship between body composition and fertility. Research discussing the positive association between higher BRI and increased risk of infertility is consistent with existing literature on the effects of obesity on reproductive health. Excess adipose tissue disrupts hormonal balance

(18–20), particularly affecting the hypothalamic-pituitary-ovarian axis, which is critical for ovulation (21). The superior diagnostic efficacy of BRI compared to body mass index (BMI) highlights its potential role in the clinical setting. BMI does not differentiate between muscle and fat mass (22), nor does it consider fat distribution. In contrast, BRI provides a more nuanced assessment (23) that better identifies women at risk of infertility due to metabolic complications.As an emerging health indicator, BRI has broad applicability in various contexts. A large retrospective study has demonstrated that an elevated BRI represents a significant risk factor for cardiovascular disease, and the correlation between the two remains statistically significant even after adjusting for potential confounding factors (24). Additionally, inadequate cardiovascular health may result in insufficient blood and nutrient supply to the ovaries and uterus, potentially affecting fertility (25). This provides further evidence for the existence of complex and interrelated pathophysiological mechanisms linking obesity, cardiovascular health, and reproductive function, which are worthy of further research.

The present study should be recognized as having certain limitations. First, its cross-sectional nature limits causal inferences; a longitudinal study is necessary to determine the temporal relationship between BRI and infertility. Secondly, it should be noted that, although the present study focused on the effect of BRI on infertility, it is acknowledged that the occurrence of infertility is a complex phenomenon involving a multitude of factors in both men and women. Consequently, the failure to consider both factors in their entirety represents a limitation of this study. In addition, reliance on self-reported factor measures may have introduced information bias in our findings. Although we adjusted for some confounders, residual confounders from unmeasured variables such as diet or physical activity could not be excluded.

## Conclusion

The results of the study indicate that an elevated BRI is associated with an increased risk of infertility. This association is stronger than that observed between BMI and the risk of infertility. BRI, as a novel indicator of obesity, may serve as a valuable reference indicator for individuals at elevated risk of infertility.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

## Ethics statement

The studies involving humans were approved by The National Center for Health Statistics (NCHS) Ethics Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

## Author contributions

ML: Data curation, Investigation, Software, Writing – original draft, Methodology. YZ: Data curation, Investigation, Methodology, Software, Writing – original draft. JL: Funding acquisition, Resources, Supervision, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Maternal environmental enrichment affects the corpora lutea and progesterone levels in pregnant mice

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**Introduction:** Maternal lifestyle impacts reproductive performance. Previously, we demonstrated that maternal environmental enrichment promotes pregnancy success in BALB/c mice. As progesterone regulates gestation, we decided to study the effect of maternal environmental enrichment on ovarian physiology during early gestation.

**Methods:** For this, six-week-old female mice were housed in enriched or control cages for six weeks and then mated with control fertile males. Females with a mucus plug were returned to their respective control or enriched cages. Pregnant mice were euthanized on day 7 of pregnancy, and ovaries and progesterone levels were investigated.

**Results:** Hematoxylin and eosin slices showed no differences in the area ($\mu m^2$) of the ovaries between control and enriched females. Also, the number of primordial, primary, preantral, antral, and atretic follicles was similar for both treatments. However, the number and area ($\mu m^2$) of corpora lutea were increased in the ovaries from the enriched group. Moreover, enriched females presented higher progesterone serum levels and increased 3β-HSD expression.

**Discussion:** Therefore, maternal environmental enrichment regulates ovarian physiology, and this could promote the benefits previously reported.

KEYWORDS

**maternal enriched environment, gestation, ovary, progesterone, corpora lutea**

# 1 Introduction

The corpus luteum is a transient ovarian endocrine gland generated after ovulation. It produces progesterone, necessary for pregnancy establishment and maintenance. In humans, once it has developed, the placenta becomes the primary source of progesterone. In contrast, the corpus luteum is the main producer of progesterone throughout gestation in rodents (1, 2). Progesterone exerts a crucial role during pregnancy by regulating different processes including the differentiation of the endometrium, the quiescence of the myometrium and the modulation of the immune system (3). Therefore, alterations in the levels of progesterone are associated with difficulties in conceiving and an increased risk of early pregnancy loss or miscarriage (4, 5).

Several studies have shown that lifestyle significantly influences both physiological and pathological events. Moreover, the perinatal environment could impact the quality of gestation, as well as fetal and offspring health (6). In particular, we reported that exposing mice to an enrichment protocol which includes social, physical, and sensory stimuli that enhance their natural behavior, has a beneficial effect on gestation (7). We observed that maternal environmental enrichment regulates uterine physiology, promotes vascular remodeling during early gestation, and prevents pregnancy loss in mice (7). Furthermore, we demonstrated that enrichment of the maternal environment prevents preterm birth in mice, and improves offspring's health (8, 9). Also, other authors informed that environmental enrichment affects the placental response to stress mediators and protects the fetus against an inflammatory stimulus (10). Altogether, this evidence suggests that modifying the maternal environment could improve pregnancy outcome.

Based on our previous results, and considering that many uterine processes are regulated by ovarian activity, we propose that maternal enrichment could also modulate ovarian physiology. We hypothesize that environmental enrichment may enhance oocyte development or the activity of corpora lutea, thereby contributing to improved pregnancy success. Therefore, we decided to study the effect of maternal environmental enrichment on ovarian morphology and progesterone production during early gestation.

# 2 Materials and methods

## 2.1 Ethical statement

The present study was performed under the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The Committee on the Ethics of Animal Experiments of the School of Medicine, University of Buenos Aires (CICUAL; permit number 2547/2019) approved this protocol. Animals were obtained from Bio Fucal S.A. (Buenos Aires, Argentina).

## 2.2 Animals

Six-week-old BALB/c female mice were randomly assigned to either a control or an enriched environment protocol as previously described (7), with 10 animals per group. Control cages (43 × 27 × 17 cm) housed 3 to 4 animals. The enriched cage was larger (64 × 42 × 20 cm) than the control cage and housed 10 females to promote social interaction. The enriched cage contained stairs, tunnels, shelters, running wheels, and other toys made from different materials, which were replaced and rearranged weekly. Additionally, the feeding box was relocated each week to stimulate foraging and exploratory behaviors. This protocol combines non-invasive visual, cognitive, and social stimuli with voluntary physical activity. The animals were housed in control or environmentally enriched cages for 6 weeks. Afterward, females were mated with fertile males of the same strain housed under control conditions. The morning when the vaginal plug was detected was considered day 0 of pregnancy and the females were returned to their respective control or enriched cage. Pregnant animals were euthanized on day 7 of gestation by decapitation. Whole blood was collected and the ovaries were extracted.

## 2.3 Ovarian histology

Ovaries were fixed in Bouin solution overnight, dehydrated in ethanol, and embedded in paraffin. Slices of 5 μm were obtained and separated by 50 μm intervals to avoid counting the same follicle twice. Slices were stained with hematoxylin and eosin to analyze and classify the follicles and corpora lutea under a light microscope (Nikon Eclipse 200).

The structures were classified according to previously established criteria (11, 12) into the following categories: primordial, primary, preantral, antral, and atretic follicles and corpora lutea. The number of follicles and corpora lutea was determined in five sections per ovary and one ovary per animal was analyzed.

Primordial follicles were defined as intact oocytes surrounded by a single layer of flattened follicular cells. Primary follicles presented one layer of cuboidal granulosa cells. Preantral follicles presented two or more layers of granulosa cells and no apparent space between granulosa cells. Antral follicles were characterized by presenting several layers of granulosa cells and the antral cavity. Corpora lutea were characterized by comprising luteal cells, endothelial cells, and immune cells. Atretic follicles were defined as those that exhibited degeneration and detachment of the granulosa cell layer, pyknotic nuclei, and oocyte degeneration. Data are expressed as the total number of each follicle type or corpus luteum counted per ovary. The area of the ovary ($\mu m^2$) and each corpus luteum ($\mu m^2$) was measured using Image-Pro Plus software (version 4.5.0.29, Scion Corporation, Media Cybernetics, Rockville, MD, USA). The corpus luteum area was normalized to the area of the ovary. The mean corpora lutea area per animal was calculated.

## 2.4 Progesterone serum levels measurement

Mice were decapitated and whole blood was collected, allowed to clot for 30 min at room temperature, and then centrifuged at 8000 g for 5 min to obtain serum. The progesterone levels were determined by immunochemiluminescence assay (Biomedical laboratory Dr. Rapela, Buenos Aires, Argentina).

## 2.5 Western blot analysis

Western blot was performed as previously described (7) with minor modifications. One ovary per animal was used. Sixty µg of protein were loaded in each lane and separated in 12,5% SDS-PAGE. The antibodies used were: anti-3β-hydroxysteroid dehydrogenase (3β-HSD) (1:200, Santa Cruz Biotechnologies, catalog #sc-28206), anti-steroidogenic acute regulatory protein (StAR) (1:1000, Novus Biologicals, catalog #NBP1-33485), and peroxidase-conjugated goat anti-rabbit antibody (1:7500, Jackson ImmunoResearch, code 111-035-003). Results were expressed as the relative optical density to total transferred protein determined with Ponceau S Staining Solution.

## 2.6 Statistical analysis

Data was analyzed with t-tests or Mann-Whitney tests using the statistical software Infostat (Facultad de Ciencias Agropecuarias, Universidad de Córdoba, Argentina). Normality and homoscedasticity were assessed using the Shapiro–Wilk and Levene tests, respectively. Mann-Whitney tests were conducted when the data exhibited non-homoscedasticity. One ovary per animal was used for each determination. Four to six mice were used for histological analysis and progesterone quantification, and six to seven for protein studies. Differences were considered statistically significant when the p-value was equal to or less than 0.05.

# 3 Results

First, the effect of maternal environmental enrichment on ovarian structure and size was analyzed. For this, the ovaries were extracted and sectioned for histological studies. The structure of the ovary was conserved in both experimental groups (Figure 1A). The ovaries exhibited distinct borders, well-developed follicles and corpora lutea, and no signs of fibrotic or hemorrhagic tissues. Besides, no differences in size were observed (control: 4.2 $mm^2$ ± 0.2; enriched: 4.3 $mm^2$ ± 0.2).

The number of primordial, primary, preantral, antral, and atretic follicles was analyzed and no differences were detected between the groups (Figures 1B–F). However, ovaries from enriched females presented more corpora lutea than control females (p=0.017) (Figures 1G, H). Also, the size of the corpora lutea was larger in enriched mothers (p=0.049) (Figure 1I).

Subsequently, the progesterone level was determined in maternal serum. Enriched females presented higher progesterone levels than the control group (p=0.038) (Figure 2A).

The ovarian levels of proteins involved in steroidogenesis were determined by western blot. StAR, a rate-limiting protein in steroid production, did not change its expression (p=0.066) (Figure 2B). Nevertheless, the level of 3β-HSD, responsible for progesterone synthesis, was found to be elevated in enriched mothers (p=0.049) (Figure 2C).

# 4 Discussion

In the present study, we demonstrated that female mice exposed to an enriched environment present more corpora lutea, higher progesterone levels, and increased protein expression of 3β-HSD on day 7 of gestation. These results suggest that maternal lifestyle can affect the physiology of the ovary and modulate the production of progesterone, a crucial hormone for maintaining pregnancy.

Results in other animal models support our observations. It has been reported that housing conditions could affect ovarian physiology. Social interaction modulates progesterone levels in non-pregnant mice (13). Also, it was reported that the environment in which gilts are housed modulates the mRNA levels of enzymes involved in steroidogenesis, like StAR and 3β-HSD (14).

In line with these observations, in the present work, we report that enriched females exhibit a higher number of corpora lutea compared to the control group. In mice, the corpus luteum is the principal source of progesterone throughout pregnancy, and it is well-described that progesterone is necessary for maintaining gestation (1). Previously, we reported that enriched females exhibit a higher pregnancy rate than control mothers (7). We described that maternal environmental enrichment improves the maintenance of gestation and prevents pregnancy loss. Therefore, we hypothesize that the higher progesterone serum levels could explain this increase in pregnancy rate, as progesterone is essential to prevent the contraction of the uterine muscles that could interfere with embryo implantation and early gestation. Also, progesterone stimulates decidualization and the secretory activity of the uterine glands necessary for the developing embryo (15).

Additionally, steroid hormones promote uterine vascular remodeling and modulate uterine artery contractility, impacting uterine blood flow during pregnancy (16). Specifically, uterine arteries express progesterone and estrogen receptors and are reactive to these steroids (17). Previously, we observed that these steroids promote the acquisition of the human first-trimester trophoblast endovascular phenotype (18). Also, progesterone stimulates nitric oxide synthase activity and, specifically, endothelial nitric oxide synthesis in endothelial cells (19). Recently, we reported that the maternal environmental enrichment protocol used in the present work induces changes in the micro and macrovasculature that are paralleled by the modulation of molecules involved in vascular processes, like VEGF-A, nitric oxide, and endoglin (7). Therefore, the increase in

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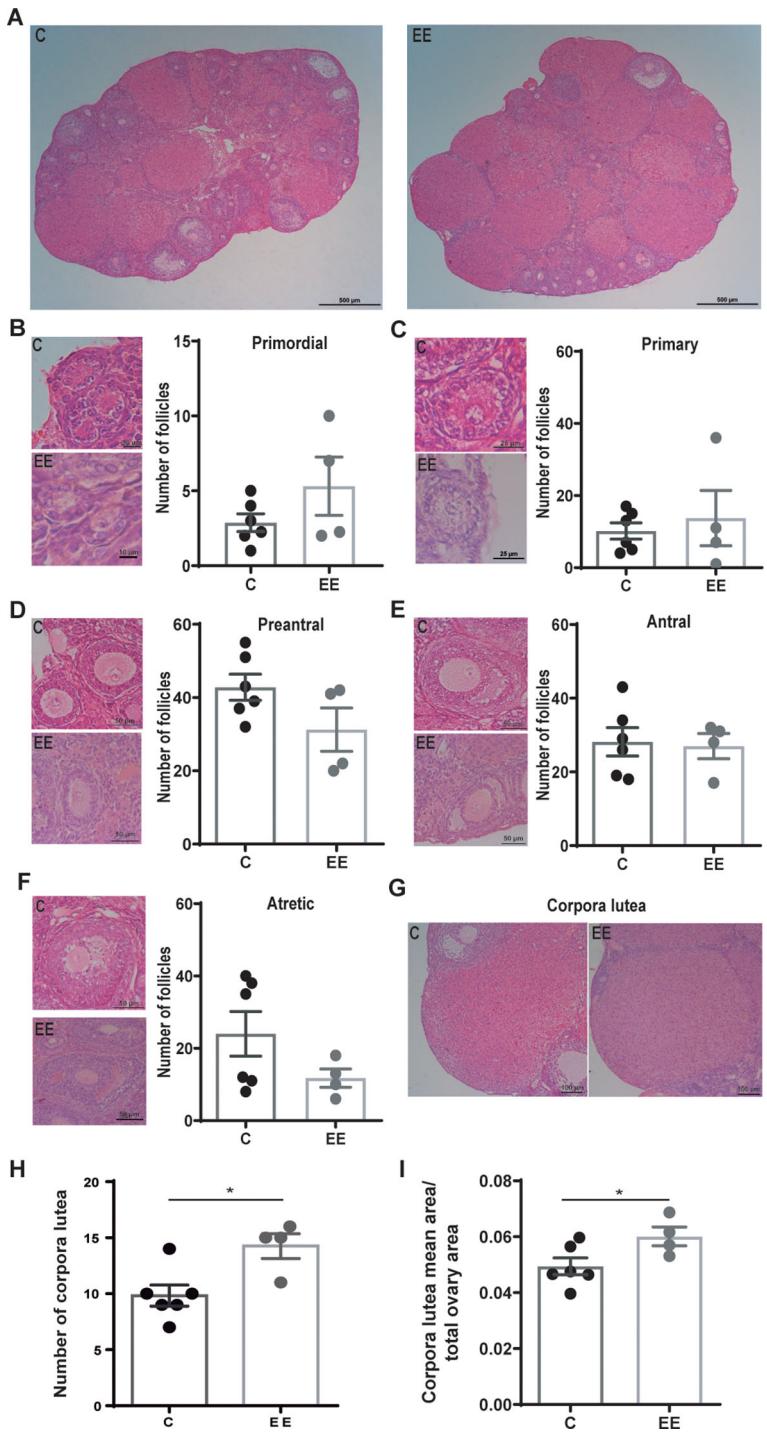


FIGURE 1
Histological analysis of the ovaries. **(A)** Representative images of d7 ovaries from control and enriched environment animals. Representative images and the number of **(B)** primordial, **(C)** primary, **(D)** preantral, **(E)** antral, and **(F)** atretic follicles. **(G)** Representative images of the corpora lutea. **(H)** Number of corpora lutea per ovary. **(I)** The mean area of corpora lutea normalized to the total area of the ovary. Data are expressed as the total number of follicles or corpora lutea per ovary. The area represents the average area of corpora lutea per animal. One ovary per animal was analyzed. N= 6 control and 4 enriched environment animals. Results are expressed as mean ± s.e.m. T-test was performed for the **(D-F, H, I)** panels. Mann-Whitney test was performed for **(B, C)** panels. *Significant statistical differences. C, control; EE, enriched environment.

serum progesterone levels accompanies the stimulation of the vascular adaptations previously observed in enriched females.

Progesterone metabolism could be regulated, among others, by StAR and 3β-HSD proteins. For its part, StAR plays a pivotal role in steroidogenesis, as it is the rate-limiting step in steroid production. It is responsible for transporting cholesterol to the inner membrane of the mitochondria, where the enzymes involved in steroidogenesis are located. This protein is primarily expressed by granulosa and

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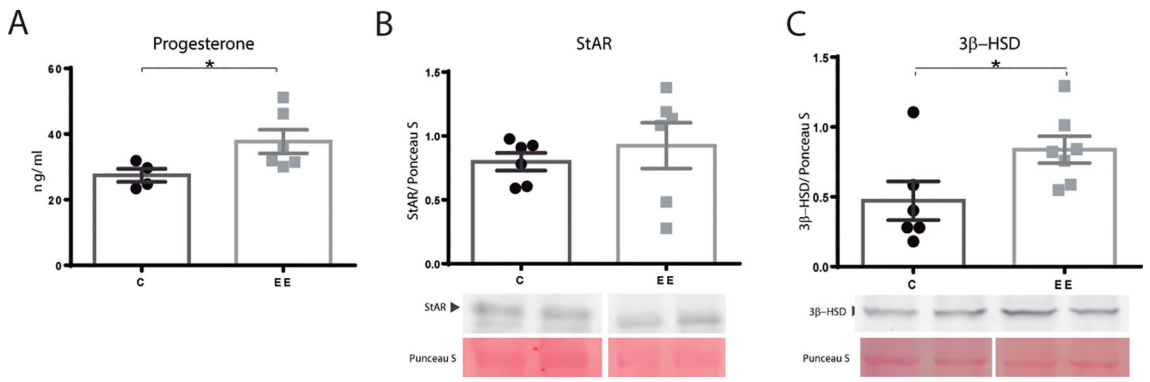


FIGURE 2
Progesterone levels and steroidogenic enzymes. **(A)** Progesterone serum levels. N= 4 control and 6 enriched environment animals. **(B)** StAR and **(C)** 3β-HSD protein levels N=6 control and 7 enriched environment animals. One ovary per animal was analyzed for western blot. C, control; EE, enriched environment. Results are expressed as mean ± s.e.m. T-test. *Significant statistical differences.

theca cells in the follicle (20). In our work, no differences in the levels of this protein were detected. However, enriched females presented higher protein levels of 3β-HSD. This enzyme converts pregnenolone into progesterone and is expressed by granulosa and theca cells in the follicle and luteal cells in the corpus luteum (20). The increase in the protein levels of 3β-HSD could explain the increment in progesterone levels observed in the present work.

The modulation of ovarian physiology is regulated by the hypothalamus and the limbic system, which are sensitive to external stimuli. They respond by releasing neurotransmitters that modulate the hypothalamus-pituitary-gonadal axis. This axis regulates reproductive processes by hormones that affect the ovaries and stimulate follicular development. The intricate interplay between the hypothalamus, the limbic system, and the hypothalamus-pituitary-gonadal axis is vital to maintaining proper reproductive function (21). Another possible mechanism linking how changes in the environment affect ovarian physiology could be the regulation of neurotrophins such as VIP or BDNF. The nervous system produces neurotrophins in response to environmental stimuli. Concerning this, neurotrophin levels are modified by exercise and other lifestyle-related factors (22). Moreover, it is reported that they participate in follicular development, oocyte maturation, ovulation, and maintenance of the corpus luteum. Also, they contribute to the stimulation of steroid hormone production in the ovaries (23, 24). Therefore, we propose that these mechanisms might be involved in the modulation of the ovarian physiology in our model.

In conclusion, our results demonstrate that the enrichment of the maternal environment regulates ovarian physiology. The increase in corpora lutea number and size, as well as progesterone serum levels, provide support for the idea that exposure to an enriched environment exerts a protective role in gestation, preventing embryo loss. Even considering the limitations of an animal model, the positive effects of the enriched environment support the idea that a maternal enrichment protocol could be recommended for women seeking pregnancy. This non-pharmacological intervention might be advantageous, particularly for women undergoing fertility treatments.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The animal study was approved by Committee on the Ethics of Animal Experiments of the School of Medicine, University of Buenos Aires. The study was conducted in accordance with the local legislation and institutional requirements.

## Author contributions

FD: Formal Analysis, Investigation, Writing – original draft. JB: Writing – review & editing, Formal Analysis. JS: Methodology, Writing – review & editing. AS: Resources, Writing – review & editing. FP: Resources, Validation, Writing – review & editing. AF: Funding acquisition, Resources, Writing – review & editing. MR: Conceptualization, Funding acquisition, Project administration, Resources, Writing – original draft, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Explore factors influencing residents' green lifestyle: evidence from the Chinese General Social Survey data

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**Introduction:** This paper explores the multifaceted dynamics and various determinants impacting residents' green lifestyles in China, focusing on drop-off recycling, low-carbon purchasing, and civic advocating.

**Methods:** Using data from the 2021 Chinese General Social Survey and the Chinese Statistical Yearbook, we provide an integrated paradigm to position green lifestyles in a hierarchical framework, explore the individual-level mediating mechanisms nested within macro influences, and elucidate the relative strengths of determinants.

**Results and discussion:** Firstly, the random coefficient regression models show that environmental knowledge and environmental protection intention significantly positively affect green lifestyles. Adopting traditional media could enhance civic advocating, and using new media may bolster low-carbon purchasing tendencies. A higher per capita GDP, increased public expenditure on environmental protection, and a reduced proportion of secondary industry are significant factors that promote drop-off recycling and low-carbon purchasing. Secondly, the multilevel mediational effects identify that people's environmental protection intention is a salient mediating variable within the control of regional macro factors. Additionally, the sheaf coefficient clustering models underscore the prominence of ecological awareness and macro-level factors in shaping drop-off recycling and civic advocating.

KEYWORDS

**green lifestyle, drop-off recycling, low-carbon purchasing, civic advocating, environmental protection intention**

## 1 Introduction

The global environment underwent a large-scale and rapid transformation with the widespread use of fossil fuels, urban expansion, rapid population growth, and the spirit of conquering nature, which have given rise to a series of ecological and environmental problems since the Industrial Revolution (1, 2). A bulk of research has analyzed the immediate and long-term consequences of environmental pollution, ecological degradation, and climate change on mental and physical health (3, 4). This has led to a call for the universal adoption of environmentally friendly behavior and green lifestyles (GL) (5, 6).

GL is defined as a pattern of living that contains thoughts about the uncertain environmental impacts of daily practices and corresponding personal reflections and adaptions (7). It refers to a combination of conservation-oriented behaviors and sustained participation performed by individuals spontaneously and consciously for pro-environmental reasons (8, 9). GL has abundant environmental and sociological

connotations. On the one hand, GL implies one's perception of social status, constituting a facet of identity and self-expression in a specific context, delineated by social class and access to informational resources. On the other hand, GL shows people's subjective proactivity to cope with ecological insecurity, which means that people do not merely adopt isolated and fragmented behavioral decisions but aspire toward multiple systematizations within the spheres of family, occupation, community, online interactions (7, 10). This conceptualization bears significant theoretical and practical implications. The exploration of GL should not be confined to the existing utilitarian perspectives centered around acquiring commodities and services (11); instead, it should be expanded to encompass the ongoing processes.

Previous studies analyze the factors influencing GL from multiple aspects. A meta-analysis of 54 empirical studies organized three categories that pose an impact on green purchase intention, including cognitive factors, consumer characteristics, and social factors (12). Similarly, a systematic review of 151 empirical studies identified that individual concerns, cultural norms, political factors, psychographic traits, ethical values, and product-related variables were significantly correlated with green purchase behaviors and intentions (13). These studies provide foundational variables for understanding GL with a particular focus on the consumption dimension. While important, green consumption represents only one aspect of GL. Other critical components, such as low-barrier everyday environmental behaviors (e.g., recycling or energy-saving habits) and higher-demand civic green political participation (e.g., advocacy or policy engagement), also warrant analysis and discussion to fully capture the multidimensional nature of GL.

Citizens of China show a regrettable inadequacy in environmental knowledge, consciousness, and behaviors, revealing a conspicuous reliance on government interventions to reduce environmental degradation, which is often described as "government-oriented-dependent" (14). However, the government usually lags in mitigating ecological challenges (15). Chinese residents are encouraged to develop and embrace a sustainable and ecologically GL to foster and mobilize public engagement, but they encounter challenges including underdevelopment of green markets, limited availability of green products, poorly constructed resource recycling systems, and barriers to achieving green transformation (16). Studies about single domains of GL aim to analyze how individuals participate in "4 Rs" activities (reducing, reusing, recycling, and purchasing), the extent to which they participate, and their following impacts and group differences (17, 18).

Currently, empirical work in this area predominantly concentrates on advanced industrialized nations and regions (19), leaving a considerable gap in the literature concerning the examination of GL among Chinese citizens. The country offers a social context of collectivism, socialist cultural values and ethics, and government-led environmental management (17). Also, the narrow focus in the previous studies of single pro-environmental behaviors usually implies limited beneficial impact (9), so an organizing taxonomy of social, economic, and political green actions could be applied to explain the incorporation of past/current choices and future decisions.

This paper delves into the contemporary landscape and the various determinants impacting China's GL and contributes to green behavior studies in three ways. First, we adopt an integrated paradigm to position GL in a hierarchical framework with multiple domains. The framework allowed for exploring an individual's engagement in a spectrum of green behaviors, ranging from elementary to intricate, to form a process of gradual life change. Second, we analyzed the multilevel influences stemming from micro and macro factors that shape GL, explored the individual-level mediating mechanisms nested within macro influences, and elucidated the relative strengths of different determinants. It provided empirical support for understanding GL's group differentiation and influence mechanisms. Third, we extended the research scope to the specific context of China, highlighting the regional differences in the governmental role of environmental protection. We also tried to offer practical insights for policymakers seeking to promote sustainable, responsible behavior among Chinese citizens.

## 2 Literature background and conceptual framework

The environmental psychological and sociological paradigms have evolved, developed, and amalgamated from them in response to the increasing attention to prominent green behaviors and lifestyles.

### 2.1 The environmental psychological paradigm

The environmental psychological paradigm examines how individual-level psychological factors influence environmental behaviors. The following three representative theories progress from the generation of environmental awareness, to the development of behavioral patterns, and finally to the activation of specific actions, highlighting psychological drivers and normative influence on individuals' green behaviors.

*The norm activation theory* aims to predict whether personal normative beliefs will motivate individuals to participate in pro-social or altruistic behaviors (20). These normative beliefs often stem from personal education and relevant knowledge, activated by awareness of behavioral consequences and assumed responsibility, serving as the basis for shaping their values to altruism (21). Current investigations concentrate on elements, such as environmental knowledge, self-regulatory emotions, and environmental grievances, in shaping attitudes about green consumption (22–25).

*The value-belief-norm theory* starts from an individual's general code of conduct to lead to the formation of beliefs, which enable people to perceive the threat posed by the possible adverse behavioral outcomes, further evolving into a sense of environmental responsibility and specific eco-friendly behaviors (26–29). Political consciousness is a factor of interest in this theory, as individuals with liberal political values often exhibit stronger

intentions to engage in green behavior and environmental concerns than conservatives (30).

*The theory of planned behavior* posits that behavioral intentions for various actions could be predicted by three key factors: attitude toward the behavior, subjective aspiration, and perceived behavioral control (31). People usually choose a goal and make plans on a continuum from intrinsic to extrinsic, influencing the actual behaviors (32, 33). A two-wave study of Italian workers demonstrated a strong association between intentions and green energy-saving behaviors (34).

We extend the above theories from explaining single environmental behaviors to encompassing an overall GL, and propose the following hypotheses:

**Hypothesis 1.1**: Personal environmental knowledge has a positive relationship with GL.

**Hypothesis 1.2**: Personal environmental protection intention has a positive relationship with GL.

**Hypothesis 1.3**: Personal environmental risk perception has a positive relationship with GL.

Nevertheless, research at the individual level faces criticism of the inconsistency between attitudes and behaviors that people may have positive intentions to recycle, but only a few of them would adopt green practices (35, 36). It is essential to consider the external influences of social factors to gain a more comprehensive understanding of GL.

## 2.2 The environmental sociological paradigm

The environmental sociological paradigm transcends the constraints of individual psychological components, focusing on the external influences of the social environment. Two widely-discussed hypotheses are grounded in the framework of social constructivism, with shared focus on human-environment dynamics and the divergent consequences of social development.

*Constructivism* believes that an environmental phenomenon turns into a social problem when it is constructed by society and accepted by the public through propaganda and social media (37). Especially for Generation Z, the Internet and social media can be leveraged to garner interest and spread information about leading GL, such as accommodating green behavior, recycling, cutting back on electricity use, and eliminating paper use in the workplace (38–40). However, sometimes social media was ineffective in bringing about large-scale behavioral change (41), so, the role of conventional media is still worthy of observation.

*The prosperity hypothesis of economic development* suggests that in societies with higher economic prosperity, the public tends to have greater environmental awareness and engagement in green practices (42). Sixty six studies from 28 countries discovered that in industrialized countries, the intention to behave environmentally was more likely to translate into actual conduct to lead a sustainable lifestyle (43). But some studies also indicate that compared to the citizens of impoverished countries, those in prosperous nations may even be less likely to engage in green consumption and environmental conservation actions due to tax concerns and moral inclination (44, 45).

*The driver hypothesis of environmental pollution* contends that individual environmental behaviors are determined by objective environmental conditions and adaptive actions (46, 47). The more adverse the objective conditions, the more people are awakened to environmental consciousness and behaviors reacting to pollution stimuli and cues (48). The impact was found to be limited, considering the varied environmental knowledge of the public, the imperceptible contaminations, and the national political conditions (49, 50).

We propose the following hypotheses to test the macro-level factors' correlations with residents' GL:

**Hypothesis 2.1**: Regional economic development has a positive relationship with GL.

**Hypothesis 2.2**: Regional pollution conditions have a positive relationship with GL.

**Hypothesis 2.3**: Regional environmental governance expenditures have a positive relationship with GL.

## 2.3 The integrated paradigm in the current research

This paper integrates the environmental psychological and sociological paradigm to address the research gaps identified in the existing literature. We proposed three critical extensions:

First, previous studies on environmental behavior have been frequently separated into the private and public spheres (51, 52) and environmental activism vs. conservation actions (36, 53), or just treats GL as single environmental behaviors, emphasizing consumption, transportation, and energy-saving behaviors. This paper emphasizes the holistic nature of individual engagement in environmental actions by merging environmental behavior into the context of GL. The range comprises low-barrier-to-entry activities such as drop-off recycling, subjectively motivated and decided low-carbon purchases, and civic advocacy that demands a particular degree of self-cultivation achievement.

Second, environmental threats often have latency and imperceptibility when situated within the framework of a risk society (54). The ongoing social changes make it hard to examine the underlying mechanisms of GL. Previous studies have insights in the translation from environmental knowledge to pro-environmental behaviors and found mediating factors of behavioral intentions (18), environmental values (55), and green commitment (56). However, most of these mechanism studies primarily focus on the impact limited to the individual level. This study builds on the theory of planned behavior and takes a step further to explore the pathways and efficacy of individual-level mechanisms structured within macro-level factors. We propose the following hypothesis:

**Hypothesis 3**: Nested within the macro structural influence, personal environmental protection intention mediates the relationship between environmental knowledge and GL.

Third, in light of China's societal transformation, we focus on the role played by government initiatives in improving environmental quality, combating pollution, and encouraging resource conservation. Influenced by the social system and fiscal structure, disparities exist in the public expenditures on

environmental protection between central and local governments in different regions (57). Incorporating government management and the protection hypothesis of public governance into the framework can highlight regional differences in China's environmental management.

We suggest the research framework to analyze the factors of a GL, including three parts: drop-off recycling, low-carbon purchasing, and civic advocating (see Figure 1). Based on the norm activation theory, the theory of planned behavior, and the value-belief-norm theory, we focus on the environmental awareness module and political awareness module at the individual level. The former module includes three variables of environmental knowledge, environmental protection intention, and environmental risk perception, while the latter module consists of democratic awareness, democratic engagement, and governance evaluation. Based on the prosperity hypothesis of economic development, the driver hypothesis of environmental pollution, and the protection hypothesis of public governance, it comprises environmental governance, pollution circumstances, and economic development at the macro level.

Specifically, the present study seeks to address four research questions: (1) What is the current level of GL of Chinese residents? (2) What factors affect living a GL? (3) How do these elements affect GL through specific mechanisms? (4) Is there any difference in the intensity of the factors' influences?

# 3 Research design

## 3.1 Sample

The data utilized in this study were sourced from the 2021 Chinese General Social Survey (CGSS). The CGSS, a nationwide and comprehensive survey in China, systematically collects information from individuals aged 18 and above concerning various aspects, including social and environmental attitudes and actions. Its primary objective is to examine the characteristics and societal trends within the local context and engage in discourse on issues of both theoretical and practical significance. The survey's methodology involved a multi-stage stratified sampling procedure, wherein counties served as the primary sampling units, urban communities, and rural villages as secondary sampling units, and households were selected randomly utilizing a mapping sampling method (58).

The year 2021 was chosen as the focus due to the availability of the latest data pertaining to environmental knowledge, awareness, attitudes, and actions. Of the survey participants, approximately 33.6 percent ($N = 8{,}148$) were randomly assigned to respond to the environmental section, resulting in a final sample size of 2,741. We also included data on provincial environmental conditions from the 2022 China Statistical Yearbook, published by the National Bureau of Statistics of China, to match 2021 individual data with relevant contextual information. The dataset offers comprehensive insights into the evolution of the national society over the preceding calendar year.

## 3.2 Measures

### 3.2.1 Green lifestyle

The measurement of a GL comprises three dimensions. The first is drop-off recycling, measured by how individuals intentionally sort materials such as glass, aluminum cans, plastic, or paper for recycling. The second dimension is low-carbon purchasing, measured by the frequency of individuals

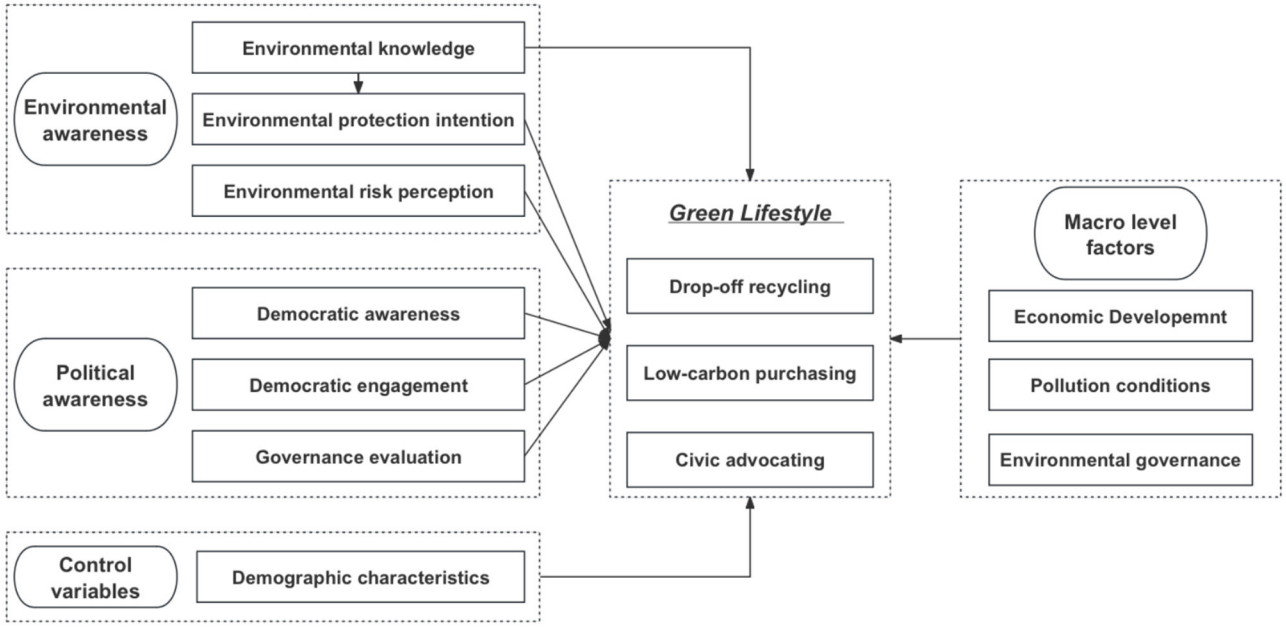


FIGURE 1
The research framework.

deliberately refraining from purchasing certain products for carbon dioxide emission reduction. Both drop-off recycling and low-carbon purchasing behaviors employed positive coding, meaning that the higher the variable values assigned, the more frequent the occurrences of GL. The third dimension is civic advocating, assessed using four binary questions in the CGSS questionnaire: "In the past five years, have you joined any environmental organizations to protect the environment, signed petitions related to environmental issues, made donations to environmental organizations, or participated in protests concerning environmental issues?" The categories were constructed as 0 = No and 1 = Yes, then, these four items were aggregated. A higher total score indicates a more frequent engagement in civic advocating within the context of a GL.

### 3.2.2 Independent variables

#### 3.2.2.1 Environmental awareness

The environmental awareness module comprises three variables: environmental knowledge, environmental risk perception, and environmental protection intention. The environmental knowledge measurement includes ecological civilization, ecological compensation, evaluation and assessment of ecological civilization construction goals, atmospheric pollution prevention and control action plans, and more. Each item was measured into the options: "unaware (=0)," "somewhat aware (=1)," "moderately aware (=2)," "very aware (=3)." Environmental risk perception was measured by the respondents' perception of the severity of 16 types of environmental issues in their residential areas, encompassing air pollution, water pollution, soil pollution, noise pollution, biodiversity degradation, extreme weather events, resource waste, and more. Response options were structured using a 5-point Likert-type scale. For environmental protection intention, the measurement was derived from the question, "What are you willing to do to address various challenges in waste disposal?," with the answers of recycling and reusing household items, being willing to discuss waste classification plans with other residents, proactively engaging with environmental organizations, government agencies, experts, and waste management authorities. Response options were also structured using a 5-point Likert-type scale. In the encoding process, scores for various contents within each variable were summed up so that higher scores denoted greater levels of environmental knowledge, environmental risk perception, and environmental protection intention.

#### 3.2.2.2 Political awareness

The political awareness module also consists of three variables: democratic awareness, democratic engagement, and governance evaluation. Democratic awareness was measured by the statement "The government should not interfere with individual freedom of speech, reproductive freedom, and freedom of work and life." And the answers were coded separately using a 5-point Likert scale. Governance evaluation measured respondents' overall assessment of the central government and local government's performance in addressing environmental issues in China also using a 5-point Likert scale. Higher values for democratic awareness and governance evaluation demonstrate stronger democratic consciousness and more favorable evaluations of government governance by the respondents. The democratic engagement was measured through a binary question of whether to vote in the last neighborhood committee/city village committee election, with the value of 1 denoting actual participation and 0 revealing none.

### 3.2.3 Macro-level variables

Three modules make up the macro-level variables. Economic development was measured using per capita regional gross domestic product (GDP). Indicators of environmental pollution include the share of the secondary industry and major air pollution (the total emissions of sulfur dioxide, nitrogen oxides, and particulate matter). Investments in pollution control and public expenditure on environmental protection are two variables of environmental governance indicators derived from the governmental fiscal spending data in the 2022 China Statistical Yearbook.

Environmental pollution indicators encompass major air pollutant emissions (total emissions of sulfur dioxide, nitrogen oxides, and particulate matter) and the share of the secondary industry. Environmental governance indicators include pollution control investments and public fiscal expenditures on environmental protection.

### 3.2.4 Control variables

Social demographic variables selected in the analysis included gender, age, education, marriage status (coded as married and not married), political identity (coded as member of the Communist Party of China and others), ethnic group (coded as *Han* Chinese and minorities), *hukou* status (coded as urban *hukou* and rural *hukou*), and annual family income (log-transformed). We had a particular focus on media usage variables, explicitly measuring the usage of traditional media (newspapers, magazines, radio, and television) and new media (the Internet and customized mobile messages). The sum of these two constitutes total media usage.

Table 1 provides a summary of the measurements of the main variables in the current analysis.

## 3.3 Analytic strategies

The primary statistical analysis is divided into four main components using Stata 17.0. Firstly, we used descriptive analysis and *t*-tests to illustrate the current state of GL among Chinese residents and to identify key demographic differences.

Secondly, utilizing multilevel linear models, we estimated the influencing factors of drop-off recycling, low-carbon purchasing, and civic advocating behaviors separately, accounting for the effects of macro-level factors. Due to the hierarchical structure of the combined data, the multilevel linear models are appropriate to partition variance at the individual and regional levels, providing insights into how contextual and individual factors influence GL while controlling for intra-regional variability. The random coefficient regressions were set up as follows:

Individual level:

$$Y_{ik} = \delta_{0k} + \delta_{1k}X_{ik} + \delta_z Z_{ik} + \omega_{ik}$$

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TABLE 1 Descriptive statistics ($N = 2,741$).

| | Variables | Mean/ proportion | SD | Range/ categories |
|---|---|---|---|---|
| **Individual level** | | | | |
| Environmental awareness module | Environmental knowledge | 3.48 | 4.83 | [0, 36] |
| | Environmental risk perception | 28.34 | 16.64 | [0, 80] |
| | Environmental protection intention | 22.60 | 4.47 | [6, 30] |
| Political awareness module | Democratic awareness | 8.64 | 2.75 | [0, 15] |
| | Democratic engagement | 0.49 | 0.50 | 0 = not engaged, 1 = engaged |
| | Governance evaluation | 7.20 | 2.32 | [0, 10] |
| Control variables | Gender | 0.46 | 0.50 | 0 = female, 1 = male |
| | Age | 51.60 | 17.62 | [18, 94] |
| | Education | 9.28 | 4.72 | [0, 19] |
| | Marriage status | 0.70 | 0.46 | 0 = not married, 1 = married |
| | Political identity | 0.12 | 0.33 | 0 = others, 1 = CPC |
| | Ethnic group | 0.92 | 0.26 | 0 = minorities, 1 = *Han* |
| | *Hukou* status | 0.41 | 0.49 | 0 = rural, 1 = urban |
| | Income | 10.40 | 2.43 | [0, 16.12] |
| | Use of new media | 3.34 | 2.53 | [0, 8] |
| | Use of traditional media | 4.43 | 2.67 | [0, 16] |
| | Use of all media | 7.77 | 3.82 | [0, 24] |
| **Macro level** | | | | |
| Economic development module | GDP per capita | 8.86 | 3.69 | [4.10, 18.40] |
| Pollution module | Major air pollution | 58.09 | 33.14 | [8.89, 161.95] |
| | Share of the secondary industry | 39.29 | 7.45 | [18, 49.6] |
| Environmental governance module | Investment in pollution control | 11.26 | 9.12 | [0.635, 37.69] |
| | Public expenditure on environmental protection | 198.90 | 70.45 | [47.71, 351.65] |

Provincial level:

$$\delta_{0k} = \sigma_{00} + \vartheta_{0k}$$
$$\delta_{1k} = \sigma_{10} + \vartheta_{1k}$$

where $\omega_{ik}$ represents the individual error term.

Thirdly, employing multilevel mediational modeling methods (59), we investigated the mediating effect of environmental protection intention on the relationship between environmental knowledge and GL after controlling for macro-level factors in the clustered data. The bootstrap method is used with a 95% confidence interval (CI) to distinguish direct effects, indirect effects, and total effects.

Lastly, the sheaf coefficients analysis was conducted after multilevel linear models to compare the differential impact of core explanatory variables at both macro and micro levels. The methodology aims to directly compare impact intensities among factors based on standardized treatments (60).

TABLE 2 Descriptive statistics of green lifestyles.

| Environmental behavior | Sample size | Mean | SD | Range |
|---|---|---|---|---|
| Drop-off Recycling | 2,741 | 2.34 | 1.09 | [1, 4], continuous |
| Low-carbon Purchasing | 2,741 | 2.01 | 0.97 | [1, 4], continuous |
| Civic Advocating | 2,741 | 0.17 | 0.49 | [0, 4], continuous |

# 4 Empirical results

## 4.1 The current state of GL among Chinese residents

The mean values and standard deviations for Chinese citizens' drop-off recycling, low-carbon purchasing, and civil advocacy are shown in Table 2. In sustainable practices, a

TABLE 3 The group differences of green lifestyles.

| | | Drop-off recycling | | Low-carbon purchasing | | Civic advocating | |
|---|---|---|---|---|---|---|---|
| | | Mean (SD) | *T*-value | Mean (SD) | *T*-value | Mean (SD) | *T*-value |
| Gender | Female | 2.30 (1.10) | 1.92* | 2.01 (0.95) | 0.25 | 0.16 (0.47) | 1.15 |
| | Male | 2.38 (1.09) | | 2.02 (0.98) | | 0.18 (0.50) | |
| Political identity | CPC | 2.55 (1.05) | 3.81*** | 2.18 (0.97) | 3.37*** | 0.24 (0.58) | 2.55** |
| | Others | 2.31 (1.10) | | 1.99 (0.96) | | 0.16 (0.47) | |
| Marriage status | Married | 2.38 (1.12) | 3.16*** | 2.00 (0.98) | 1.22 | 0.16 (0.47) | 2.39** |
| | Not married | 2.24 (1.03) | | 2.05 (0.93) | | 0.21 (0.52) | |
| Ethnic group | *Han* Chinese | 2.34 (1.09) | 1.25 | 2.01 (0.97) | 0.03 | 0.16 (0.09) | 3.02** |
| | Minorities | 2.25 (1.10) | | 2.01 (0.98) | | 0.27 (0.62) | |

Significance levels: $^{+}p < 0.1$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (Two-tailed test).

discernible pattern emerges, showcasing the varying frequencies of environmentally conscious behaviors. Drop-off recycling demonstrated the most prevalent and frequent occurrence ($mean = 2.34$, $SD = 1.09$), and low-carbon purchasing followed closely but with slightly less frequency ($mean = 2.01$, $SD = 0.97$). Civic advocating was observed to be the least frequent of GL ($mean = 0.17$, $SD = 0.49$), for it involves a myriad of complex factors, encompassing public environmental inclusivity, civic behavioral norms, and civic traditions (61). This hierarchy of environmentally responsible actions reflects an intriguing spectrum of engagement with ecological concerns, highlighting the diverse approaches individuals undertake to pursue a greener, more sustainable world.

Additionally, we looked at the demographic variations between the three eco-friendly lifestyles (see Table 3). Men had a significantly higher frequency of drop-off recycling than women $(t = 1.92, p < 0.05)$, while significant gender disparities were not evident in the case of the other two GLs. Members of CPC were more prone to practicing GL. The mean value of drop-off recycling $(t = 3.81, p < 0.001)$, low-carbon-purchasing $(t = 3.37, p < 0.001)$, and civic advocating $(t = 2.55, p < 0.01)$were significantly higher than that of non-affiliated individuals, which might be closely connected with the CPC norms and standards. Compared with not married people, married people placed more emphasis on drop-off recycling $(t = 3.16, p < 0.001)$ but less likely on civic advocating $(t = 2.39, p < 0.01)$. The minorities were more likely to participate in civic advocating $(t = 3.02, p < 0.01)$ than the *Han Chinese*.

## 4.2 Factors influencing GL

We performed multilevel models to scrutinize the interplay between macro-level and individual-level factors in shaping GL. With a specific focus on drop-off recycling (see Table 4), environmental knowledge emerged as a pivotal driver, with statistically significant coefficients ranging from 0.007 to 0.012. The correlation between environmental protection intention and drop-off recycling was strong and robust across models, with the coefficients ranging from 0.055 to 0.060. In Models 1–2, the influence of all media usage on drop-off recycling was significant $(\beta = 0.023, p < 0.001)$. Models 1–3 displayed the negative correlation between democratic awareness and drop-off recycling in the political awareness module $(\beta = -0.022, p < 0.01)$, and the relationship was consistent in the Models 1–7 ($\beta = -0.021$, $p < 0.05$). The influence of economic development, as represented by GDP per capita, aligned with the prosperity hypothesis, revealing a positive correlation with drop-off recycling in Models 1–4 $(\beta = 0.056, p < 0.001)$, and the influence was still significant in the full model. Similarly, the protection hypothesis of public governance was also supported by Models 1–6 that a higher allocation of the public expenditure on environmental protection was related to more drop-off recycling $(\beta = 0.002, p < 0.05)$. The driver hypothesis of environmental pollution was not supported by Models 1–5, since the share of the secondary industry was negatively associated with drop-off recycling $(\beta = -0.017, p < 0.05)$.

In the random effects, the models revealed consistently significant variances, ranging from 0.160 to 0.277, indicating that the variations in macro-level factors could explain about one-fifth of the provincial differences in personal drop-off recycling practices.

In the context of low-carbon purchasing (as depicted in Table 5), it demonstrated positive of new media usage on low-carbon purchasing $(\beta = 0.028, p < 0.01)$, signifying the potential of contemporary communication platforms in fostering eco-conscious consumer behavior. Within the sphere of political awareness, a distinctive pattern emerged in contrast to our prior findings in drop-off recycling. Here, low-carbon

TABLE 4 The multilevel models of drop-off recycling (Groups = 19).

| Variables | Model 1-1 | Model 1-2 | Model 1-3 | Model 1-4 | Model 1-5 | Model 1-6 | Model 1-7 |
|---|---|---|---|---|---|---|---|
| **Fixed effects** | | | | | | | |
| Environmental knowledge | 0.012* (0.005) | 0.009+ (0.005) | 0.009+ (0.005) | 0.011* (0.005) | 0.012* (0.005) | 0.012* (0.005) | 0.007 (0.005) |
| Environmental risk perception | 0.001 (0.001) | 0.001 (0.001) | 0.001 (0.001) | 0.001 (0.001) | 0.001 (0.001) | 0.001 (0.001) | 0.001 (0.001) |
| Environmental protection intention | 0.060*** (0.005) | 0.058*** (0.005) | 0.057*** (0.005) | 0.060*** (0.005) | 0.060*** (0.005) | 0.060*** (0.005) | 0.055*** (0.005) |
| Use of all media | | 0.023*** (0.007) | | | | | 0.021** (0.007) |
| Democratic awareness | | | −0.022** (0.008) | | | | −0.021* (0.008) |
| Democratic engagement | | | 0.035 (0.048) | | | | 0.030 (0.048) |
| Governance evaluation | | | 0.005 (0.011) | | | | 0.004 (0.011) |
| GDP per capita | | | | 0.056*** (0.014) | | | 0.030+ (0.017) |
| Major air pollution | | | | | −0.001 (0.001) | | −0.001 (0.002) |
| Share of secondary industry | | | | | −0.017* (0.009) | | −0.013+ (0.007) |
| Investment in pollution control | | | | | | −0.006 (0.007) | 0.002 (0.006) |
| Public expenditure on environmental protection | | | | | | 0.002* (0.001) | 0.001* (0.001) |
| Control variables | Y | Y | Y | Y | Y | Y | Y |
| **Random effects** | | | | | | | |
| Variance | 0.272*** (0.051) | 0.270*** (0.051) | 0.277*** (0.052) | 0.185*** (0.042) | 0.229*** (0.045) | 0.232*** (0.045) | 0.160*** (0.037) |
| Log-likelihood | −3,061.27 | −3,055.87 | −2,977.77 | −3,055.82 | −3,058.53 | −3,058.53 | −2,965.27 |
| Wald $\chi^2$ | 186.27 *** | 198.08 *** | 184.86 *** | 206.63 *** | 194.47 *** | 194.19 *** | 227.64 *** |
| Observations | 2,124 | 2,124 | 2,068 | 2,124 | 2,124 | 2,124 | 2,068 |

The cells show the coefficients and standard errors of variables. The variance component in the null model is 0.087*** with an Interclass Correlation Coefficient (ICC) of 0.073. Y means the variables are controlled. Significance levels: + p<0.1, * p<0.05, ** p<0.01, *** p<0.001 (Two-tailed test). All the results in the paper are interpreted with the same standard.

purchasing exhibited a favorable correlation with governance evaluation $(\beta = 0.024,\ p < 0.05)$. Similar to drop-off recycling, the prosperity hypothesis of economic development and the conservation hypothesis of public governance were supported as higher levels of GDP per capita $(\beta = 0.038,\ p < 0.001)$ and public expenditure on environmental protection $(\beta = 0.002,\ p < 0.01)$ were associated with increasing low-carbon purchasing. Except for the full model, the reduction of variance in random effects was the most in the economic development module (Models 2–4), signifying the importance of using economic conditions to explain the regional variations in low-carbon purchase behaviors.

Moving to civic advocating, as presented in Table 6, it is noteworthy that three variables within the environmental awareness module exhibited significant and positive correlations with increased civic advocacy. Compared to the coefficients associated with drop-off recycling and low-carbon purchasing, the coefficients relating to environmental protection intention and its impact on civic advocacy were observed to be more moderate $(\beta = 0.012,\ p < 0.001)$. Traditional media usage was positively correlated to civic advocating $(\beta = 0.010,\ p < 0.05)$. When considering political awareness, a higher degree of democratic engagement was significantly associated with increased civic advocacy $(\beta = 0.057,\ p < 0.01)$. However, in contrast to the outcomes mentioned above, the influence of macro-level factors on civic advocacy did not yield statistically significant results.

The random effects echoed the descriptive statistics results of three GLs. While the variances were statistically significant, the coefficients were relatively small. It suggests that a substantial portion of the variance by other relevant factors and should be taken into account in further research.

TABLE 5 The multilevel models of low-carbon purchasing (Groups = 19).

| Variables | Model 2-1 | Model 2-2 | Model 2-3 | Model 2-4 | Model 2-5 | Model 2-6 | Model 2-7 |
|---|---|---|---|---|---|---|---|
| **Fixed effects** | | | | | | | |
| Environmental knowledge | 0.019*** (0.004) | 0.018*** (0.005) | 0.016** (0.005) | 0.019*** (0.004) | 0.019*** (0.004) | 0.019*** (0.004) | 0.015** (0.005) |
| Environmental risk perception | 0.004** (0.001) | 0.004** (0.001) | 0.004** (0.001) | 0.004** (0.001) | 0.004** (0.001) | 0.004** (0.001) | 0.004** (0.001) |
| Environmental protection intention | 0.059*** (0.005) | 0.058*** (0.005) | 0.056*** (0.005) | 0.059*** (0.005) | 0.059*** (0.005) | 0.060*** (0.005) | 0.056*** (0.005) |
| Use of new media | | 0.028** (0.010) | | | | | 0.027** (0.010) |
| Democratic awareness | | | −0.000 (0.007) | | | | 0.000 (0.007) |
| Democratic engagement | | | 0.053 (0.043) | | | | 0.053 (0.042) |
| Governance evaluation | | | 0.024* (0.009) | | | | 0.024* (0.009) |
| GDP per capita | | | | 0.038*** (0.009) | | | −0.002 (0.029) |
| Major air pollution | | | | | −0.002 (0.001) | | −0.002+ (0.000) |
| Share of secondary industry | | | | | −0.012* (0.005) | | −0.013 (0.012) |
| Investment in pollution control | | | | | | −0.006 (0.004) | 0.001 (0.003) |
| Public expenditure on environmental protection | | | | | | 0.002** (0.000) | 0.002*** (0.001) |
| Control variables | Y | Y | Y | Y | Y | Y | Y |
| **Random effects** | | | | | | | |
| Variance | 0.176*** (0.036) | 0.176*** (0.036) | 0.175*** (0.036) | 0.109*** (0.032) | 0.131*** (0.032) | 0.126*** (0.030) | 0.040* (0.036) |
| Log-likelihood | −2,790.07 | −2,786.14 | −2,714.49 | −2,784.71 | −2,786.26 | −2,785.15 | −2,697.28 |
| Wald $\chi^2$ | 283.30*** | 292.21*** | 278.91*** | 306.37*** | 296.83*** | 300.37*** | 382.63*** |
| Observations | 2,124 | 2,124 | 2,068 | 2,124 | 2,124 | 2,124 | 2,068 |

Significance levels: $^{+}p < 0.1$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (Two-tailed test).

## 4.3 The mechanism of conducting GL

In our preceding regression analysis, we refrained from presenting the outcomes of introducing independent variables sequentially in order to conserve space. Actually, when these variables were systematically integrated into the model, the magnitude of environmental knowledge's influence on GL diminished after the inclusion of the environmental protection intention variable. This observation underscores the pivotal role of environmental protection intention as a significant mediating variable through which environmental knowledge exerts its influence on GL. Also, the result aligns with the propositions of the theory of planned behavior, which suggests that individuals' environmental knowledge is activated by their environmental protection intention, thereby translating into actual GL practices. The further question is whether this mechanism still holds when embedded within the influence of external environmental factors.

In contrast to conventional mediation analysis, the multilevel mediation approach directs its attention to the hierarchical structure inherent in the dataset. It not only accommodates the clustered nature of the data but also endeavors to illuminate the intricate mechanism between variables at the micro-level within the broader macro-context (59). As such, it is crucial to emphasize the specific advantages and nuances of multilevel mediation analysis and highlight its relevance in offering a more sophisticated statistical framework.

The veracity of mediation was substantiated through the results of the bootstrap tests (see Table 7). The indirect effects of environmental protection intention on drop-off recycling, low-carbon purchasing, and civic advocating have all demonstrated statistical significance. The most substantial proportion of the total effects mediated pertained to drop-off recycling, accounting for 43.44%. Regarding low-carbon purchasing, 29.0% of the total effect was mediated by environmental protection intention. Civic advocacy, in comparison, exhibited a proportion of total effect

TABLE 6 The multilevel models of civic advocating (groups = 19).

| Variables | Model 3-1 | Model 3-2 | Model 3-3 | Model 3-4 | Model 3-5 | Model 3-6 | Model 3-7 |
|---|---|---|---|---|---|---|---|
| **Fixed effects** | | | | | | | |
| Environmental knowledge | 0.019*** (0.002) | 0.018*** (0.002) | 0.017*** (0.002) | 0.018*** (0.002) | 0.018*** (0.002) | 0.018*** (0.002) | 0.016*** (0.002) |
| Environmental risk perception | 0.002** (0.001) | 0.002** (0.001) | 0.002** (0.001) | 0.002** (0.001) | 0.002** (0.001) | 0.002** (0.001) | 0.002** (0.001) |
| Environmental protection intention | 0.012*** (0.002) | 0.012*** (0.002) | 0.012*** (0.002) | 0.012*** (0.002) | 0.012*** (0.002) | 0.012*** (0.002) | 0.012*** (0.002) |
| Use of traditional media | | 0.010* (0.004) | | | | | 0.009* (0.004) |
| Democratic awareness | | | −0.004 (0.004) | | | | −0.003 (0.004) |
| Democratic engagement | | | 0.057** (0.022) | | | | 0.057** (0.022) |
| Governance evaluation | | | 0.007 (0.005) | | | | −0.000 (0.005) |
| GDP per capita | | | | −0.004 (0.005) | | | −0.012* (0.005) |
| Major air pollution | | | | | −0.000 (0.000) | | −0.001$^{+}$ (0.000) |
| Share of secondary industry | | | | | 0.001 (0.003) | | −0.002 (0.002) |
| Investment in pollution control | | | | | | −0.000 (0.002) | 0.002 (0.002) |
| Public expenditure on environmental protection | | | | | | −0.000 (0.000) | 0.000 (0.000) |
| Control variables | Y | Y | Y | Y | Y | Y | Y |
| **Random effects** | | | | | | | |
| Variance | 0.055*** (0.015) | 0.055*** (0.015) | 0.049*** (0.015) | 0.054*** (0.015) | 0.053*** (0.015) | 0.055*** (0.015) | 0.038* (0.015) |
| Log-likelihood | −1,422.36 | −1,419.70 | −1,358.27 | −1,421.97 | −1,421.94 | −1,422.35 | −1,353.59 |
| Wald $\chi^2$ | 179.76*** | 185.54*** | 175.18*** | 180.51*** | 180.57*** | 179.78*** | 185.77*** |
| Observations | 2,124 | 2,124 | 2,068 | 2,124 | 2,124 | 2,124 | 2,068 |

Significance levels: $^{+}p < 0.1$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (Two-tailed test).

mediated at 7.28%. Further insight into the coefficients of the mediational pathways can be seen in Figure 2.

## 4.4 The differential impact of the core factors

We employed the sheaf coefficients clustering method to investigate disparities in the intensity of influence among the three factors contributing to GL, using calibration based on a multilevel linear model (see Table 8).

Concerning drop-off recycling, variables within the environmental awareness module, the macro-variable module, and the political awareness module all exhibited statistically significant associations with environmentally friendly behavior. The influence of the first two modules was significantly greater than that of the political awareness module, with effect sizes of 4.06 and 4.03 times, respectively. For low-carbon purchasing, a significant difference was observed between all three modules. Environmental awareness had the most substantial impact, with an effect size of 4.62 times that of political awareness. For civic advocating, the coefficients of influence from each module exhibited a reduction in comparison to the other aspects of GL. The difference between the influence of macro variables and political awareness was not statistically significant $(\chi^2 = 0.01,\ p > 0.05)$.

Among the factors shaping environmental behavior, environmental awareness yielded the most potent influence, while political awareness had the weakest impact. The macro-environment exerted more influence on drop-off recycling and low-carbon purchasing compared to civic advocating.

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TABLE 7 The multilevel mediation effects of environmental protection intention.

| | | Coefficients (Standard Error) | Bootstrap 95%CI | | Proportion of total effect mediated |
|---|---|---|---|---|---|
| | | | LL | UL | |
| Drop-off Recycling | Total effect | 0.015** (0.006) | 0.004 | 0.026 | 43.44% |
| | Direct effect | 0.009 (0.006) | −0.002 | 0.020 | |
| | Indirect effect | 0.007*** (0.001) | 0.004 | 0.009 | |
| Low-carbon Purchasing | Total effect | 0.026*** (0.004) | 0.019 | 0.033 | 29.10% |
| | Direct effect | 0.018*** (0.004) | 0.011 | 0.025 | |
| | Indirect effect | 0.008*** (0.001) | 0.005 | 0.010 | |
| Civic advocating | Total effect | 0.019*** (0.004) | 0.012 | 0.026 | 7.28% |
| | Direct effect | 0.018*** (0.004) | 0.011 | 0.025 | |
| | Indirect effect | 0.001** (0.000) | 0.001 | 0.002 | |

Bootstrap was performed in each group with repeated sampling of 100 times. Significance levels: $^{+}p < 0.1$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (Two-tailed test).

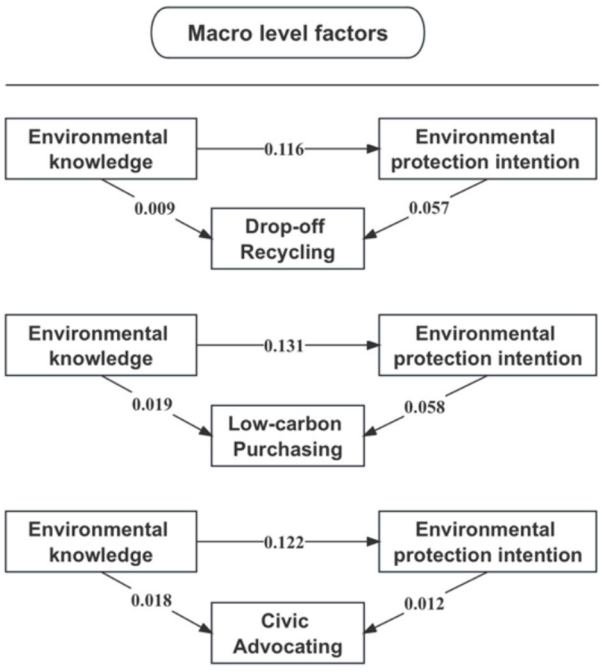


FIGURE 2
The multilevel mediation effects of environmental protection intention on green lifestyles.

## 5 Discussion

This study, grounded in the persistent contradiction between ongoing economic development and escalating environmental pollution, scrutinized the influencing factors on residents' GL at micro and macro levels, elucidated the functioning mechanisms, and assessed the relative strengths of these influences, all aimed at augmenting people's adoption of a GL. The key findings of this research can be summarized as follows.

Firstly, residents exhibited a moderately low level of GL practices, with civic advocating being notably limited, considerably lower than those in Europe and Japan, where people have multiple carbon footprint reduction potential of lifestyle choices (62, 63). The members of the CPC demonstrated significantly higher levels of engagement across the three GL dimensions compared to non-party members, whose discrepancy can be attributed to income and educational attainment, along with social capital and network.

Secondly, environmental knowledge and environmental protection intentions consistently displayed significant effects among the micro-level factors influencing residents' GL. When controlling for macro-level factors, converting environmental knowledge into tangible green behaviors primarily hinged on elevating environmental protection intentions. While a meta-analysis focusing on international research in 2004–2014 finds that in developed and individualistic countries, environmental protection intentions are more likely to translate into actual behavior (43), the current study provides evidence for developing and collectivist countries.

Education offered an institutional framework (39) within which individuals were inspired by environmental knowledge to expand the scale and quality of GL. Media utilization served as a non-institutional means (40). It showed variations in shaping different forms of GL: new media increased low-carbon purchasing,

TABLE 8 The comparison of factors of green behaviors (sheaf coefficients).

| | | Drop-off recycling | Low-carbon purchasing | Civic advocating |
|---|---|---|---|---|
| Environmental awareness module (M1) | | 0.252*** (0.024) | 0.282*** (0.022) | 0.112*** (0.012) |
| Political awareness module (M2) | | 0.062** (0.024) | 0.061** (0.021) | 0.030** (0.011) |
| Macro factors module (M3) | | 0.250*** (0.050) | 0.180*** (0.023) | 0.032* (0.014) |
| Differences | M1 vs. M2 | $\chi^2 = 27.29^{***}$ | $\chi^2 = 44.19^{***}$ | $\chi^2 = 22.62^{***}$ |
| | M1 vs. M3 | $\chi^2 = 0.00$ | $\chi^2 = 10.04^{**}$ | $\chi^2 = 19.78^{***}$ |
| | M2 vs. M3 | $\chi^2 = 11.78^{***}$ | $\chi^2 = 14.43^{***}$ | $\chi^2 = 0.01$ |

Significance levels: $^{+}p < 0.1$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (Two-tailed test).

while traditional media promoted engagement in civic advocating. Media propaganda fostered rational and scientific perceptions of environmental issues and events and disseminated information from non-governmental organizations. It would be beneficial to call for green consumption behaviors through new media while capitalizing on the expertise and credibility of traditional media to establish public participation channels. As for the influence of political awareness, its impact was multifaceted, reflecting the nascent stage of political democratic awareness and behavior among the Chinese population. Unlike liberalism's positive effect on green purchase behavior and political engagement in socio-ecological issues (13), conservative attitudes and reliance on government intervention rendered their effect on GL rather subtle.

Thirdly, within macro-level factors, the significantly positive impact of financial expenditures and economic development levels underscored the need to strengthen environmental governance, promote social development, and fully leverage government fiscal protective roles. Nevertheless, it is imperative to assess the extent of government involvement in environmental protection endeavors to circumvent potential infringements on individual agencies within the public domain.

We acknowledge several limitations of the study. Firstly, the discussion of macro-level factors and their internal linkages could be more extensive to explore potential macro-micro moderating effects. Additional pathways such as social norms and post-materialism ideology could assist in a deeper understanding of the mechanism underlying GL. A richer and more nuanced framework including moderating and mediating effects would provide a more holistic perspective on GL. Secondly, our study was limited to establishing correlational relationships rather than causal analyses. Future studies could build on the findings by leveraging longitudinal datasets when they become accessible. The cross-sectional data in the present study lack a temporal dimension and therefore cannot analyze the dynamic relationships between variables over time, which is a key strength of random effects models in longitudinal data. Thirdly, we believe that seeking the spatiotemporal patterns of GL within the country or internationally is a promising field of research and is worthy of further analysis. It would capture more cultural, institutional, and social differences and expand the applicability of our findings in China. Moreover, we must approach the results derived from post-COVID-19 data with caution. The pandemic likely caused both temporary and long-term shifts in behavior, such as a reduction in carbon footprints, disruption of social norms, and rise of digital and remote behaviors. Future studies could explore these impacts by comparing pre- and post-pandemic data to understand how GL has evolved in response to the pandemic.

There is a need for targeted environmental education campaigns to improve environmental knowledge and foster green lifestyles at the individual level with the assistance of NGOs, public welfare organizations, and online communities. Meanwhile, recognizing the differential impact of traditional and new media on civic engagement and daily practices, policymakers should leverage both forms of media and establish partnerships with media outlets to enhance the power of media influence. They can form partnerships with Internet influencers to normalize sustainable consumption behaviors among younger generations and increase online green communities. As for the macro-level implications, policymakers should prioritize economic diversification and allocate sufficient funds to environmental protection initiatives, including optimizing green taxation and subsidies, adopting region-specific sustainable strategies, promoting innovative carbon-reduction technologies, reducing reliance on secondary industry, and fostering a transition for a greener economy. Future studies might explore how specific green policies, such as waste sorting and recycling initiatives, influence residents' GL across provinces and districts. It would address gaps in spatiotemporal patterns or causal analysis by incorporating fine-grained spatial and longitudinal data.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

## Author contributions

YL: Conceptualization, Formal analysis, Visualization, Writing – original draft. JL: Conceptualization, Methodology, Formal analysis, Writing – original draft. CL: Funding acquisition, Supervision, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Generative AI statement

The author(s) declare that no Gen AI was used in the creation of this manuscript.

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# Deciphering the association between the Life's Essential 8 and infertility: insights into depression, inflammation, and metabolic mediation

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**Background:** The Life's Essential 8 (LE8) score has been associated with various health outcomes, but its relationship with female infertility remains unclear.

**Methods:** This study investigated the relationship between LE8 and infertility in women aged 20-45 years using the National Health and Nutrition Examination Survey (NHANES) data from 2013 to 2020. Weighted multifactorial logistic regression models were utilized to examine the association between the LE8 factors and their two subgroups [health behavior score (HBS) and health factor score (HFS)], as well as depressive status and infertility. Nonlinear relationships were examined using weighted restricted cubic spline (RCS) regression. Subgroup analysis and mediation analysis of depression, metabolic, and inflammation further elucidated the relationships. Sensitivity analysis was conducted to ensure the robustness of the findings.

**Results:** A total of 2,182 participants were included in this study, with 304 in the infertility cohort. Multifactorial regression analysis revealed significant negative correlations between LE8 and its subgroup HFS with infertility. Among the eight subscales, scores for sleep health, body mass index, and blood glucose were significantly negatively correlated with depression, while Patient Health Questionnaire-9 (PHQ-9) scores showed a positive correlation with infertility. Weighted RCS regression modeling indicated no nonlinear relationships between LE8, depression, HFS, HBS, and infertility. Mediation analyses suggested that depression scores, systemic immune inflammation index (SII), and uric acid (UA) mediated the association between LE8 and infertility.

**Conclusion:** Higher LE8 scores, indicating better cardiovascular health, are associated with lower depression scores and reduced levels of SII and UA. These factors collectively contribute to a lower risk of infertility in women. Targeted interventions aimed at enhancing cardiovascular health may potentially mitigate infertility risk through these pathways.

KEYWORDS

**cardiovascular health, infertility, Life's Essential 8, inflammation, depression, metabolism**

# 1 Introduction

Infertility, characterized by the inability to conceive after at least 12 months of unprotected intercourse (1), affects approximately 15% of couples worldwide (2–4). Beyond its central role in reproductive health, infertility profoundly impacts the physical and mental well-being of affected women, leading to distress, depression, and declining birth rates (5–7). The etiology of infertility is multifaceted, encompassing conditions such as premature ovarian insufficiency (4, 8), polycystic ovary syndrome (PCOS) (1), uterine fibroids, endometriosis (9, 10), and endometrial polyps. Recently, lifestyle factors have gained attention for their potential role in impairing fertility through inflammatory responses (11, 12).

Infertility extends beyond reproductive health, significantly affecting the overall physical, emotional, and social well-being of individuals and families. Shared risk factors between infertility and cardiovascular disease (CVD) include tobacco use, diet quality, and obesity. Thus, linking infertility with these factors could provide valuable markers for early cardiovascular health (CVH) screening and preventive efforts. CVD remains the leading cause of mortality in the United States (13). PCOS, a known cause of infertility, is associated with impaired glucose tolerance and CVD (14). Approximately 25% of female infertility cases are attributed to anovulation related to PCOS, with many cases remaining unexplained. Previous evidence has uncovered a notable correlation between infertility and CVH (15). However, the relationship between CVH metrics and infertility lacks solid evidence, warranting further exploration to elucidate the implications of CVD prevention for women grappling with infertility.

In 2022, the American Heart Association (AHA) introduced Life's Essential 8 (LE8), incorporating sleep health and expanding upon the previous Life's Sample 7 (LS7) health domains (16). LE8 categorizes individuals into poor, intermediate, and ideal levels based on eight CVD risk factors and behaviors: smoking, physical activity, BMI, total cholesterol, fasting glucose, blood pressure, diet, and sleep. Research by Wang et al. has shown an inverse relationship between CVH and infertility (17). A recent study also indicated an association between a history of infertility and lower overall and biomedical CVH scores (15). However, the relationship between LE8 and infertility remains underexplored.

This study aims to investigate the relationship between LE8 and infertility among women aged 20-45 in the United States. We hypothesize that inflammation, depression and metabolism mediate this relationship. Through a dissection of the interplay among LE8, inflammation, metabolism, and infertility, our aim is to explicate the roles of inflammation, depression and metabolism in this relationship. Understanding these connections will not only aid in preventing and managing cardiovascular diseases but also inform comprehensive infertility treatment strategies, thereby enhancing overall health outcomes for affected women.

# 2 Methods

## 2.1 Data sources

This study was conducted based on data from the National Health and Nutrition Examination Survey (NHANES) database, a continuous, multistage, cross-sectional health survey program manage by the Centers for Disease Control and Prevention (CDC) to evaluate the health status and nutritional intake of the US population (18). The NHANES program adhered to strict uniform protocols and standards, and researchers received specialized training to ensure the high quality of questionnaire and physical examination data. The NHANES protocol was approved by the National Center for Health Statistics (NCHS) Ethics Review Board, and all participants provided written informed consent (19).

## 2.2 Study design

As a cross-sectional study, we strictly followed to the STROBE Statement for cross-sectional studies. Participants from the 2013 to 2020 NHANES were selected for this study. Out of the 44,960 subjects enrolled in NHANES during these years, 5,812 female participants aged 20-45 years were selected based on study requirements. Due to missing infertility diagnosis data, 888 participants were excluded. An additional 2,574 subjects were excluded due to incomplete LE8 score data. Furthermore, 168 participants were excluded for missing covariate information, such as the Patient Health Questionnaire-9 (PHQ-9) scale information, marital status, family economic status, and education. Following these screening criteria, a total of 2,182 participants with complete information were included in the study. Among these participants, 304 had a history of infertility (Figure 1).

## 2.3 Data collection and variable definitions

### 2.3.1 CVH scores

In this study, CVH was assessed according to the LE8 score proposed by the AHA in 2022 (16). The LE8 consists of two subgroups, the health behavior score (HBS) and the health factor score (HFS), which include eight domains: namely, the four HBS domains of diet, physical activity, nicotine exposure, and sleep health, as well as the four HFS domains of body mass index (BMI), lipids, glucose, and blood pressure. Scores for each CVH metric ranged from 0 to 100, and the overall LE8 score was derived by calculating the unweighted mean of the eight CVH metrics (see Supplementary Table S1 for details of the calculation). Based on the LE8 score, participants were categorized into three cardiovascular fitness level groups, with 80-100 categorized as a high CVH group, 50-79 as a medium CVH group, and 0-49 as a low CVH group. The same cut-off values were used to define HBS and HFS.

### 2.3.2 PHQ-9 measures and depressive states

The PHQ-9 is a self-administered tool used to assess depressive symptoms and diagnose depression. It consists of nine items, each scored from 0 (not at all) to 3 (nearly every day), resulting in a total score ranging from 0 to 27. Higher scores indicate more severe depressive symptoms. The scoring classification of the PHQ-9 scale for depressive symptoms is as follows:0-4: no depressive state, 5-9:

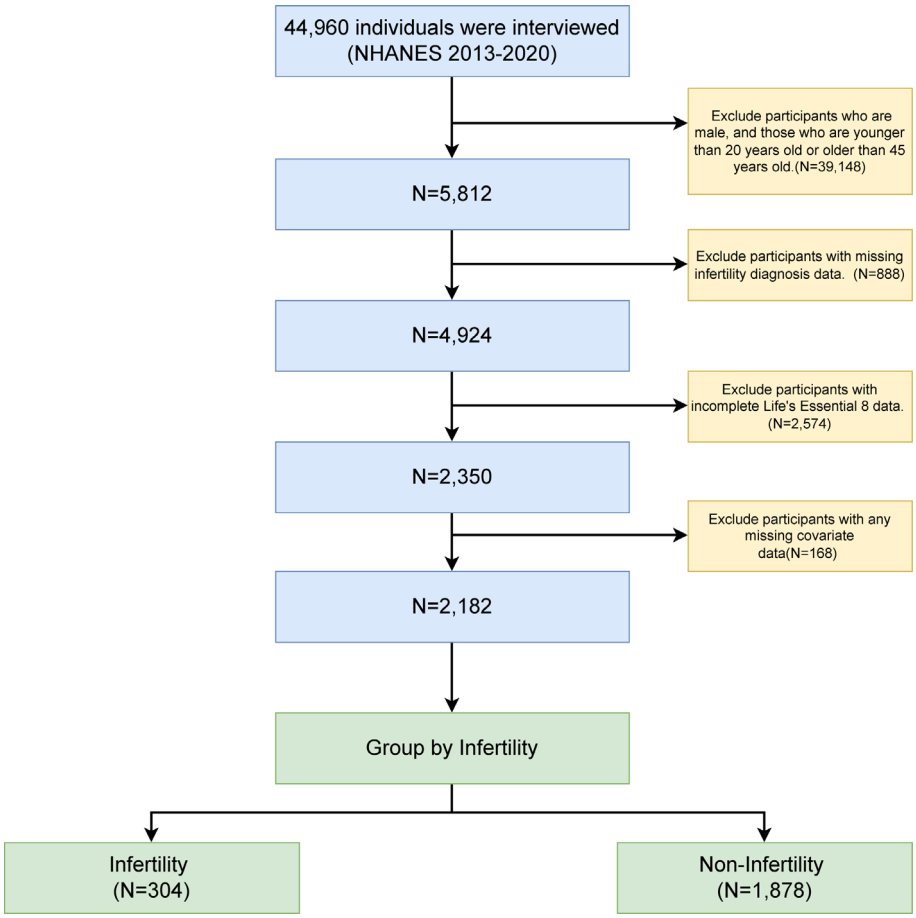


FIGURE 1
Participant flowchart.

mild depression,10 or more: moderate to severe depressive state (20).

### 2.3.3 Covariates

Demographic information was collected through household interviews using a standardized questionnaire, which included details on age, race, education, marital status, household income status (PIR, categorized according to poverty income ratios: less than 1.3 for low, 1.3 to less than 3.5 for intermediate, and 3.5 and above for high), and alcohol consumption (g/day). Metabolic indicators included uric acid (UA) levels (mg/dL). The selection of UA as a metabolic indicator is based on its relevance in assessing various health risks. Elevated UA levels, known as hyperuricemia, have been linked to several metabolic disorders and health conditions. These include cardiovascular diseases such as hypertension, coronary artery disease, and stroke, as well as metabolic syndrome components like obesity, insulin resistance, hyperglycemia, hypertension, and dyslipidemia. Moreover, hyperuricemia is associated with an increased risk of chronic kidney disease and can exacerbate complications in patients with diabetes. By measuring UA levels, we aim to comprehensively evaluate participants' metabolic health status and identify potential health risks (21). Inflammation indicators were selected from the Systemic Immune Inflammation Index (SII), which was calculated from platelet count x neutrophil count/lymphocyte count and expressed as $10^9$ cells per microliter (22).

### 2.3.4 Definition of outcomes

The diagnosis of infertility history typically hinges on responses to specific inquiries, focusing on two primary aspects: 1. Duration of attempts to conceive: Women are queried about whether they have endeavored unsuccessfully to conceive for a minimum of one year. 2. Medical consultation: Participants are also asked if they have sought medical advice due to difficulties in conceiving. In line with the NHANES questionnaire design, women are classified as having a history of infertility if they respond affirmatively to either of these questions.

## 2.4 Statistical analysis

Statistical analysis was conducted using R software version 4.30. Two-sided tests were employed for statistical inference, with significance set at $P \leq 0.05$. Clinical baseline data were summarized using frequencies (weighted percentages) for categorical variables, with group differences assessed using chi-

square tests. Continuous variables were presented as mean ± standard error. For normally distributed data, one-way ANOVA was applied, while the Wilcoxon test was used for non-normally distributed data.

Weighted multifactorial logistic regression models were constructed to investigate the association between CVH, HBS, HFS, depressive state, and infertility. To explore potential nonlinear relationships, weighted restricted cubic spline (RCS) regression models were employed. Likelihood ratio test was used to test nonlinearity. The number of nodes was determined based on the lowest value of the Akaike information criterion (AIC).Subgroup analyses were conducted based on race, education level, age, household income, and marital status, with interactions assessed via likelihood ratio tests.

Mediation analyses were performed to evaluate the mediating effect of LE8 scores on infertility, considering depression (PHQ-9), oxidative stress (UA), and inflammation (SII) variables. We used a similar approach to assess the relationships between LE8 and PHQ-9, UA, and SII, as well as the relationships among PHQ-9, UA, SII, and infertility. The R packages 'mediator' and 'lavaan' were employed to estimate the independent and joint mediating effects of SII, UA, and PHQ-9, respectively. A total of 5,000 replicate simulations were conducted to obtain more accurate estimates of the mediating effects and their confidence intervals. The results included the effect (β), indirect effect (βindirect, β-IE), direct effect (βdirect, β-DE), total effect (Total β), and p-values. Mediation effects were analyzed based on LE8 scores (per 10-point increase).

Two sensitivity analyses were conducted to verify the robustness of the findings. Sensitivity analysis 1 reanalyzed the data using unweighted methods to ensure that results were not dependent on the weighting approach. Sensitivity analysis 2 made further adjustments for variables associated with CVH and infertility, including total bilirubin, creatinine, CVD, albumin, SII, and UA. These additional corrections in sensitivity analysis 2 aimed to account for potential confounding factors.

# 3 Results

## 3.1 Baseline characteristics

The study encompassed 2,182 participants, including 304 individuals in the infertile group. The mean age of participants in the non-infertile group was 32.17 years, significantly lower than the infertile group (35.23 years, $p < 0.001$). Marital status analysis revealed a higher proportion of individuals in the infertile group living as part of a couple (Coupled) compared to the non-infertile group ($p < 0.001$). Additionally, infertility prevalence was notably higher among participants in the middle- and high-income brackets ($p = 0.008$). Regarding physical health indicators, the infertile group exhibited significantly lower scores in HFS, BMI, blood lipids, blood pressure, blood glucose, and sleep health compared to the non-infertile group ($p < 0.05$). The infertile group also had a higher proportion of low scores in CVH and HFS, along with significantly higher levels of UA and depression scores (PHQ-9). Notably, severe depression was more prevalent in the infertile group compared to the non-infertile group (18.37% vs. 9.8%) (Table 1).

## 3.2 Multifactorial logistic regression analysis

The results of multifactorial logistic regression analysis revealed a significant negative association between the HFS within LE8 and infertility ($p=0.004$), both overall and in the BMI score($p=0.002$) and blood glucose score($p=0.030$) subgroups. The OR and 95% CI for each 10-point increase in HFS was 0.855 (0.767, 0.954). This indicates that higher cardiovascular fitness, as reflected in higher LE8 scores and increased levels of healthy physical activity, may mitigate the risk of infertility. Conversely, the association between the HBS score and infertility did not reach statistical significance ($p=0.077$). Furthermore, scores for sleep health, BMI, and blood glucose were significant negative correlations with infertility. In contrast, there was a notable positive correlation observed between depressive symptoms, as measured by PHQ-9 scale scores, and infertility ($p<0.001$), with an OR and 95% CI of 1.073 (1.031,1.116) for each point increase in PHQ-9, indicating that individuals reporting higher levels of depression symptoms are more prone to infertility (Table 2, Supplementary Table S2).

## 3.3 Dose response relationships

RCS analysis indicated that all non-linear associations were found to be statistically non-significant (P-nonlinear > 0.05). LE8 and HFS demonstrated a linear negative correlation (P-overall < 0.05), while PHQ-9 scores showed a linear positive correlation. There was no significant correlation observed with HBS (P-overall > 0.05) (Figure 2).

## 3.4 Subgroup analysis

The association between LE8 and its subgroup HFS with infertility varies depending on age, with a significant association observed in the 20-34 age group. Additionally, the association between PHQ-9 scores and infertility varies across different stratifications by marital status and income levels. Specifically, a significant association between depression scores and the risk of infertility is found among coupled individuals and those with intermediate income levels, while other groups do not show significant associations (Figure 3).

## 3.5 Mediation analysis

LE8 (per 10-point increase) showed a negative correlation with depression score, SII (1/10 SII), and UA (β-depression=-0.812, $p<0.001$; β-SII=-3.174, $p<0.001$; β-UA=-0.207, $p<0.001$). Additionally, depression score and UA were positively correlated

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TABLE 1 Baseline characteristics according to infertility.

| Variable | Total | Non-infertility | Infertility | *P* value |
|---|---|---|---|---|
| Age, year | 32.63 ± 0.25 | 32.17 ± 0.24 | 35.23 ± 0.59 | < 0.001 |
| Education, n (%) | | | | 0.659 |
| Less than high school | 91 (2.63) | 81 (2.77) | 10 (1.85) | |
| High school | 630 (26.56) | 542 (26.59) | 88 (26.39) | |
| Some college or above | 1461 (70.80) | 1255 (70.63) | 206 (71.76) | |
| Ethnicity, n (%) | | | | 0.278 |
| White | 771 (57.02) | 643 (55.97) | 128 (62.92) | |
| Black | 463 (12.98) | 400 (13.08) | 63 (12.42) | |
| Mexican | 371 (12.12) | 323 (12.36) | 48 (10.82) | |
| Hispanic | 211 (6.92) | 191 (7.29) | 20 (4.81) | |
| Other | 366 (10.96) | 321 (11.30) | 45 (9.04) | |
| Marital Status, n (%) | | | | < 0.001 |
| Coupled | 1276 (60.39) | 1056 (57.88) | 220 (74.54) | |
| Single or separated | 906 (39.61) | 822 (42.12) | 84 (25.46) | |
| Family Income, n (%) | | | | 0.008 |
| Low | 778 (28.86) | 680 (30.03) | 98 (22.32) | |
| Intermediate | 813 (36.55) | 697 (34.92) | 116 (45.70) | |
| High | 591 (34.59) | 501 (35.05) | 90 (31.98) | |
| LE8 | 74.60 ± 0.57 | 75.34 ± 0.58 | 70.44 ± 1.34 | 0.001 |
| HBS | 69.07 ± 0.70 | 69.57 ± 0.75 | 66.25 ± 1.57 | 0.058 |
| HFS | 80.14 ± 0.63 | 81.12 ± 0.67 | 74.63 ± 1.56 | < 0.001 |
| Body Mass Index | 59.45 ± 1.42 | 61.34 ± 1.39 | 48.83 ± 3.57 | 0.001 |
| Blood Lipids | 79.83 ± 0.81 | 80.55 ± 0.86 | 75.75 ± 2.05 | 0.033 |
| Blood Pressure | 87.50 ± 0.67 | 88.15 ± 0.71 | 83.82 ± 1.92 | 0.042 |
| Blood Glucose | 93.78 ± 0.47 | 94.43 ± 0.51 | 90.12 ± 1.33 | 0.005 |
| Sleep Health | 84.51 ± 0.68 | 85.12 ± 0.70 | 81.09 ± 1.81 | 0.038 |
| Nicotine Exposure | 75.36 ± 1.22 | 76.19 ± 1.27 | 70.67 ± 3.06 | 0.095 |
| Diet | 38.56 ± 1.27 | 38.97 ± 1.35 | 36.24 ± 2.42 | 0.288 |
| Physical Activity | 77.84 ± 1.22 | 77.99 ± 1.23 | 77.00 ± 3.33 | 0.772 |
| CVH, n (%) | | | | < 0.001 |
| Low (LE8<50) | 141 (5.90) | 108 (4.93) | 33 (11.36) | |
| Moderate [50-79) | 1245 (53.63) | 1055 (52.57) | 190 (59.64) | |
| High (≥80) | 796 (40.46) | 715 (42.50) | 81 (29.01) | |
| HBS, n (%) | | | | 0.083 |
| Low (HBS<50) | 381 (15.63) | 314 (14.85) | 67 (20.06) | |
| Moderate [50-79) | 1101 (49.12) | 946 (48.72) | 155 (51.41) | |
| High (≥80) | 700 (35.24) | 618 (36.44) | 82 (28.53) | |
| HFS, n (%) | | | | < 0.001 |
| Low (HFS<50) | 142 (5.67) | 109 (4.88) | 33 (10.12) | |

*(Continued)*

TABLE 1 Continued

| Variable | Total | Non-infertility | Infertility | *P* value |
|---|---|---|---|---|
| Moderate [50-79) | 863 (37.53) | 714 (35.79) | 149 (47.34) | |
| High (≥80) | 1177 (56.80) | 1055 (59.33) | 122 (42.53) | |
| SII | 540.72 ± 8.09 | 541.93± 9.30 | 533.90 ± 16.43 | 0.682 |
| UA, mg/dl | 4.57 ± 0.03 | 4.54 ± 0.04 | 4.78 ± 0.09 | 0.017 |
| PHQ-9 | 3.59 ± 0.13 | 3.39 ± 0.13 | 4.76 ± 0.45 | 0.006 |
| Depression Status, n (%) | | | | 0.004 |
| Non-Depression | 1568 (72.58) | 1372 (74.20) | 196 (63.47) | |
| Mild-Depression | 386 (16.33) | 324 (16.00) | 62 (18.17) | |
| Depression | 228 (11.09) | 182 (9.80) | 46 (18.37) | |

CVH, Cardiovascular health; LE8, Life's essential 8; HBS, Health behaviors; HFS, Health factors; SII, Systemic immune-inflammation index; UA, Uric acid.

with infertility, while SII was negatively correlated with infertility (β-depression=0.004, p=0.023; β-SII=0.0003, p=0.031; β-UA=0.024, p=0.001). Mediation analyses indicated that depression score, SII, and UA collectively mediated 25.9% of the relationship between LE8 and infertility, with individual mediations of 11.1%, 3.7%, and 18.5%, respectively (p<0.001) (Figure 4).

## 3.6 Sensitivity analysis

Sensitivity analysis confirmed the robustness of our results. Both the primary analysis and sensitivity analysis 2 utilized weighted data, while sensitivity analysis 1 employed unweighted data. Adjustments in the primary analysis and sensitivity analysis 1 were made for age, ethnicity, income, education, and marital status, with further adjustments for PHQ-9 scores in cardiovascular health associations and for cardiovascular health metrics in depression associations. Sensitivity analysis 2 included additional adjustments for total bilirubin, creatinine, albumin, uric acid, systemic immune-inflammation index, and history of cardiovascular disease. The consistent findings across these analyses affirm the reliability and stability of the observed associations (Table 3).

# 4 Discussion

Our study uncovered a novel negative correlation between LE8 and infertility, indicating that elevated cardiovascular fitness levels may mitigate the risk of infertility. We further found that HFS, a subgroup of LE8, was inversely associated with infertility. Among

TABLE 2 Association of CVH and depression with infertility.

| Variable | Model 1 | | Model 2 | | Model 3 | |
|---|---|---|---|---|---|---|
| | *OR* (95% CI) | *P* value | *OR* (95% CI) | *P* value | *OR* (95% CI) | *P* value |
| Cardiovascular Health | | | | | | |
| Low (LE8<50) | 1.000 (1.000,1.000) | | 1.000 (1.000,1.000) | | 1.000 (1.000,1.000) | |
| Moderate [50-79) | 0.493 (0.311,0.782) | 0.003 | 0.577 (0.358,0.928) | 0.025 | 0.575 (0.349, 0.947) | 0.031 |
| High (≥80) | 0.297 (0.167,0.528) | <0.001 | 0.366 (0.205,0.651) | 0.001 | 0.361 (0.198, 0.660) | 0.002 |
| LE8 (per 10) | 0.791 (0.697,0.899) | <0.001 | 0.821 (0.725,0.929) | 0.003 | 0.816 (0.712, 0.935) | 0.005 |
| *p* for trend | | <0.001 | | 0.002 | | 0.003 |
| Health Behaviors | | | | | | |
| Low (HBS<50) | 1.000 (1.000,1.000) | | 1.000 (1.000,1.000) | | 1.000 (1.000,1.000) | |
| Moderate [50-79) | 0.781 (0.517,1.179) | 0.232 | 0.767 (0.508,1.159) | 0.201 | 0.817 (0.529, 1.260) | 0.348 |
| High (≥80) | 0.579 (0.328,1.024) | 0.060 | 0.565 (0.324,0.987) | 0.045 | 0.603 (0.332, 1.093) | 0.093 |
| HBS (per 10) | 0.916 (0.839,1.001) | 0.052 | 0.916 (0.841,0.998) | 0.045 | 0.926 (0.842, 1.018) | 0.107 |
| *p* for trend | | 0.054 | | 0.041 | | 0.077 |

*(Continued)*

TABLE 2 Continued

| Variable | Model 1 | | Model 2 | | Model 3 | |
|---|---|---|---|---|---|---|
| | *OR* (95% CI) | *P* value | *OR* (95% CI) | *P* value | *OR* (95% CI) | *P* value |
| **Health Factors** | | | | | | |
| Low (HFS<50) | 1.000 (1.000,1.000) | | 1.000 (1.000,1.000) | | 1.000 (1.000,1.000) | |
| Moderate [50-79) | 0.638 (0.404,1.007) | 0.053 | 0.752 (0.459,1.230) | 0.248 | 0.722 (0.440, 1.186) | 0.190 |
| High (≥80) | 0.346 (0.196,0.611) | <0.001 | 0.441 (0.239,0.814) | 0.010 | 0.431 (0.232, 0.801) | 0.009 |
| HFS (per 10) | 0.819 (0.744,0.902) | <0.001 | 0.854 (0.769,0.950) | 0.005 | 0.855 (0.767, 0.954) | 0.007 |
| *p* for trend | | <0.001 | | 0.004 | | 0.004 |
| **Depression** | | | | | | |
| Non-Depression | 1.000 (1.000,1.000) | | 1.000 (1.000,1.000) | | 1.000 (1.000,1.000) | |
| Mild-Depression | 1.327 (0.830,2.121) | 0.230 | 1.457 (0.910,2.334) | 0.114 | 1.533 (0.934,2.516) | 0.088 |
| Depression | 2.191 (1.368,3.509) | 0.002 | 2.301 (1.464,3.617) | <0.001 | 2.549 (1.599,4.065) | <0.001 |
| PHQ-9 (per 1) | 1.063 (1.023,1.104) | 0.003 | 1.067 (1.027,1.108) | 0.001 | 1.073 (1.031,1.116) | <0.001 |
| *p* for trend | | 0.004 | | 0.001 | | <0.001 |

Model 1: adjusts for none.
Model 2: adjusts for age and ethnicity.
Model 3: adjusts for age, ethnicity, income, education and marital status. In the model3 that associates cardiovascular health, health behaviors and health factors with infertility, we also adjusted for the PHQ-9. In the association between depression and infertility, we further adjusted for cardiovascular health.
LE8, Life's essential 8; HBS, Health behaviors; HFS, Health factors; OR, odds ratio; CI, confidence interval.

the eight subscales of LE8, scores for sleep health, BMI, and blood glucose were significantly negatively correlated with depression scores, whereas depressive state and PHQ-9 scores were positively associated with infertility. Furthermore, SII, UA and depression were identified as mediators in the relationship between LE8 and infertility, highlighting their significant mediating roles.

Previous research has identified a relationship between CVH and infertility, highlighting increased susceptibility to

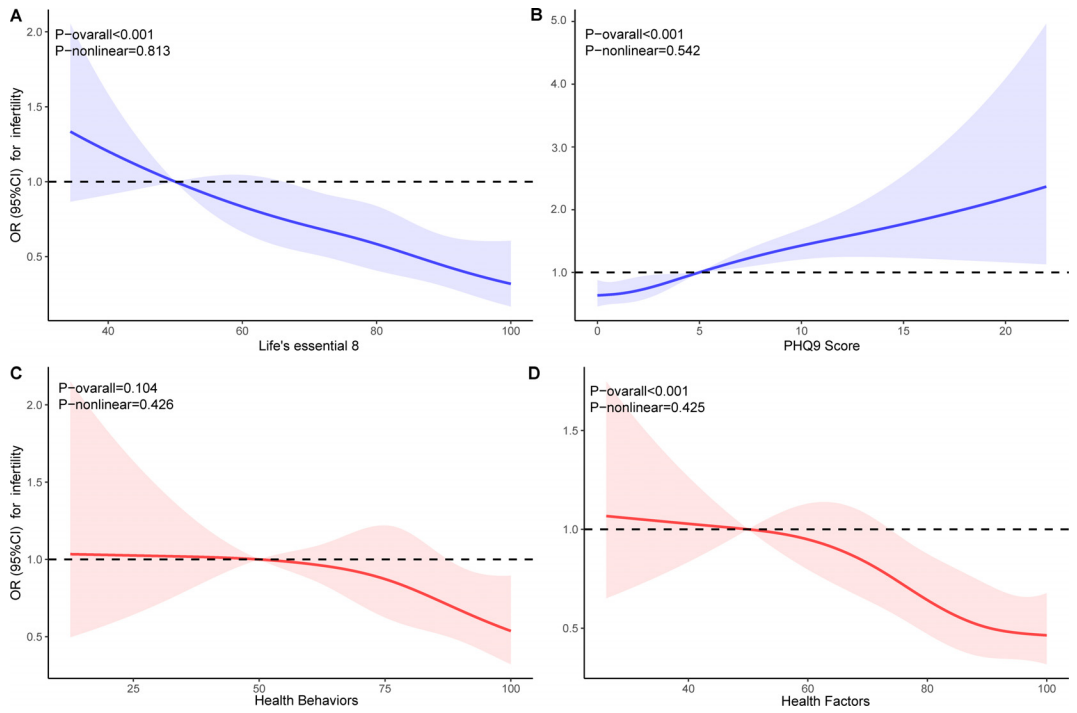


FIGURE 2
Dose-response relationships for Life's Essential 8 **(A)**, PHQ-9 **(B)**, Health Behaviors **(C)**, and Health Factors **(D)** with infertility. OR, odds ratio; CI, confidence interval.

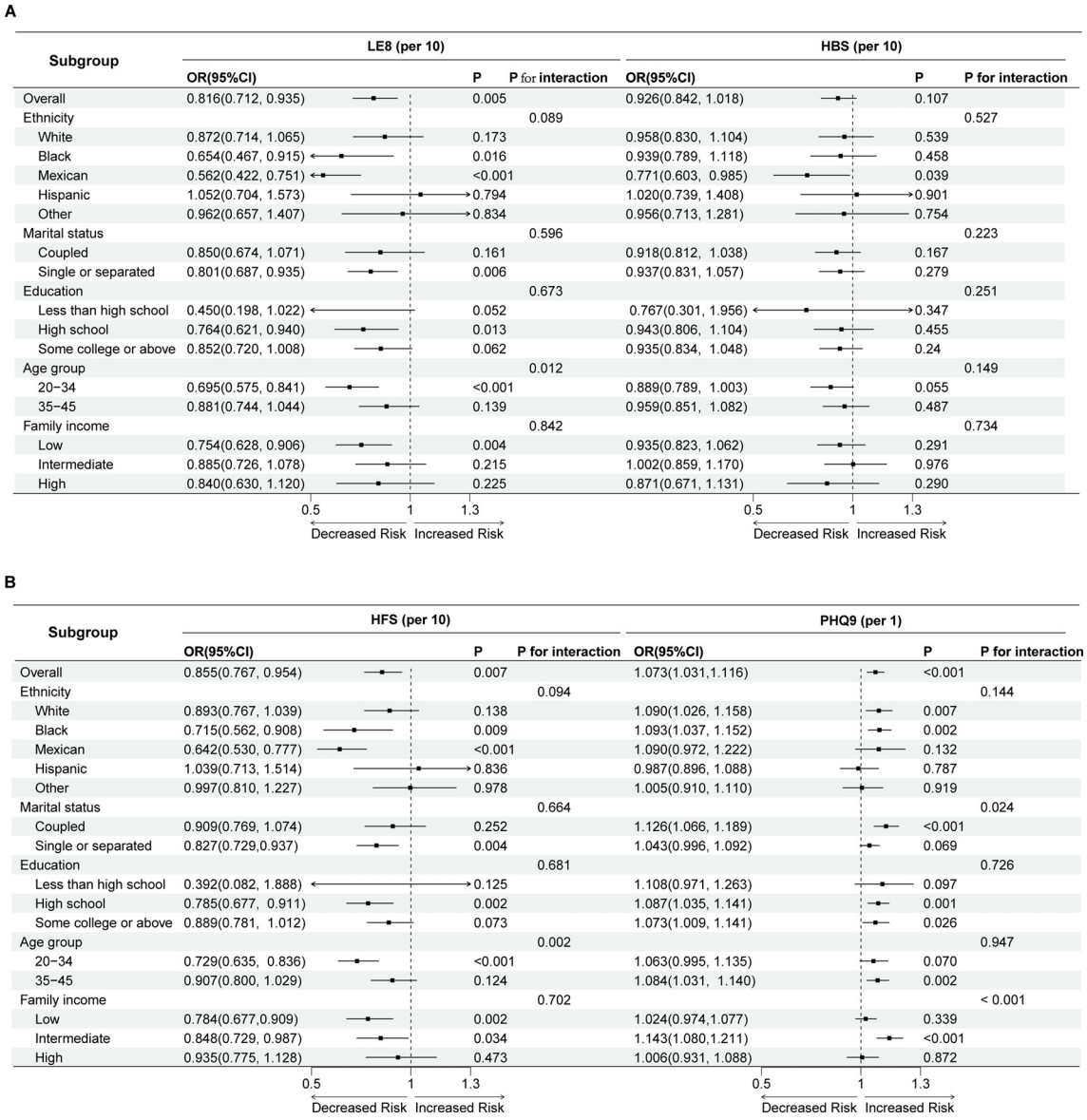


FIGURE 3
Forest plot of subgroup analysis and interaction tests in Life's Essential 8, Health Behaviors **(A)**, Health Factors and PHQ-9 **(B)** for the risk of infertility. LE8, Life's Essential 8; HBS, Health Behaviors; HFS, Health Factors; OR, odds ratio; CI, confidence interval.

cardiovascular issues among infertile women. A recent prospective cohort study supported this link by demonstrating lower LE8 scores in association with infertility (15). Furthermore, infertility has been linked to heightened risks of various cardiovascular diseases in women, including CVD events, cardiovascular mortality, coronary heart disease, stroke, and heart failure. Despite these observations, the specific impact of LE8 on infertility remains underexplored. Our study contributed by revealing a negative association between LE8 scores and infertility, suggesting that optimal cardiovascular health may lower infertility risk in women. Research evidence has indicated that specific indicators within the LE8 framework are associated with infertility, including BMI (23), smoking (24, 25), physical activity (26, 27), diet quality (28–30), and dietary fiber (31). Our study further validated associations of sleep health score, blood glucose score, and BMI score with infertility. Consistent with a previous study by Wang et al. (17), fertility was positively correlated with LS7 scores, suggesting reduced infertility odds with improved cardiovascular health. Notably, LE8's impact on sleep was more significant compared to LS7, aligning with findings that shorter sleep duration increases infertility risk (26, 32, 33). Therefore, LE8 presents promising prospects for managing cardiovascular risks and potentially enhancing fertility outcomes among affected individuals.

Our further analysis revealed a higher prevalence of major depression in the infertility group. In-depth examination demonstrated a significant positive correlation between depressive states and PHQ-9 scale scores with infertility. These findings were consistent with studies in Hungary and Pakistan, which reported higher rates of depression and anxiety among infertile women (34, 35). Infertile women typically exhibit more symptoms of depression and anxiety compared to their partners (36). The

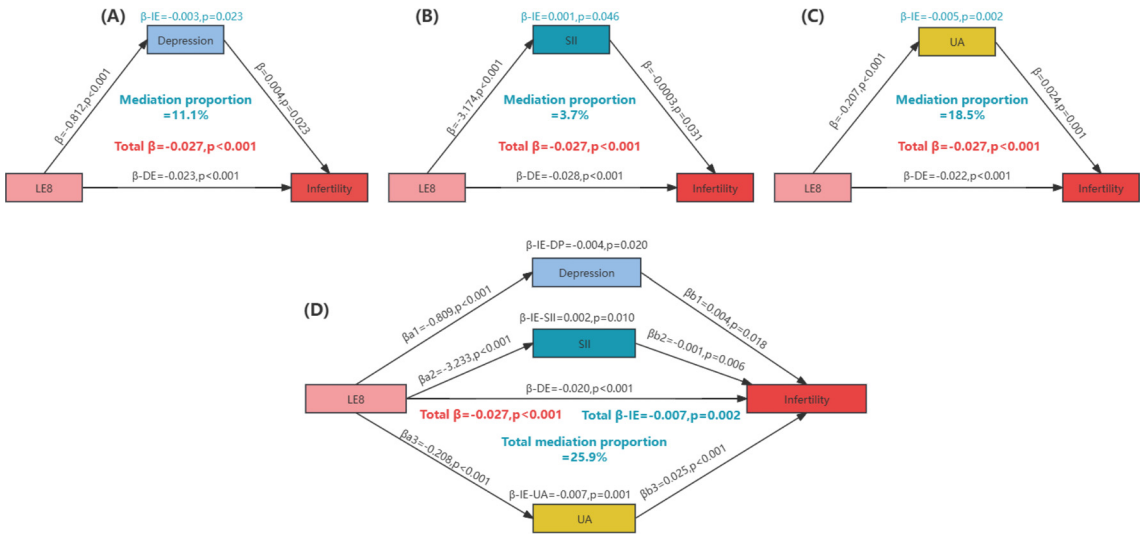


FIGURE 4
Quantitative assessment of depression, oxidative stress, and inflammation as mediators in the association between life's Essential 8 and infertility. **(A)** The mediating effect of depression; **(B)** The mediating effect of SII; **(C)** The mediating effect of UA; **(D)** Joint mediating effects of depression, SII and UA. Mediation proportion = β-IE/Total β. IE, indirect effect; DE, direct effect; LE8, Life's Essential 8; SII, systemic immune-inflammation index; UA, uric acid.

Iranian study highlighted that infertile women often experience psychological issues, such as sexual dysfunction, due to depression and anxiety (37). Additionally, research indicates that PHQ-9 scores may influence infertility by impacting BMI (38). Infertility presents significant emotional challenges, leading to various psychological issues, including anxiety, depression, eating disorders, and low self-esteem. Interventions like psychological counseling and therapy for infertile couples have demonstrated substantial reductions in these psychological issues and have been shown to enhance conception rates (39). Thus, providing psychological support and treatment for infertile women is crucial.

PCOS is a leading cause of infertility often linked with insulin resistance and cardiometabolic issues like dyslipidemia, hypertension, impaired glucose tolerance, diabetes mellitus, and

TABLE 3 Sensitivity analysis of the association of the CVH and depression with infertility.

| Variable | Primary Analysis | | Sensitivity Analysis 1 | | Sensitivity Analysis 2 | |
|---|---|---|---|---|---|---|
| | *OR* (95% CI) | *P* value | *OR* (95% CI) | *P* value | *OR* (95% CI) | *P* value |
| Cardiovascular Health | | | | | | |
| Low (LE8<50) | 1.000 (1.000,1.000) | | 1.000 (1.000,1.000) | | 1.000 (1.000,1.000) | |
| Moderate[50-79) | 0.575 (0.349, 0.947) | 0.031 | 0.666 (0.431,1.050) | 0.073 | 0.640 (0.380, 1.077) | 0.090 |
| High (≥80) | 0.361 (0.198, 0.660) | 0.002 | 0.418 (0.256,0.692) | <0.001 | 0.435 (0.219, 0.864) | 0.020 |
| LE8 (per 10) | 0.816 (0.712, 0.935) | 0.005 | 0.814 (0.741,0.894) | <0.001 | 0.853 (0.743, 0.978) | 0.025 |
| *p* for trend | | 0.003 | | <0.001 | | 0.027 |
| Depression | | | | | | |
| Non-Depression | 1.000 (1.000,1.000) | | 1.000 (1.000,1.000) | | 1.000 (1.000,1.000) | |
| Mild-Depression | 1.533 (0.934,2.516) | 0.088 | 1.423 (1.027,1.951) | 0.031 | 1.494 (0.906,2.465) | 0.111 |
| Depression | 2.549 (1.599,4.065) | <0.001 | 1.879 (1.280,2.719) | 0.001 | 2.662 (1.622,4.368) | <0.001 |
| PHQ-9 (per 1) | 1.073 (1.031,1.116) | <0.001 | 1.051 (1.024,1.079) | <0.001 | 1.075 (1.030,1.121) | 0.002 |
| *p* for trend | | <0.001 | | <0.001 | | <0.001 |

The primary analysis and sensitivity analysis 2 used weighted data, while sensitivity analysis 1 used unweighted data.
Primary analysis and sensitivity analysis 1: adjusts for age, ethnicity, income, education and marital status. In the association with cardiovascular health, further adjustments were made for the PHQ-9. In the association with Depression, further adjustments were made for the cardiovascular health.
Sensitivity analysis 2: based on the primary analysis, further adjustments were made for total bilirubin, creatinine, albumin, uric acid, systemic immune-inflammation index, uric acid and cardiovascular disease history.
LE8, Life's essential 8; OR, odds ratio; CI, confidence interval.

metabolic syndrome (MetS), increasing cardiovascular disease risk in affected women. Research has shown that obesity significantly raises the risk of infertility. Obese individuals frequently exhibit functional hyperandrogenism and hyperinsulinemia, which, together with insulin resistance, impair fertility (40). Additionally, obesity triggers oxidative stress and ovarian inflammation, stimulating the production of androgens, while enhancing peripheral aromatization of estrogen and reducing the production of sex hormone-binding globulin (SHBG) in the liver. These disruptions lead to follicular atresia and anovulatory cycles (41). Studies have also demonstrated that women of normal or overweight BMI have significantly higher fertility rates compared to obese women, suggesting that obesity may not only impair oocyte quality but also negatively affect uterine receptivity, further compounding fertility challenges (42).

Furthermore, elevated blood glucose scores were found to be inversely associated with infertility risk. Higher levels of blood glucose and hemoglobin A1c have been linked to an increased likelihood of infertility in women. Appropriate glucose levels are essential for the maturation of oocytes and successful embryo development. During the nuclear and cytoplasmic maturation of oocytes, abnormal glucose concentrations can disrupt these processes, leading to poor embryo development after fertilization and reduced fertility (43). Our study identified a negative association between blood glucose scores and infertility risk. Observational studies suggest impaired glucose tolerance links to both cardiovascular risk and fertility problems. Elevated blood glucose levels correlate with pregnancy complications, infertility, and miscarriage risk if poorly managed. Shared pathogenic mechanisms between infertility and cardiovascular disease include activation of the hypothalamic-pituitary-adrenal (HPA) axis and neuroendocrine stress responses. Hyperuricemia indeed represents a hallmark of the metabolic syndrome (44). UA played an 18.5% mediating role between LE8 scores and infertility in our mediation analysis, underscoring metabolism's critical role linking cardiovascular health and infertility. Infertility thus serves as a potential indicator for cardiometabolic disorders, warranting timely interventions to mitigate their impacts. Women with PCOS may develop adverse cardiovascular traits during reproductive years, affecting both maternal and offspring health outcomes.

Inflammation and immune responses are critical factors in the development of female infertility. Our study investigated the relationship between the SII within LE8 scores and infertility. We found a negative correlation between SII and infertility, consistent with previous research (45). Mediation analysis indicated that SII mediated 3.7% of the association between LE8 scores and infertility. Chronic endometritis, often detected during screening for secondary amenorrhea and infertility (46), may contribute to infertility by disrupting menstrual cycles and impairing implantation. Anti-inflammatory dietary interventions, such as the Mediterranean diet rich in monounsaturated fatty acids, n-3 polyunsaturated fatty acids, and flavonoids, and low in red and processed meat, have shown promise in improving male fertility and sperm quality (12). Implementing an anti-inflammatory diet as an adjunctive treatment for fertility may enhance reproductive outcomes and potentially reduce the need for more intensive medical interventions.

Importantly, the findings suggested that targeted interventions aimed at improving CVH, as indicated by LE8 scores, could have offered potential pathways to mitigate infertility risk. For instance, strategies focused on improving sleep quality, maintaining a healthy BMI, and controlling blood glucose levels might have reduced depression and inflammation, which were significant mediators in the LE8-infertility association. Future research should investigate how specific interventions targeting these health domains could directly influence reproductive outcomes. Moreover, our findings paved the way for interdisciplinary studies to explore how improving cardiovascular health metrics could potentially benefit both cardiovascular and reproductive health. Developing comprehensive health programs that integrated cardiovascular health with fertility treatment might offer new avenues for reducing infertility risk and improving overall women's health outcomes.

We utilized the NHANES database to conduct our study, leveraging its large sample size to enhance statistical power and result generalizability. Employing multifactorial regression and restricted cubic spline modeling, we explored the association between LE8 scores and infertility, conducting subgroup and mediation analyses to uncover physiological and metabolic mechanisms. Despite the robustness of NHANES data, limitations include potential biases from missing or incomplete data and reliance on self-reported information and physiological measurements, which could introduce errors and biases affecting study accuracy. Given the constraints of observational studies, we couldn't establish causality between LE8 scores and infertility, necessitating future long-term follow-up or intervention studies. These studies should investigate whether improving cardiovascular health via LE8 scores can mitigate infertility risks. It's recommended that future interventions target cardiovascular health improvements and assess their long-term impacts on physical and psychological health in infertile patients. Collaborative interdisciplinary efforts are crucial to advancing understanding of cardiovascular disease and infertility mechanisms, supporting the development of effective clinical strategies. In conclusion, while our NHANES-based study provides insights into LE8 scores and infertility, further research is essential to address limitations and translate findings into clinical applications for enhancing women's health and reproductive management.

## 5 Conclusion

Our study utilizing data from the NHANES database highlights that higher LE8 scores were significantly associated with lower depression scores and reduced levels of SII and UA. These factors collectively contributed to a decreased risk of infertility in women. Notably, improvements in specific LE8 health factors—such as sleep health, BMI and blood glucose management—were shown to have potential for lowering infertility risk by reducing associated mediators like depression and inflammation. Based on the findings of this study, we recommend the development of health

policies focused on weight management and glycemic control to help prevent infertility.

## Data availability statement

The study's original contributions are publicly available. You can access this data at the NHANES website: https://www.cdc.gov/nchs/nhanes.

## Ethics statement

Ethical approval was not required for the studies involving humans because the data utilized in this study were sourced from publicly accessible databases and were managed in accordance with approved ethical exemptions. The studies were conducted in accordance with the local legislation and institutional requirements. The study's original contributions are publicly available. This data can be accessed from the NHANES website: https://www.cdc.gov/nchs/nhanes Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements.

## Author contributions

SC: Formal Analysis, Methodology, Writing – original draft, Writing – review & editing. LL: Data curation, Formal Analysis, Writing – review & editing. WQ: Formal Analysis, Methodology, Writing – original draft. WL: Conceptualization, Methodology, Writing – original draft. XZ: Formal Analysis, Project administration, Supervision, Validation, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2025.1451030/full#supplementary-material

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# Association between urinary arsenic and the prevalence of endometriosis in women in the United States

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**Background:** Endometriosis affects up to 15% of women of reproductive age and can lead to various symptoms. More than 200 million people worldwide are at risk of higher than safe levels of arsenic exposure through drinking water. Studies investigating the relationship between arsenic and endometriosis are very limited and have yielded inconsistent results. This study aimed to explore the relationship between total urinary arsenic, arsenic species (Urinary arsenous acid, Urinary Arsenic acid, Urinary Arsenobetaine, Urinary Arsenocholine, Urinary Dimethylarsinic acid, Urinary Monomethylarsonic acid) and endometriosis.

**Methods:** We utilized a nationally representative dataset from the National Health and Nutrition Examination Survey (NHANES) from 2003 to 2006. A total of 650 participants were included. We examined the association between total urinary arsenic and different arsenic species with endometriosis using weighted multivariate logistic regression models.

**Results:** Urinary arsenous acid and urinary monomethylarsonic acid (MMA) were positively correlated with endometriosis ($p < 0.05$). After adjusting for potential confounding factors, the positive correlation of urinary MMA remained significant (OR: 1.317, 95%CI: 1.074–1.615). Subgroup analyses and interaction tests indicated that this association was not dependent.

**Conclusion:** Our research underscores a significant positive association observed between factors urinary MMA and endometriosis. Future research is needed to elucidate the specific mechanisms behind this association.

KEYWORDS

**endometriosis, arsenic, NHANES, gynecology, chronic inflammatory disease**

## 1 Introduction

Endometriosis is characterized by the proliferation of endometrial-like tissue, including glandular cells and stroma, beyond the uterine cavity. The clinical presentation typically encompasses cyclical hemorrhage, dysmenorrhea, and dyspareunia (1). Epidemiological data suggests a prevalence rate of 10–15% in the female population of childbearing age (2).

At present, the pathogenesis of endometriosis remains elusive. Proposed etiological hypotheses comprise retrograde menstruation, vascular or lymphatic dissemination, aberrant coelomic metaplasia, immune system dysregulation, and factors of cellular determinism, along with hereditary and environmental influences (2).

Arsenic is one of the most extensively studied elements in the field of metal toxicity, second only to lead (Pb) (3). Arsenic, a metalloid, is ubiquitously present in water, soil, and air, originating from both natural processes and human activities, existing in both inorganic and organic forms (4). Arsenic exposure through drinking water represents the primary mechanism of human contact, with over 200 million individuals worldwide facing the threat of encountering arsenic levels surpassing safety thresholds (5). Arsenic, a well-known metalloid, can cause endocrine disruption and reproductive disorders in women (6). Existing studies have linked arsenic exposure to ovarian dysfunction and infertility (7, 8). Exposure to high doses of arsenic during pregnancy increases the risk of miscarriage, fetal defects, and toxicity to the fetus (9, 10).

However, the relationship between arsenic exposure and endometriosis remains under-researched and is marked by contradictory findings (11, 12). Previous studies have not investigated the correlation between arsenic forms and endometriosis. Consequently, our research is designed to investigate the link between urinary total arsenic and different arsenic (urinary arsenous acid, urinary arsenic acid, urinary arsenobetaine, urinary arsenocholine, urinary dimethylarsinic acid, urinary monomethylarsonic acid) forms with endometriosis.

# 2 Methods

## 2.1 Data source

In this cross-sectional investigation, we employed data from the National Health and Nutrition Examination Survey (NHANES), which utilizes a sophisticated, multi-stage, stratified probability sampling method, ensuring samples that are representative of the national populace. The National Center for Health Statistics Institutional Review Board approved the data collection methods employed in the NHANES study, securing informed consent from all contributors. NHANES data are publicly available, enhancing transparency and facilitating scientific research across various fields. People can access extensive NHANES datasets, comprehensive operational manuals, consent forms, and periodical guides on the NHANES portal.[1]

Abbreviations: NHANES, National Health and Nutrition Examination Survey; MMA, Monomethylarsonic acid; HPLC, High-Performance Liquid Chromatography; LLOD, Lower limit of detection; DMA, Dimethylarsinic acid; PIR, Poverty income ratio; BMI, Body Mass Index; RCS, Restricted cubic splines; OR, Odds ratios; ERα, Estrogen receptor-alpha; ROS, Reactive oxygen species; GPx, Glutathione peroxidase; SOD, Superoxide dismutase.

1 https://www.cdc.gov/nchs/nhanes

## 2.2 Study participants

This study analyzed data from two NHANES cycles (2003–2004 and 2005–2006), and all groups were initially included; the male group, those with missing information on endometriosis, those with urinary arsenic levels, and relevant covariates were excluded in turn, and 650 participants were included in the final analysis (Figure 1).

## 2.3 Urinary arsenic and creatinine measurement

This analysis used NHANES datasets for arsenic from 2003–2004 (L06UAS_C) and 2005–2006 (UAS_D), utilizing High-Performance Liquid Chromatography (HPLC) for species separation, followed by ICP-DRC-MS for detection of total urinary arsenic and species concentrations. The following arsenic species and their respective lower limit of detection (LLOD) were analyzed: Urinary total arsenic 0.74 μg/L, urinary arsenous acid 1.2 μg/L, urinary arsenic acid 1.0 μg/L, urinary arsenobetaine 0.4 μg/L, urinary arsenocholine 0.6 μg/L, urinary dimethylarsinic acid (DMA) 1.7 μg/L, and urinary monomethylarsonic acid (MMA) 0.9 μg/L. Concentrations below detection limits were substituted with LLOD/ $\sqrt{2}$ values. To correct for urine arsenic in urine cadmium concentration, we used urinary creatinine concentration (mg/dl) data from the same dataset.

## 2.4 Endometriosis status

The diagnosis of endometriosis was determined through a reproductive health questionnaire for women aged 20–54 years, asking, "Has a doctor or other health professional ever told you that you had endometriosis?"

## 2.5 Covariates assessment

Drawing from existing research and clinical insights, our analysis included the following covariates to minimize potential confounding effects. We gathered data on demographic aspects such as age, ethnicity (Non-Hispanic Black, Non-Hispanic White, Other), educational level (high school and below, above high school), marital status (married/living with a partner, widowed/divorced/separated, never married), and the poverty income ratio (PIR). The Body Mass Index (BMI), determined by the individual's mass in kilograms divided by the square of their height in meters, was classified into either normal or underweight (BMI < 25 kg/m$^2$) and overweight or obese (BMI ≥ 25 kg/m$^2$). We queried participants about their smoking status with the question, "Have you smoked at least 100 cigarettes in your lifetime?" and measured alcohol consumption by asking, "In the past 12 months, have you consumed 4 to 5 or more drinks per day?" Histories of diabetes and hypertension were acquired from self-reports in our health survey questionnaire. Additional data on age at menarche, fertility status were gleaned from the reproductive health questionnaire.

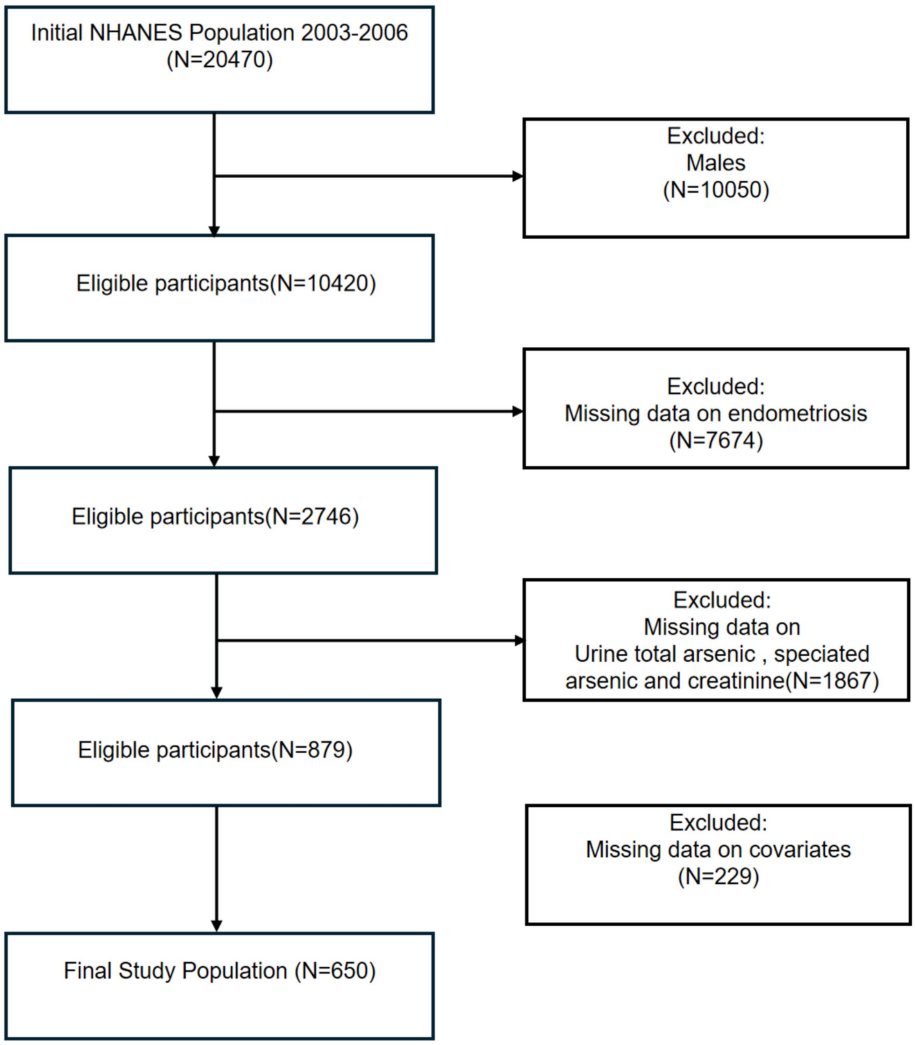


FIGURE 1
Flow chart of eligible participants' selection.

## 2.6 Statistical analysis

To delineate the variations in baseline characteristics between individuals with and without endometriosis, categorical variables were represented as weighted proportions, and continuous variables as weighted means with standard deviation or weighted medians with interquartile ranges. Prior to conducting logistic regression analyses, we evaluated the correlations between different arsenic species using Spearman's correlation coefficients. To avoid potential multicollinearity issues, we examined the variance inflation factors for all arsenic species. The variance inflation factors values were all below 5, indicating no significant multicollinearity among the arsenic species. Based on these analyses, we conducted separate logistic regression models for each arsenic species to avoid the potential confounding effects of inter-species correlations. Weighted logistic regression analyses, both univariate and multivariate, were applied to gauge the relationship between six arsenic species and endometriosis. Model 1 did not control for any covariates, Model 2 was adjusted for age and ethnicity, and Model 3 further controlled for marital status, educational attainment, smoking status, BMI, PIR, menarche onset, alcohol use, hypertension, diabetes, fertility status and creatinine levels in urine. The adoption of restricted cubic splines (RCS) served to address the nonlinear relationship between MMA and endometriosis. Finally, statistical $p$ for interaction among covariates and endometriosis prevalence rate was assessed, and subgroup analyses were performed to enhance understanding of these findings. In addition, age-stratified analysis using 40 years as the cutoff point was conducted, taking into account both the natural course of endometriosis and the potential cumulative effects of arsenic exposure. Besides, the multiple imputation (MI) approaches were applied by us to fill in the missing values that exist in the data through the "MICE" package of the R software. R and RStudio (version 4.3.0) facilitated all statistical computations, with $p$-values below 0.05 denoting statistical significance.

TABLE 1 Characteristics of NHANES participants included in the analysis.

| Characteristics | Non-endometriosis (*N* = 606) | Endometriosis (*N* = 44) | *p* value |
|---|---|---|---|
| Age (years) | 39.20 (9.42) | 41.08 (6.40) | 0.049 |
| Age at menarche (years) | | | 0.710 |
| <12 | 143 (22.7%) | 11 (17.7%) | |
| 12–13 | 301 (50.1%) | 22 (54.8%) | |
| ≥14 | 162 (27.2%) | 11 (27.5%) | |
| PIR | 2.96 (1.59) | 3.27 (1.65) | 0.249 |
| BMI (kg/m$^2$) | | | 0.039 |
| <25 | 173 (33.6%) | 20 (52.1%) | |
| ≥25 | 433 (66.4%) | 24 (47.9%) | |
| Smoked ≥100cigarettes | | | 0.971 |
| Yes | 217 (40.6%) | 14 (41.0%) | |
| No | 389 (59.4%) | 30 (59.0%) | |
| Race/ethnicity | | | 0.003 |
| Non-Hispanic White | 263 (66.4%) | 32 (89.2%) | |
| Non-Hispanic Black | 162 (15.7%) | 7 (5.2%) | |
| Other | 181 (18.0%) | 5 (5.7%) | |
| Marital status | | | 0.233 |
| Married/Living with partner | 434 (75.9%) | 28 (64.0%) | |
| Never married | 79 (15.0%) | 5 (24.9%) | |
| Widowed/Divorced/Separated | 93 (9.1%) | 11 (11.1%) | |
| Education level | | | 0.153 |
| High school and below | 153 (18.4%) | 4 (8.1%) | |
| Above high school | 453 (81.6%) | 40 (91.9%) | |
| Hypertension | | | 0.801 |
| Yes | 116 (20.5%) | 11 (22.6%) | |
| No | 490 (79.5%) | 33 (77.4%) | |
| Diabetes | | | 0.077 |
| Yes | 38 (6.3%) | 0 (0.0%) | |
| No | 568 (93.7%) | 44 (100.0%) | |
| Alcohol intake | | | 0.822 |
| Yes | 359 (65.8%) | 26 (63.8%) | |
| No | 247 (34.2%) | 18 (36.2%) | |
| Fertility status | | | 0.420 |
| Nulliparous | 39 (7.5%) | 5 (11.3%) | |
| ≥one birth | 567 (92.5%) | 39 (88.7%) | |

Continuous variables were presented as median (interquartile range) or mean (standard deviation). Categorical variables were presented as frequency (percentage).

# 3 Results

## 3.1 Characteristics of the participants

The baseline characteristics and weighted estimates of the 650 participants were detailed in Table 1. Out of the total participants, 44 were afflicted with endometriosis, while 606 remained unaffected. Substantial variations ($p < 0.05$) were evident between the endometriosis and non-endometriosis cohorts concerning age, BMI, ethnicity. There were no significant differences ($p > 0.05$) in menarche age, PIR, marital status, smoking status, education level. The distribution of laboratory measurement results among participants was displayed in Table 2, with MMA being the only indicator significantly associated with endometriosis. Individuals with endometriosis were more likely to have urinary MMA concentrations at or above the detection limit.

## 3.2 Weighted logistic regression and subgroup analyses

Weighted univariate and multivariate logistic regression analyses were utilized to examine the correlation between six types of arsenic

TABLE 2 Laboratory measures of NHANES participants included in the analysis.

| Characteristics | Non-endometriosis (*N* = 606) | Endometriosis (*N* = 44) | *p*-value |
|---|---|---|---|
| Creatinine, urine (mg/dL) | 103.00 (64.00, 161.65) | 108.96 (40.04, 146.53) | 0.540 |
| Urinary arsenic, total (μg/L) | 7.96 (3.79, 17.19) | 8.22 (2.42, 13.89) | 0.192 |
| Urinary arsenous acid (μg/L) | | | 0.778 |
| At or above detection limit | 15 (2.2%) | 1 (3.1%) | |
| Below lower detection limit | 591 (97.8%) | 43 (96.9%) | |
| Urinary arsenic acid (μg/L) | | | 0.867 |
| At or above detection limit | 38 (9.5%) | 4 (8.8%) | |
| Below lower detection limit | 568 (90.5%) | 40 (91.2%) | |
| Urinary arsenobetaine (μg/L) | | | 0.775 |
| At or above detection limit | 432 (68.8%) | 32 (71.0%) | |
| Below lower detection limit | 174 (31.2%) | 12 (29.0%) | |
| Urinary arsenocholine (μg/L) | | | 0.958 |
| At or above detection limit | 12 (2.9%) | 1 (3.1%) | |
| Below lower detection limit | 594 (97.1%) | 43 (96.9%) | |
| Urinary DMA (μg/L) | | | 0.487 |
| At or above detection limit | 515 (83.8%) | 36 (78.6%) | |
| Below lower detection limit | 91 (16.2%) | 8 (21.4%) | |
| Urinary MMA (μg/L) | | | 0.010 |
| At or above detection limit | 168 (30.1%) | 21 (55.1%) | |
| Below lower detection limit | 438 (69.9%) | 23 (44.9%) | |

Continuous variables were presented as median (interquartile range) or mean (standard deviation). Categorical variables were presented as frequency (percentage). DMA, dimethylarsinic acid; MMA, monomethylarsonic acid.

TABLE 3 Unadjusted and adjusted odds ratios (OR) and 95% confidence intervals (CI) of the association between arsenic and endometriosis NHANES 2003–2006.

| Speciated urinary arsenic | OR (95% CI) *p* | | |
|---|---|---|---|
| | Model 1 | Model 2 | Model 3 |
| Urinary arsenous acid (μg/L) | 1.974 (1.136–3.430)<br>0.018 | 1.898 (1.072–3.362)<br>0.029 | 1.741 (0.961–3.154)<br>0.065 |
| Urinary arsenic acid (μg/L) | 0.958 (0.612–1.501)<br>0.847 | 0.905 (0.536–1.526)<br>0.697 | 0.842 (0.443–1.602)<br>0.576 |
| Urinary arsenobetaine (μg/L) | 0.997 (0.989–1.005)<br>0.405 | 0.997 (0.990–1.004)<br>0.356 | 0.997 (0.992–1.003)<br>0.313 |
| Urinary arsenocholine (μg/L) | 0.317 (0.009–11.461)<br>0.031 | 0.242 (0.005–11.500)<br>0.457 | 0.363 (0.018–7.463)<br>0.484 |
| Urinary dimethylarsinic acid (DMA) (μg/L) | 1.000 (0.952–1.050)<br>0.992 | 1.013 (0.970–1.058)<br>0.550 | 1.005 (0.961–1.051)<br>0.825 |
| Urinary monomethylarsonic acid (MMA) (μg/L) | 1.341 (1.127–1.595)<br>0.002 | 1.356 (1.106–1.663)<br>0.005 | 1.317 (1.074–1.615)<br>0.012 |

Model 1 did not control for any covariates, Model 2 was adjusted for age and ethnicity, and Model 3 further controlled for marital status, educational attainment, smoking status, BMI, PIR, menarche onset, alcohol use, hypertension, diabetes, fertility status and creatinine levels in urine.

and endometriosis. As depicted in Table 3, Model 1 did not adjust for covariates, Model 2 adjusted for age and race, and Model 3 was subjected to further adjustments incorporating marital status, education level, smoking status, BMI, PIR, age at menarche, alcohol use, hypertension, diabetes, fertility status and urine creatinine levels. Associations were reported using odds ratios (OR) with 95% confidence intervals (CI). Higher concentrations of arsenous acid and MMA in urine were positively correlated with endometriosis ($p < 0.05$). Although the positive correlation of arsenous acid weakened after adjusting for potential confounding factors, MMA remained significantly correlated with endometriosis (MMA: OR: 1.317, 95%CI: 1.074–1.615).

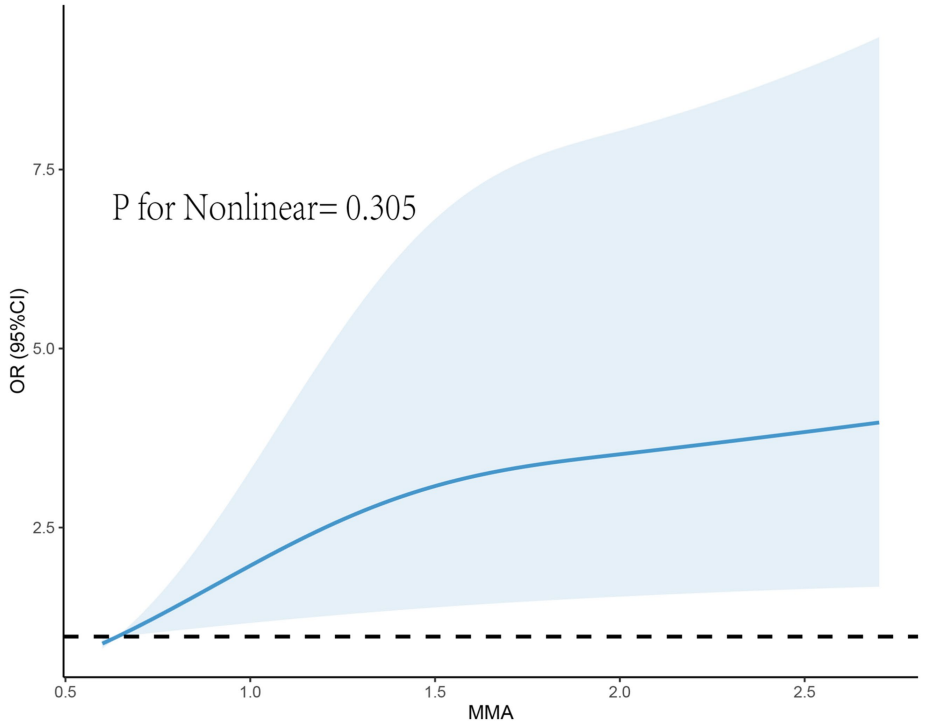


FIGURE 2
Restricted cubic spline plot of the association between monomethylarsonic acid (MMA) and endometriosis.

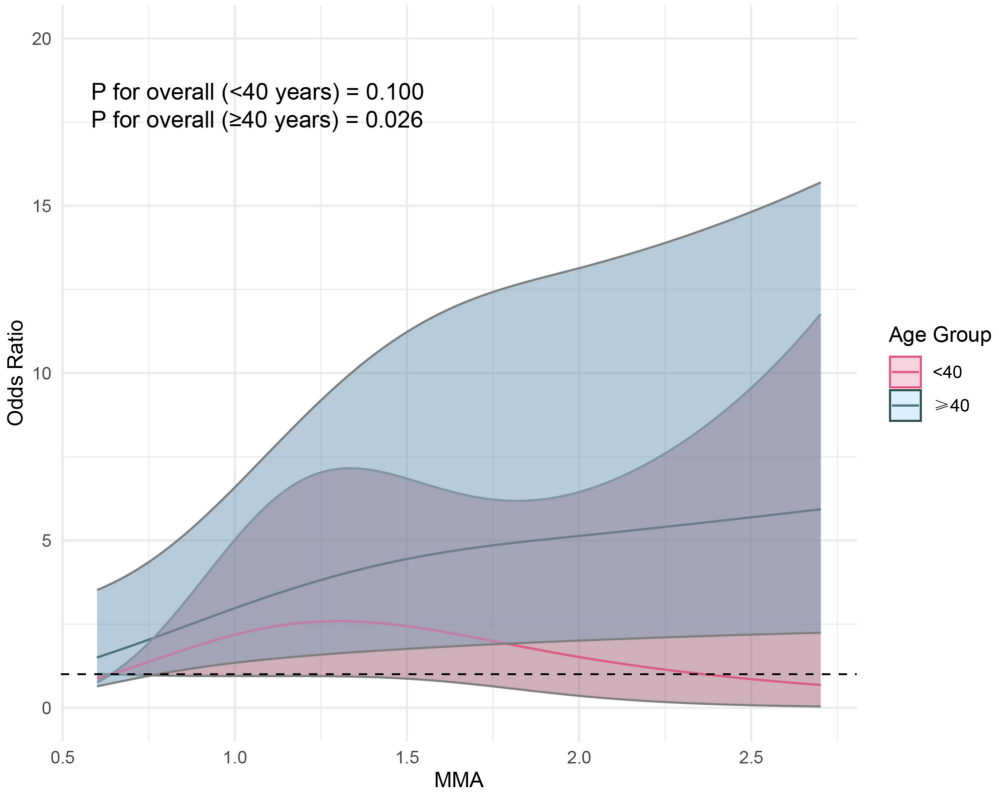


FIGURE 3
Restricted cubic spline plot of the association between monomethylarsonic acid (MMA) and endometriosis stratified by age.

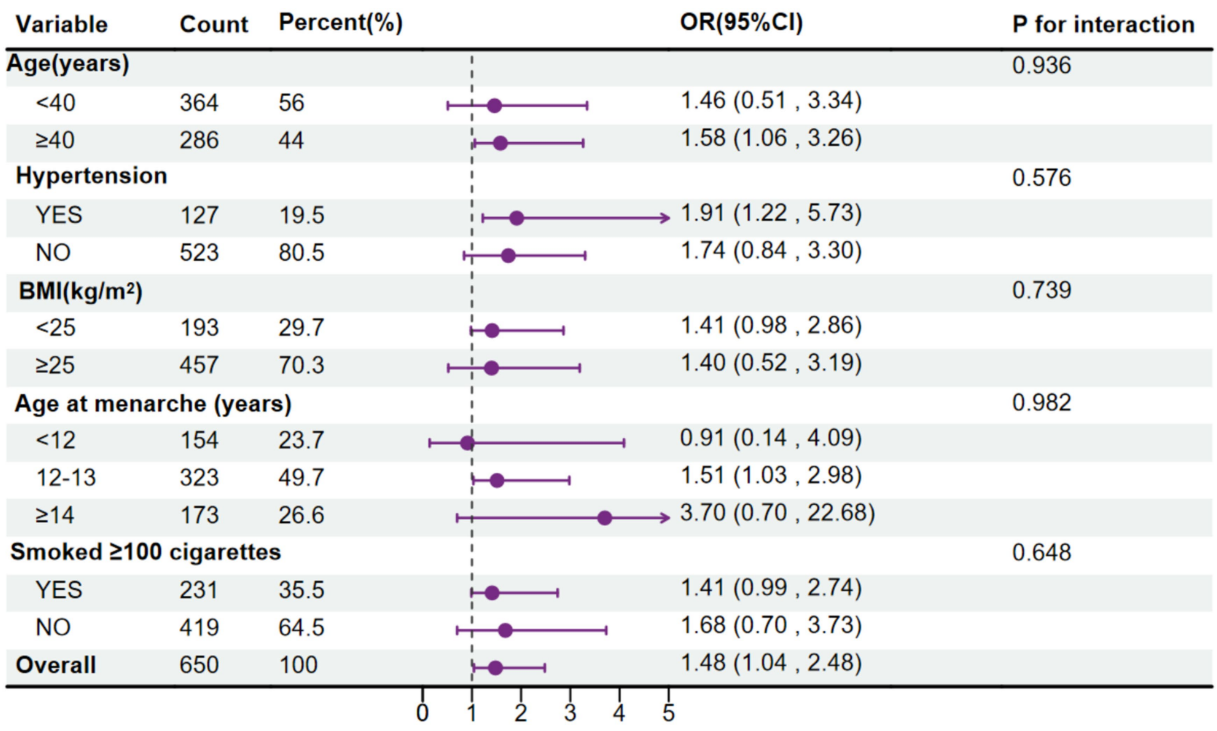


FIGURE 4
Forest plot for the association between urinary monomethylarsonic acid (MMA) and endometriosis, stratified by age, body mass index (BMI), age at menarche, hypertension and smoking status.

After adjusting for covariates in Model 3, although RCS did not reveal a nonlinear relationship between MMA and endometriosis, the prevalence rate of endometriosis seemed to increase with higher exposure levels (Figure 2). Furthermore, we found that the correlation between MMA and endometriosis was not significant in individuals under 40 years old but was significant in those over 40 (Figure 3), possibly related to long-term arsenic exposure. The sensitivity analysis results after multiple interpolation indicate that they were still robust (Supplementary Table S1).

As illustrated in Figure 4, when observing the association between MMA and endometriosis, grouped by age, marital status, education level, BMI, age at menarche, smoking status, and race, no interaction of the related covariates was observed in this association ($p$ for interaction > 0.05), indicating that the correlation between MMA and endometriosis does not vary due to age, BMI, age at menarche, smoking status, and hypertension.

## 4 Discussion

In our nationally representative study of American women, we found a correlation between urinary MMA and self-reported physician-diagnosed endometriosis, after adjusting for potential confounders. As far as we are aware, this serves as the inaugural large-scale, nationally representative inquiry into the relationship between total urinary arsenic, arsenic species, and endometriosis, unveiling a positive correlation between urinary MMA and endometriosis. Studies on arsenic and endometriosis are limited and inconsistent; a previous cohort study involving 473 women did not observe a correlation between urinary arsenic and endometriosis (12). In China, a recent study on the association between exposure to various toxic metals and endometriosis found that the concentration of arsenic in blood or follicular fluid contributed the most, reporting a positive correlation with endometriosis (11).

As an estrogen-dependent chronic inflammatory disease, endometriosis has the highest prevalence rate between the ages of 25–35 (13). Estrogen plays a crucial role in the disease's pathogenic mechanism; increased local production of estrogen fosters the survival, implantation, and proliferation of ectopic endometrial tissue (14). The relationship between arsenic and estrogen is still controversial. A study based on adolescents showed a positive correlation between total arsenic, its metabolites, and estradiol (14), while two experimental studies indicated a significant decrease in estradiol levels after arsenic exposure (15, 16). Arsenic interacts with the ligand-binding domain of estrogen receptor-alpha (ERα) with high affinity, exhibiting potent estrogenic activity *in vitro* and *in vivo* (17). Moreover, arsenic, characterized by its properties as a metalloid with both metallic and nonmetallic attributes, predominantly manifests its toxic effects through oxidative stress, widely acknowledged as the principal mechanism of arsenic toxicity (18, 19). Reactive oxygen species (ROS) production can alter redox balance, ultimately leading to reproductive abnormalities such as miscarriage, endometriosis, and polycystic ovary syndrome (20, 21). Studies have found that antioxidant defense mechanisms in the uterus of mice, Including enzymes like peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT), are reduced by 2.5, 2.6, and 2.9 times, respectively, in arsenic-exposed groups (16). MMA is a major component of arsenic metabolites, with up to 11% of total urinary arsenic excreted as MMA (22). It is one of the most toxic

forms of arsenic in the human body (22, 23), possibly due to its higher cellular uptake rate (24) and high affinity for thiol (-SH) groups (22). Although the toxicological characteristics of arsenic metabolites vary in the body, the toxicity phenotype of MMA is mostly associated with ROS production (3). Arsenic exposure also leads to an increase in pro-inflammatory cytokines such as IL-6, IL-10, and TNF-$\alpha$, which are closely related to inflammatory responses (25). The inflammatory pathway is also involved in the mechanism of endometriosis formation (26). The complexity of arsenic toxicity is manifested through its multifaceted nature (27). Nevertheless, owing to the scarcity of specific research examining arsenic exposure in relation to endometriosis, further experiments are warranted to elucidate the involved mechanisms.

This study has the following limitations: Firstly, as it is a cross-sectional study design, potential reverse causation cannot be ruled out. The study measures current arsenic levels but cannot account for the time elapsed between endometriosis diagnosis and arsenic measurement. Secondly, although urinary arsenic concentration is commonly used as a typical biomarker for arsenic exposure, the rapid metabolism in humans means that data from a single urine sample may limit our ability to accurately assess the average level of arsenic exposure. Additionally, the distribution of arsenic forms differs between blood and urine, with blood arsenic potentially providing a more accurate reflection of exposure levels in target tissues and organs (28). Furthermore, our analytical approach, which focused on single urinary arsenic measurements, represents a methodological limitation. More sophisticated mixed-effects analyses, such as weighted quantile sum regression or Bayesian kernel machine regression, could provide deeper insights into the complex relationships between multiple arsenic species and endometriosis risk. Lastly, most of the data used were collected through interviews or self-reported questionnaires, which could be subject to recall and reporting biases.

## 5 Conclusion

In conclusion, this investigation establishes the initial evidence of a direct link between urinary MMA and the prevalence of endometriosis. Considering the significant prevalence of endometriosis and the pervasive exposure to arsenic in the general populace, it is vital to pursue further experimental studies to validate our findings and elucidate the specific mechanisms involved.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: all the data analyzed in our study can be obtained from NHANES database.

## Author contributions

LS: Writing – original draft. YR: Writing – review & editing. RX: Writing – review & editing. SZ: Writing – review & editing. WL: Writing – review & editing. CM: Writing – review & editing. XZ: Writing – review & editing. ZD: Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Generative AI statement

The authors declare that no Generative AI was used in the creation of this manuscript.

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## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2025.1525986/full#supplementary-material

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# The association between glycated hemoglobin, physical activity and infertility: a multiple logistic regression and mediation analysis based on the NHANES database

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**Objective:** To determine the relationship between physical activity (PA) patterns and infertility among women in the United States and to ascertain whether glycated hemoglobin (HbA1c) influences this association.

**Design:** A cross-sectional study of PA patterns and infertility was conducted. The PA classification was based on two categories: recreational PA and work-related PA. The subgroups were classified as inactive, less active, or active. We conducted data analysis via both multiple logistic regression analysis and mediation analysis.

**Setting:** National Health and Nutrition Examination Survey (NHANES, 2013–2018).

**Participants:** Women aged 18–49 years (n, 3,948).

**Results:** There was no statistically significant link demonstrated between infertility and work-related PA patterns. The recreational PA group may decrease the prevalence of infertility, with its occurrence being merely 0.79 times that of inactive group. (95% CI: 0.62, 1.0; $p = 0.045$). The consistency of this discrepancy was not preserved in Model III after the mixed effect was incorporated into the recreational PA group. The stratified research findings revealed that the association between recreational PA patterns and infertility was influenced by variables such as age, BMI, and history of diabetes. Furthermore, the mediation analysis revealed that recreational PA did not have a statistically

significant direct effect on infertility ($p$ = 0.098). HbA1c serves as a mediator in this interaction (95% CI: -0.06, -0.02).

**Conclusion:** Recreational PA patterns were associated with infertility among women aged 18–49 years in the United States, which was likely due to the effects of HbA1c.

KEYWORDS

**physical activity (PA), activity patterns, infertility, glycated hemoglobin (HbA1c), mediation**

# Introduction

Infertility is characterized as the inability to conceive following 12 months of consistent, unprotected sexual intercourse (1). This condition is a major worldwide health concern that adversely affects individual well-being and human rights. The most recent guidelines from the World Health Organization (WHO) indicate that approximately 10–15% of couples in the United States experience infertility, with the frequency of infertility increasing with age (2). Infertility is influenced by a variety of complex circumstances involving both male and female contributions. WHO guidelines identify the primary contributors to female infertility as ovulatory disorders, pelvic factors, infectious factors, immunological factors, and unexplained causes. In females, age is a paramount factor influencing fertility, as the ovarian reserve decreases with advancing age, thus resulting in a reduction in both egg quality and quantity (3). Moreover, the incidence of aneuploidy (which stems from chromosomal irregularities) is more prevalent in older women, thus increasing the likelihood of miscarriage and contributing to infertility (4). This underscores the necessity for an extensive understanding of female reproductive health, especially regarding the impact of various lifestyle factors on fertility.

PA is essential for sustaining overall health. The WHO defines PA as any bodily action that expends energy through the skeletal muscular system. Regular PA has numerous advantages, such as improved cardiovascular function, effective weight management, enhanced metabolic health, and increased mental well-being (5). These factors may indirectly affect fertility by regulating hormone balance and enhancing reproductive outcomes (6, 7).

HbA1c is an essential biomarker for evaluating long-term glucose regulation, especially in individuals with associations with diabetes. Recent studies have indicated that HbA1c may also represent overall metabolic health and can act as a marker for reproductive health (8, 9). Recent studies have also shown that an increase in HbA1c levels is significantly associated with the risk of ovarian dysfunction, which may affect ovarian health by influencing insulin resistance and the inflammatory response (10). Regular PA has been shown to lower HbA1c levels, thereby improving metabolic health and improving ovarian function (11). This effect is particularly pertinent for women, due to the fact that increased HbA1c levels have been linked to unfavorable reproductive outcomes, including heightened risks of conception challenges and hazardous pregnancy problems such as gestational diabetes mellitus, early pregnancy abortion, fetal dysplasia, etc (12). Additional data have indicated correlations among metabolic health, physical fitness, and reproductive health (13). Nevertheless, there are limited findings regarding the mediation function of HbA1c in the relationship between PA and infertility. Therefore, an understanding of the relationships among PA, HbA1c levels, and infertility may yield significant insights into prospective therapeutic options for enhancing reproductive health in aging women. This article categorized PA levels into three groups, including active, less active, and inactive groups, according to PA guidelines (14–16). A stratified study was performed to examine the associations between infertility and PA levels, as well as explore the mediating role of HbA1c in the relationship between PA and infertility, with a particular focus on female reproductive health. This analysis aims to understand how lifestyle modifications can enhance fertility outcomes and inform clinical practices for those individuals who experience infertility.

# Materials and methods

## Study population

This study utilized cross-sectional survey data from the NHANES (https://wwwn.cdc.gov/nchs/nhanes/). A total of 14,948 women were included from 3 cycles of the NHANES, and 5,431 of them responded to the question on infertility. After excluding individuals aged 50 years or older who did not have exercise patterns or HbA1c indicators, the study included a total of 3,948 participants, as visually demonstrated in Figure 1. The NHANES protocols were approved by the NCHS Research Ethics Review Board, and written informed consent forms were provided by all of the participants.

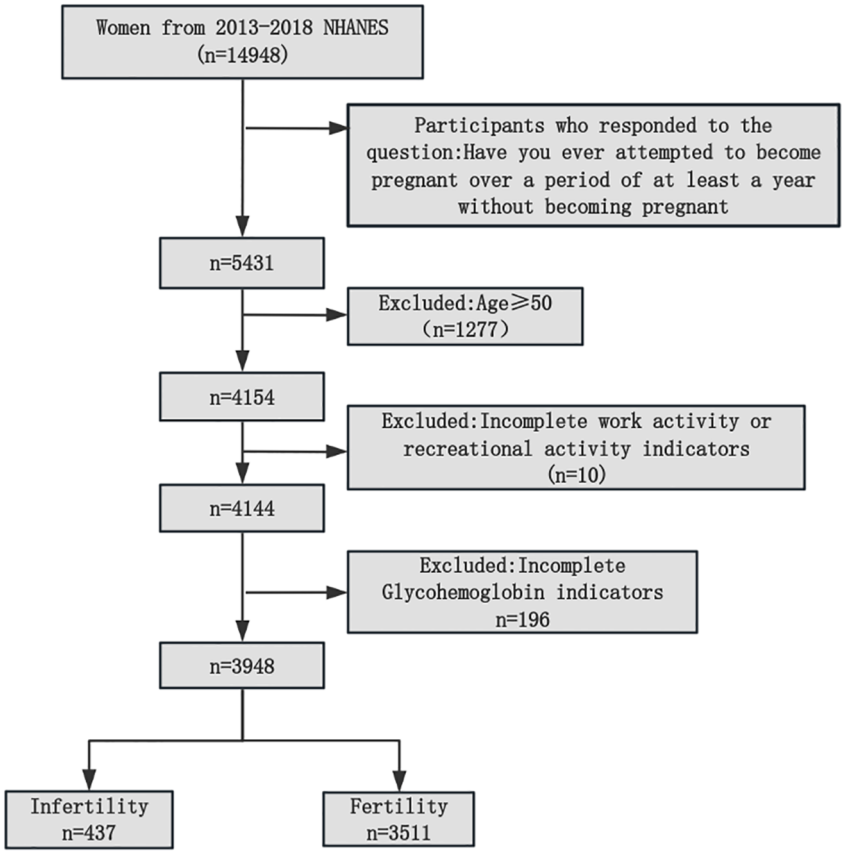


FIGURE 1
Flowchart of participants selection from the NHANES 2013–2018. NHANES, National Health and Nutrition Examination Survey.

## Assessments of PA, infertility and HbA1c

The NHANES study collected data on PA via a questionnaire, in which individuals reported their own PA levels. The questions regarding PA were as follows: "Do you participate in any vigorous exercise or fitness activity on a regular basis that leads to a significant increase in your breathing or heart rate, such as running or playing basketball for at least 10 minutes?"; and "Do you often participate in any type of moderate-intensity PA, such as brisk walking, cycling, swimming, or playing volleyball, for at least 10 minutes, which causes a slight increase in your breathing and heart rate?". This study included both work and recreational activities. According to the recommendations of WHO and national health institutions, adults aged 18–64 years need to engage in at least 150 minutes of moderate-intensity aerobic exercise (such as brisk walking or cycling) or 75 minutes of high-intensity aerobic exercise (such as running or swimming) per week to promote their health. We separately calculated the durations of both moderate- and high-intensity PA and classified PA into three categories: inactive, less active (≤ recommended amount of activity), and active (≥ recommended amount of activity) (Table 1). We reported infertility via the following question: "Have you made any attempts to conceive for a duration of at least one year without achieving pregnancy?". We classified those individuals who responded affirmatively as infertile and those who responded negatively as fertile. We classified unanswered or refused

TABLE 1 Physical activity (PA) classification.

| INACTIVE PA | LESS ACTIVE PA | ACTIVE PA |
|---|---|---|
| No high-intensity PA | Only high-intensity PA, and the weekly PA time is less than 75 minutes. | Only high-intensity PA, and the weekly PA time must exceed 75 minutes. |
| No moderate-intensity PA | Only moderate-intensity PA, and the weekly PA time is less than 150 minutes. | Only moderate-intensity PA, and the weekly PA time must exceed 150 minutes. |
| Sedentary behavior | The duration of both types of PA is less than 75 minutes for high-intensity PA and less than 150 minutes for moderate-intensity PA. | The weekly duration of high-intensity PA exceeds 75 minutes, and the moderate-intensity PA also exceeds 150 minutes minutes. |

responses as missing values. At Columbia University, Missouri, HbA1c tests were conducted. The A1c G7 HPLC Glycohemoglobin Analyzer (produced by Tosoh Medicine, Inc., 347 Oyster Pt. Blvd., Suite 201, So. San Francisco, CA 94080) was used to measure HbA1c levels.

## Other variables of interest

We conducted a thorough examination of 10 potential variables that could influence the relationship between physical activity PA and infertility. These variables included age (18–49 years), race, sex, BMI, smoking status, education, sleep, history of diabetes, poverty-to-income ratio (PIR), menstrual cycle, current pregnancy, and HbA1c. To facilitate comparative analysis, we converted continuous variables into categorical variables and divided each covariate into a reference group and additional groups for comparison. Furthermore, we designated "unknown, rejected, or undetectable" values as values that were not present or could not be determined.

## Statistical analysis

The chi-square test was used to assess the statistical disparities in confounding factors between the two groups, including fertility and infertility. A logistic regression model was used to investigate the correlation between infertility and PA. The model calculated the odds ratio (OR) between infertility and various categories of work and recreational PA, and it compared the 95% confidence interval (CI) with that of women who were inactive (the reference group). The regression model was adjusted for age, race, BMI, smoking status, education, sleep, history of diabetes, poverty-to-income ratio (PIR), menstrual cycle and current pregnancy or nonpregnancy status. In Model 1, no variables were adjusted; in Model 2, racial and educational adjustments were made; and in Model III, we accounted for all of the covariates and subsequently examined them as subgroup variables in a stratified analysis. To assess the extent to which HbA1c mediates the association between PA patterns and infertility, we performed a causal mediation analysis. Initially, we employed the causal stepwise regression test to conduct linear

TABLE 2 Baseline characteristics of study population according to fertity*.

| | Infertility (n=437) | | Fertility (n=3511) | | P Value† |
|---|---|---|---|---|---|
| | Mean/n | SE/% | Mean/n | SE/% | |
| Age (n %) | | | | | <0.001 |
| <35 | 162 | 7.7 | 1941 | 92.3 | |
| ≥35 | 275 | 14.9 | 1570 | 85.1 | |
| Race (n %) | | | | | 0.02 |
| Mexican-American | 62 | 9.0 | 629 | 91.0 | |
| Other Hispanic | 38 | 9.2 | 375 | 90.8 | |
| Non-Hispanic White | 174 | 13.2 | 1141 | 86.8 | |
| Non-Hispanic Black | 90 | 10.6 | 759 | 89.4 | |
| Other Race | 73 | 10.7 | 607 | 89.3 | |
| BMI (n %) | | | | | <0.001 |
| Normal<25 | 119 | 8.7 | 1256 | 91.3 | |
| Overweight(25-29.99) | 77 | 8.3 | 852 | 91.7 | |
| Obesity(≥30) | 234 | 14.6 | 1373 | 85.4 | |
| Education (n %) | | | | | 0.12 |
| <Highschool | 59 | 10.1 | 524 | 89.9 | |
| ≥Highschool | 371 | 12.4 | 2622 | 87.6 | |
| Smoking (n %) | | | | | 0.65 |
| Every day | 76 | 14.0 | 467 | 86.0 | |
| Some day | 16 | 12.0 | 117 | 88.0 | |
| Not at all | 66 | 15.2 | 369 | 84.8 | |

*(Continued)*

TABLE 2 Continued

| | Infertility (n=437) | | Fertility (n=3511) | | P Value† |
|---|---|---|---|---|---|
| | Mean/n | SE/% | Mean/n | SE/% | |
| Sleeping (n %) | | | | | 0.45 |
| ≤8 | 122 | 10.6 | 1028 | 89.4 | |
| >8 | 131 | 9.7 | 1221 | 90.3 | |
| Diabete history (n %) | | | | | <0.001 |
| Yes | 39 | 19.8 | 158 | 80.2 | |
| No | 388 | 10.5 | 3298 | 89.5 | |
| PIR (n %) | | | | | 0.04 |
| <1.3 | 134 | 10.1 | 1191 | 89.9 | |
| 1.30-2.99 | 117 | 10.7 | 977 | 89.3 | |
| ≥3.0 | 159 | 13.2 | 1045 | 86.8 | |
| Menstrual cycle (n %) | | | | | 0.54 |
| Regular menstruation | 385 | 11.2 | 3056 | 88.8 | |
| Irregular menstruation | 52 | 10.3 | 454 | 89.7 | |
| Is currently in a pregnant state? (n %) | | | | | 0.72 |
| YES | 18 | 14.2 | 109 | 85.8 | |
| NO | 243 | 13.0 | 1620 | 87.0 | |
| HbA1C (mmol/L) | 2.47 | 0.2 | 2.43 | 0.17 | <0.001 |

BMI, body mass index; PIR, poverty income ratio; HbA1C, glycosylated hemoglobin.
*Values presented are means and their SE for continuous variables, and as percentages for categorical variables.
†We tested for differences in baseline characteristics using weighted $\chi$ 2 tests for categorical variables and weighted ANOVA for continuous variables.

regression and logistic regression on the dependent variable with respect to the independent variable (17). We subsequently determined the regression coefficients a, b, c, and c′ in the mediation model. The RMediation package in R software was used to calculate the 95% confidence interval of Za * Zb via the cumulative distribution approach (18). The PA pattern, infertility status, and HbA1c level were used to represent the independent variables, outcome variables, and mediating variables, respectively, in our survey. A significance threshold of P<0.05 and a Za * Zb 95% CI excluding 0 guided the determination of statistical significance.

# Results

## Characteristics of participants

This study included 3,948 female participants, with an average age of 33.24 ± 9.47 years; additionally, 11.07% had previously experienced infertility. Table 2 presents a thorough summary of the fundamental characteristics of the participants. Women who were over the age of 35 years, of non-Hispanic white ethnicity, and obese,

TABLE 3 Association between PA patterns and infertility.

| | Model I | Model II | Model III |
|---|---|---|---|
| | OR 95%CI P Value | OR 95%CI P Value | OR 95%CI P Value |
| Work related PA | | | |
| Inactive | Ref | Ref | Ref |
| Less active | 0.87 (0.65-1.16) 0.33 | 0.80 (0.60-1.08) 0.15 | 0.79 (0.33-1.86) 0.59 |
| Active | 1.01 (0.80-1.28) 0.92 | 0.94 (0.74-1.20) 0.63 | 0.91 (0.46-1.79) 0.78 |
| Recreational PA | | | |
| Inactive | Ref | Ref | Ref |
| Less active | 0.89 (0.69-1.14) 0.36 | 0.84 (0.65-1.09) 0.20 | 0.66 (0.28-1.60) 0.36 |
| Active | 0.79 (0.62-1.0) 0.045 | 0.75 (0.59-0.96) 0.02 | 0.82 (0.38-1.78) 0.62 |

PA, physical activity.
Model 1 adjusted for none.
Model 2 adjusted for race and education.
Model 3 adjusted for age, race, BMI, education level, sleeping hour, smoke status, Diabete history, PIR, menstrual cycle, pregnant state.

in addition to having a high poverty-to-income ratio (PIR) and a history of diabetes, were more likely to experience infertility. There was no statistically significant disparity between the two groups in terms of education level, smoking status, sleep duration, or regularity of the menstrual cycle. Furthermore, individuals with infertility exhibited a notable increase in HbA1c levels compared to patients who did not have a history of infertility (2.47 ± 0.17 vs. 2.43 ± 0.2).

## Results of multiple logistic analysis

These findings indicated that there is no substantial association between work-related PA and infertility. However, in recreational PA, there is no substantial correlation between less active PA and infertility, whereas there is a strong association between active PA and infertility. During active PA, the likelihood of infertility is 0.79 times that during inactive PA. In Model II, after accounting for the confounding factors of race and education, the correlation remained significant (OR=0.75, 95% CI: 0.59, 0.96; $p$=0.02). However, in Model III, after adjusting for confounding factors in all of the covariates, the strength of this correlation was no longer statistically significant (OR = 0.82, 95% CI: 0.38, 1.78; $p$ = 0.62), as indicated in Table 3.

## Stratified analyses

A stratified analysis of the correlation between all of the covariates and infertility in three different modes of recreation-related phsical activity (PA) revealed that among women who engaged in active and less active PA, obese women (BMI ≥ 30) had a greater probability of infertility than women of normal weight; however, this difference was not observed in women without exercise. In addition, there was no statistically significant difference in the incidence of infertility between overweight women (BMI = 25–29.9) and normal-weight women among the three PA modes. More significantly, there was a significant correlation between a history of diabetes and infertility in women without exercise and in women with active PA; however, this correlation was not significant in women with less active PA, as indicated in Table 4.

## Mediation analysis

Mediation analysis sought to measure the extent to which HbA1c acts as a mediator in the relationship between recreational physical PA patterns and infertility. In contrast to conventional mediation research, our study specifically examined categorical variables as both independent and dependent factors. Thus, we calculated the relative mediation effects, relative direct effects, and relative total effects and computed the mediating effect value by multiplying Za and Zb, after which we assessed the 95% confidence interval of Za * Zb via the product distribution method. The results of our study indicated that engaging in recreational active PA does not have a statistically significant direct effect on infertility ($p$=0.098). However, we found that HbA1c plays a mediating role in the relationship between recreational active PA and infertility, with a mediating effect of -24.9 (95% CI: -0.06, -0.02), as shown in Figure 2.

# Discussion

As society progresses and educational levels improve, an increasing number of individuals over the age of 35 express their desire to become pregnant, and infertility is particularly common among older women. While aging is the primary cause of declining fertility, the development of assisted reproductive technology in recent years has provided the possibility of reproduction for older infertile women. As a result, physiological and individual factors play an important role for women of advanced reproductive age. Our study extended the age range of the research subjects to females aged 18 to 49, potentially increasing the universality and application of the research findings, allowing them to span a broader range of female populations and providing more thorough data support for clinical treatment. This study primarily investigated the role of HbA1c in modulating the link between PA and infertility. We performed a cross-sectional study utilizing a questionnaire survey to examine the interrelations among PA, HbA1c, and infertility within a population that willingly participated in the survey in the United States throughout the same period of time. The incidence of infertility is lower with active PA, as recommended by WHO, than with inactive PA. However, this tendency was solely evident in recreation-related PA, with no differences in infertility rates being associated with work-related PA. Moreover, HbA1c is negatively correlated with PA and positively correlated with infertility, and it may mediate the link between PA and infertility. The impact of obesity and a history of diabetes on infertility is primarily mediated by PA.

By distinguishing our study from previous studies, we specifically offer a novel perspective by categorizing PA patterns in accordance with World Health Organization(WHO) guidelines. This choice was based on the clear notion that neither high-intensity nor moderate PA encompasses the duration of the activity. Research has indicated that moderate PA enhances pregnancy rates in women with reproductive problems and that consistent moderate PA supports reproductive health maintenance (19–21). HaKimi et al. analyzed an extensive amount of literature and proposed that exceeding 60 minutes of strenuous exercise on a daily basis increases the chance of anovulation; however, 30–60 minutes of moderate-intensity exercise may mitigate ovulatory infertility. Therefore, following the WHO guidelines, we classify exercise into inactive, less active, and active exercise types while considering both exercise intensity and duration (22).

Our research indicated that the relationship between different types of physical PA and infertility is inconsistent. recreation-related PA is strongly associated with reduced infertility rates, which is consistent with the findings of previous studies that highlighted its importance for improving reproductive health in women of childbearing age (23). We propose that this effect is due to the advantageous benefits of consistent recreational activity, as recommended by WHO. This may mitigate the adverse effects of an energy deficit on fertility by increasing the body's

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TABLE 4 Recreational PA patterns – infertility subgroup analysis.

| | Inactive (n=1823) | Less active (n=893) | Active (n=1232) |
|---|---|---|---|
| | OR 95%CI P Value | OR 95%CI P Value | OR 95%CI P Value |
| **Age (Y)** | | | |
| <35 | Ref | Ref | Ref |
| ≥35 | 1.97 (1.47-2.65) <0.001 | 2.12 (1.37-3.27) <0.001 | 2.21 (1.51-3.24) <0.001 |
| **Race** | | | |
| Mexican-American | Ref | Ref | Ref |
| Other Hispanic | 1.19 (0.68-2.07) 0.54 | 1.27 (0.50-3.27) 0.62 | 0.60 (0.23-1.56) 0.29 |
| Non-Hispanic White | 1.61 (1.06-2.44) 0.03 | 1.72 (0.84-3.56) 0.14 | 1.50 (0.84-2.70) 0.17 |
| Non-Hispanic Black | 1.03 (0.65-1.63) 0.92 | 1.61 (0.73-3.58) 0.24 | 1.34 (0.70-2.60) 0.38 |
| Other Race | 1.38 (0.85-2.24) 0.19 | 1.11 (0.49-2.50) 0.81 | 1.17 (0.66-1.17) 0.66 |
| **BMI** | | | |
| Normal<25 | Ref | Ref | Ref |
| Overweight (25-29.99) | 0.87 (0.57-1.33) 0.51 | 1.35 (0.71-2.57) 0.36 | 0.80 (0.45-1.42) 0.45 |
| Obesity (≥30) | 1.29 (0.93-1.80) 0.14 | 2.51 (1.50-4.19) <0.001 | 2.30 (1.50-3.53) <0.001 |
| **Education** | | | |
| <Highschool | Ref | Ref | Ref |
| ≥Highschool | 1.32 (0.93-1.88) 0.13 | 1.04 (0.50-2.16) 0.91 | 1.76 (0.80-3.88) 0.16 |
| **Smoking** | | | |
| Every day | Ref | Ref | Ref |
| Some day | 0.97 (0.43-2.18) 0.94 | 0.83 (0.25-2.77) 0.76 | 0.78 (0.24-2.54) 0.68 |
| Not at all | 0.83 (0.49-1.40) 0.48 | 1.40 (0.64-3.07) 0.40 | 1.49 (0.72-3.07) 0.28 |
| **Sleeping (H)** | | | |
| <8 | Ref | Ref | Ref |
| ≥8 | 1.06 (0.74-1.54) 0.74 | 0.77 (0.44-1.34) 0.35 | 0.76 (0.46-1.26) 0.29 |
| **Diabete history** | | | |
| NO | Ref | Ref | Ref |
| YES | 1.98 (1.21-3.24) 0.006 | 1.43 (0.62-3.27) 0.40 | 3.22 (1.54-6.75) 0.002 |
| **PIR** | | | |
| <1.3 | Ref | Ref | Ref |
| 1.30-2.99 | 1.11 (0.80-1.58) 0.56 | 0.79 (0.43-1.45) 0.45 | 1.29 (0.77-2.19) 0.34 |
| ≥3.0 | 1.69 (1.12-2.41) 0.004 | 1.25 (0.74-2.09) 0.40 | 1.28 (0.78-2.09) 0.32 |
| **Menstrual cycle** | | | |
| Irregular menstruation | Ref | Ref | Ref |
| Regular menstruation | 1.11 (0.75-1.65) 0.59 | 1.30 (0.65-2.57) 0.46 | 1.69 (0.84-3.41) 0.14 |
| **Is currently in a pregnant state?** | | | |
| NO | Ref | Ref | Ref |
| YES | 1.04 (0.50-2.14) 0.93 | 1.63 (0.64-4.12) 0.31 | 0.71 (0.23-2.70) 0.71 |

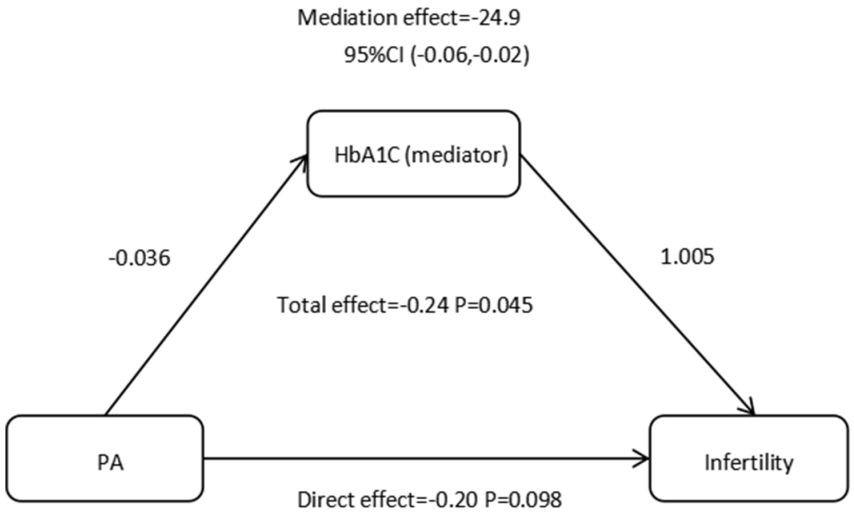


FIGURE 2
Effect of the HbA1C (mediators) on the relationship between PA (exposure) and infertility (outcome). HbA1C, glycosylated hemoglobinl; PA, physical activity.

metabolic rate, regulating endocrine function and strengthening immunity (24). However, such nonlinear associations have not been recognized between work-related PA and infertility. Research on the relationship between work-related PA and infertility has produced inconsistent findings. Some evidence indicated that increased levels of work-related PA are associated with a greater risk of infertility (25). However, other studies suggested that insufficient work-related PA may adversely affect fertility (26). Furthermore, our research revealed no substantial correlation between work-related PA and infertility. We have analyzed potential explanations and shown that PA predominantly affects female reproductive health via metabolic control, whereas work activities denote obligations that individuals must fulfill during working hours, which often entails extended and low-intensity static tasks. Unlike leisure activities, which are predominantly subjective, work-related PA may not aid in metabolic regulation. A study of Taiwanese workers also revealed no significant relationship between work-related PA and metabolic markers (27, 28). This may also be linked to endocrine regulation. Research has indicated that PA augments the body's biosynthetic response by increasing the levels of growth hormone and insulin-like growth factor, thus promoting reproductive health (29). The research by Schjeldrup Skarpsno (30) suggested a correlation between professional activity and insomnia, which clearly affects endocrine function.

Previous studies have reported that abnormal HbA1c is related to pregnancy problems. High HbA1c can increase the risk of gestational diabetes mellitus (31), and may also increase the risk of early abortion and premature birth (32, 33). Most importantly, our research suggests that HbA1c may play a role in the connection between recreation-related PA and infertility. Our data indicate that PA can downregulate HbA1c and has a negative correlation with infertility, which is consistent with the findings of previous studies (34, 35). HbA1c serves as a diagnostic tool for monitoring diabetes. Numerous studies have investigated the effects of a physically active lifestyle on individuals who are diagnosed with diabetes (36, 37). A previous study revealed that children diagnosed with type 1 diabetes who had lower levels of PA had poorer blood glucose control than did children who participated in more consistent PA. The level of HbA1c was significantly elevated in the less physically active group compared with the more physically active group (38). An analysis of a population sample of women aged 18–45 years in the United States revealed a notably positive association between HbA1c levels and infertility (39). However, this study investigated the role of HbA1c in influencing the association between PA patterns and infertility. We identified the mediating regulatory function of HbA1c. The findings of this study demonstrated that recreation-related PA does not have a statistically significant direct effect on infertility, as evidenced by a coefficient of -0.20. These findings suggest that the influence of exercise on infertility is consistently moderated by additional factors. The statistical analysis demonstrated that the mediating effect of HbA1c was significant, as evidenced by a coefficient of -24.9. These findings provide some evidence for the potential role of HbA1c in the modulation of PA on infertility, but more research is required to establish a definitive conclusion.

Finally, we unexpectedly found that the influence of obesity and a history of diabetes on infertility is mainly regulated by PA. The impact of obesity on the incidence of infertility mainly occurs in the active and less active population, thus indicating that weight loss in women who exercise is beneficial for their reproductive health, whereas weight loss alone does not promote female reproductive health in women who do not exercise. This may indicate that simply losing weight is not related to promoting reproductive health. A large amount of clinical data has confirmed that weight loss can improve reproductive function, and weight loss has become a basic component of infertility treatment for obese individuals (40). However, previous research has shown that increasing weight loss in obese or overweight women before IVF treatment does not improve reproductive outcomes (41). Our research results also confirmed this finding. In addition, among the less active population, a history of diabetes does not affect the incidence of

infertility, which indicates that less activity may be beneficial to women with a history of diabetes.

Our research provides further evidence for the negative correlation between PA and infertility; moreover, based on previous reports, we found no significant correlation between work-related PA and infertility incidence. These findings suggest that recreation-related PA has greater significance in promoting reproductive health. In addition, mediation analysis revealed potential reasons for the association between PA and infertility. Based on these data, it will be possible to provide patients with more accurate and evidence-based advice to determine the optimal frequency and intensity of exercise, thus increasing their chances of pregnancy. Finally, the findings of hierarchical analysis also suggest that, in our clinical research, the body mass index of individuals who exercise is more worthy of attention, and less active PA is more recommended for women with a history of diabetes.

However, this study has several significant drawbacks. The cross-sectional approach that was used in our study did not allow us to establish a cause-effect association between PA and infertility. To confirm this relationship, additional prospective cohort studies or randomized controlled trials are needed. Furthermore, the identification of diseases depends on self-reported responses from participants, which could lead to the introduction of recall bias and misclassification. Nevertheless, prior research has demonstrated that data obtained from the NHANES are reliable for evaluating the prevalence of infertility in the entire population (42, 43). Finally, future research might look into the reasons and treatment methods of infertility in women of different ages, as well as how to provide more individualized fertility support to women of all ages.

In conclusion, the findings of this study revealed that confounding factors had a significant influence on the correlation between the two variables. Although stratified analysis has been conducted, further inquiries are needed to explore the relationships between confounding factors and infertility. Although mediation analysis is essential, it is worth noting that the results obtained within the cross-sectional framework are still correlated and do not prove causal relationships.

## Conclusion

Our results revealed that HbA1c may play a potencial mediation role in the association between PA and infertility, thus revealing a potential mechanism underlying the association between PA and infertility, which offers valuable insights for establishing healthy PA guidelines for women. However, due to the fact that this study was a cross-sectional study, more prospective cohort studies are needed in the future to explore causality.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author/s.

## Ethics statement

The studies involving humans were approved by NCHS Ethics Review Board (ERB). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

## Author contributions

PC: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Validation, Writing – original draft. LZ: Software, Writing – review & editing. YM: Project administration, Writing – review & editing. SZ: Formal analysis, Methodology, Supervision, Writing – review & editing. GH: Funding acquisition, Resources, Supervision, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2025.1495470/full#supplementary-material

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# Flaxseed intervention and reproductive endocrine profiles in patients with polycystic ovary syndrome: an open-labeled randomized controlled clinical trial

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**Introduction:** Polycystic Ovary Syndrome (PCOS), affecting 6–15% of women of reproductive age, is characterized by hormonal imbalances and metabolic disturbances. Flaxseed, rich in omega-3 fatty acids and phytoestrogens, may offer a natural approach to improve reproductive hormone profiles in PCOS patients. This study is aimed at evaluating the effects of flaxseed supplementation on reproductive endocrine profiles in women with PCOS.

**Methods:** In this open-labeled, randomized controlled trial, 70 women with PCOS were randomly assigned to either the intervention group (n=35), receiving 30 grams of milled flaxseed daily along with lifestyle modifications, or the control group (n=35), receiving only lifestyle modifications, for 12 weeks. The primary outcome was the change in follicle-stimulating hormone (FSH) levels. Secondary outcomes included changes in serum concentrations of luteinizing hormone (LH), the LH/FSH ratio, anti-Müllerian hormone (AMH), estradiol, dehydroepiandrosterone sulfate (DHEAS), and androstenedione.

**Results:** After 12 weeks, the flaxseed group showed a significant increase in FSH levels compared to the control group. FSH levels increased from 9.72 ± 11.95 μU/mL at baseline to 10.59 ± 12.14 μU/mL after the intervention ($p = 0.027$). The mean treatment effect was 0.87 (95% CI: 0.086 to 1.75). The LH/FSH ratio also significantly decreased in the flaxseed group (mean treatment effect: -0.341, 95% CI: -0.63 to -0.08, $p = 0.031$). No significant changes were observed in AMH, LH, estradiol, DHEAS, or androstenedione levels.

**Conclusion:** Flaxseed supplementation may benefit women with PCOS by increasing FSH levels and decreasing the LH/FSH ratio, suggesting its potential as a complementary intervention for managing hormonal disturbances in PCOS. Further studies are needed to confirm these findings and elucidate the underlying mechanisms.

KEYWORDS

**flaxseed, polycystic ovary syndrome (PCOS), reproductive endocrine profile, follicle-stimulating hormone (FSH), nutrition**

# Introduction

PCOS, a common endocrine condition in women of childbearing years, afflicts between 6 and 15 percent of this demographic (1, 2). It is primarily characterized by irregular menstrual cycles, hyperandrogenism, and polycystic ovarian morphology on ultrasound (3, 4). PCOS is associated with an increased likelihood of developing metabolic syndrome, type 2 diabetes, and cardiovascular disease (2, 5, 6). Although the exact cause of PCOS remains unclear, obesity and insulin resistance (IR) are believed to play significant roles in its pathogenesis (7).

Metabolic irregularities, characterized by heightened concentrations of LH, normal or diminished levels of FSH, and an increased LH/FSH ratio, are frequently observed in individuals with PCOS and can have lasting effects on their overall health (8). Also, a key feature of PCOS is hyperandrogenism, driven by elevated testosterone levels due to stimulation of ovarian theca cells (8).

PCOS treatment is currently symptomatic and prescribed to reduce insulin resistance, lower androgen levels, alleviate inflammatory symptoms, and regulate menstrual cycles. Pharmacological treatment includes contraceptive hormones and other insulin-sensitizing agents, such as metformin (9, 10). However, PCOS treatment should include lifestyle changes such as dietary adjustments, increased physical activity, and behavioral interventions (11). Finally, there is a growing interest in the use of medicinal herbs as a complementary or alternative therapeutic approach for women with PCOS (12, 13).

Nutritional intervention is emerging as a promising strategy for managing PCOS (14, 15). Omega-3 polyunsaturated fatty acids (PUFAs) have demonstrated a range of health advantages, including anti-inflammatory properties, increased insulin sensitivity, and improved ovulation (16–18). Flaxseed, a plant-based food, is abundant in beneficial nutrients such as α-linolenic acid, phytoestrogenic lignans, and fiber (19, 20). Due to lignan's structural similarity to sex hormones, this substance could potentially reduce the production of estrogen and increase the levels of sex hormone-binding globulin (SHBG) in adipose tissue and the liver (21).

Despite existing research on the effects of flaxseed on some metabolic and hormonal parameters in women with PCOS (22, 23), there is limited data on its impact on FSH, the LH/FSH ratio, AMH, estradiol, DHEAS, and androstenedione in PCOS patients. Therefore, a recent systematic review and meta-analysis on flaxseed and sex hormones (21), concluded that more RCTs of flaxseed supplementation on sex hormones are necessary to elucidate their complex relationship. Hence, this study aims to evaluate the effects of flaxseed on reproductive endocrine profiles in women with PCOS.

# Materials and methods

## Trial design

The PICO study (flaxseed and its impact on markers of fertility) was a monocentric, randomized, open-labeled controlled clinical trial. The study was approved by the local ethics review board at Ahvaz Jundishapur University of Medical Sciences in Iran (IR.AJUMS.REC.1396.619). The study followed ethical standards set by the Declaration of Helsinki and Good Clinical Practice. Additionally, the study was registered with the Iranian Registry of Clinical Trials (www.irct.ir) under the identifier IRCT20120704010181N11 (Registration date: 2018-01-21). To ensure transparency and rigor in reporting the trial, the researchers adhered to the Consolidated Standards of Reporting Trials (CONSORT) guidelines for randomized clinical trials. Prior to enrollment, all participants provided written informed consent. Participant recruitment commenced on 7 April 2019 and concluded on 3 March 2022. The study was temporarily suspended for a seven-month period during the COVID-19 pandemic.

## Sample size

We used a randomized clinical trial sample size calculation formula, where type one (α) and type two (β) errors were set at 0.05

**Abbreviations:** ALA, α-linolenic acid, AMH, Anti Mullerian Hormone, CVD, Cardiovascular Disease, DHEAS, Dehydroepiandrosterone Sulfate, FSH, Follicle-stimulating Hormone, IR, Insulin Resistance, LH, Luteinizing Hormone, PCOS, Polycystic Ovary Syndrome, PUFAs, Polyunsaturated Fatty Acids, SHBG, Sex Hormone-binding Globulin.

and 0.20, respectively (power = 80%). Based on a previous trial (2), we estimated that a minimum of 35 participants per group would be required to detect a significant difference in FSH levels between the intervention and control groups.

## Participants

Patients were recruited at their first diagnosis of PCOS at the Endocrinology Clinic of Golestan Hospital, Ahvaz, Iran, after agreeing to participate and signing the informed consent form. Diagnosis of PCOS was based on the Rotterdam criteria (24) and the updated criteria available at the time of study initiation (25). At least two of the following criteria were required for the diagnosis: (1) clinical and/or biochemical hyperandrogenism, (2) ovulatory dysfunction (oligomenorrhea, amenorrhea), and (3) polycystic ovarian morphology on ultrasound according to the updated criteria (4). All participants were premenopausal women aged ≥18 years. Before establishing a diagnosis of PCOS, they were screened for conditions with overlapping clinical manifestations, including congenital adrenal hyperplasia, androgen-secreting tumors, Cushing's syndrome, thyroid disorders, and hyperprolactinemia, to ensure accurate diagnosis and study eligibility.

Patients were excluded if they had any of the following criteria: pregnancy; overlapping hyperandrogenism causes; current use of supplements such as vitamin E, omega-3, or vitamin D; following restrictive diets (e.g, ketogenic or weight-loss diets); chronic conditions (cancer, diabetes, kidney disease, hypertension, inflammatory disorders, and autoimmune disease); allergy to flaxseed or any herbal product; and current hormonal treatments, including the use of combined oral contraceptive pills (COCs) in the three months prior to enrollment (26).

## Trial procedure and randomization

Subjects were randomly allocated (1:1) to either the flaxseed or control groups for a three-month intervention. The first group (n=35) consumed 30 grams of brown milled flaxseed daily in conjunction with lifestyle modifications. The control group (n=35) received lifestyle modifications alone. For the lifestyle modification program, a series of general nutritional and physical activity recommendations were explained to all patients in both groups in the same manner. Bulk flaxseed was acquired and stored in a cool, dark environment prior to the commencement of the study.

Randomization assignment was carried out using computer-generated random numbers. To ensure balanced groups, age (<30 and ≥30 years) and BMI (<25 and ≥25 kg/m$^2$) were utilized as stratification factors. A randomization schedule was generated by an independent statistician affiliated with the Ahvaz Jundishapur University of Medical Sciences in Ahvaz, Iran. This sequence was subsequently integrated into the REDCap platform for implementation (27). The randomized allocation sequence, participant enrollment, and group allocation were conducted by trained staff at the clinic. Upon confirmation of eligibility, participants were randomly assigned to groups. Due to the nature of the study, it was not possible to blind the patients and the research team, but the laboratory operators who performed the biochemical tests were blinded to the intervention and control groups.

A week before distribution to participants, the flaxseed underwent a milling process and was subsequently packaged into 30-gram portions. The nutritional composition of the flaxseed powder was determined by the Ahvaz Food and Nutrition Research Laboratory. Each 100-gram serving contained 465 kilocalories, 39 grams of fat, 21 grams of alpha-linolenic acid (ALA), 18 grams of protein, 29 grams of carbohydrates, and 28 grams of dietary fiber. To monitor any changes in nutrient content, subsequent analyses were conducted at three-week intervals over a 12-week period.

At the initial visit (baseline), participants provided demographic and clinical data. Subsequently, the intervention group received a three-week allotment of milled flaxseed. Participants in this group were instructed to incorporate flaxseed into their daily diet by adding it to salads, yogurt, or cold beverages. Participants followed a comprehensive lifestyle intervention during the trial period, which included both nutritional guidance and physical activity recommendations. The nutritional component of the program focused on the dietary guidance aligned with recommendations from the American Heart Association (28), while the physical activity recommendations aimed to patients physical activity, aiming for at least half an hour of moderate-intensity exercise on three or more days each week.

## Compliance

To improve adherence to the study protocol, participants in the flaxseed group received daily text message reminders to consume the flaxseed powder. Participant compliance was assessed by measuring the quantity of returned flaxseed powder. Participants who consumed less than 90% of the recommended flaxseed were not included in the final data analysis.

## Trial outcomes

The primary outcome of this study was to assess the variation in the blood concentration of FSH during the study period in the intervention and control groups. Secondary outcomes included evaluating the effect of flaxseed compared to control on changes in other hormones including LH, LH/FSH ratio, AMH, estradiol, DHEAS and androstenedione.

For biochemical evaluations, 10 cc of fasting blood were taken from all patients after 10–12 hours of fasting at 8–9 in the morning at baseline (the day before starting the intervention) and at the end of the 12-week intervention. All blood samples were preserved for future analysis by storing them at a temperature of -80 degrees

Celsius in the Ahvaz Jundishapur University of Medical Sciences Biobank. AMH, androstenedione, FSH, LH, 17β-estradiol, DHEAS and other biochemical parameters levels were measured at two time points: baseline (pre-intervention) and at the end of the study (post-intervention, after three months).

Hormonal assessments, including FSH, LH, AMH, estradiol, androstenedione, and DHEAS, were performed at specific points in the menstrual cycle to account for physiological fluctuations. In ovulatory participants, blood samples were collected in the early follicular phase (days 2–5 of the menstrual cycle) at both baseline and post-intervention. For anovulatory participants, samples were collected at random time points but remained consistent for each individual across both assessments to minimize variability. These procedures align with current clinical guidelines for hormonal assessment in PCOS.

FSH and LH levels were quantified using an enzyme-linked immunosorbent assay (ELISA) kit (Monobind Inc., USA). 17β-estradiol levels were quantified using IMMULATE® CLIA assays (Siemens Healthcare Diagnostics Products Ltd., Glyn Rhonwy, UK). AMH was evaluated using an ELISA kit (Beckman Coulter, France). DHEAS levels were determined using an ELISA kit (Labor Diagnostika Nord, Nordhorn, Germany), and androstenedione levels were measured using an ELISA kit (Siemens Healthcare Diagnostics Products Ltd., Glyn Rhonwy, UK).

Serum insulin values were determined by the use of available ELISA kit (DiaMetra, Milano, Italy) with inter- and intra-assay coefficient variances (CVs) of 3.5 to 5.1%, respectively. Enzymatic kits of Pars Azmun (Tehran, Iran) were used to evaluate fasting blood sugar (FBS), serum triglycerides, total-, LDL- and HDL cholesterol values.

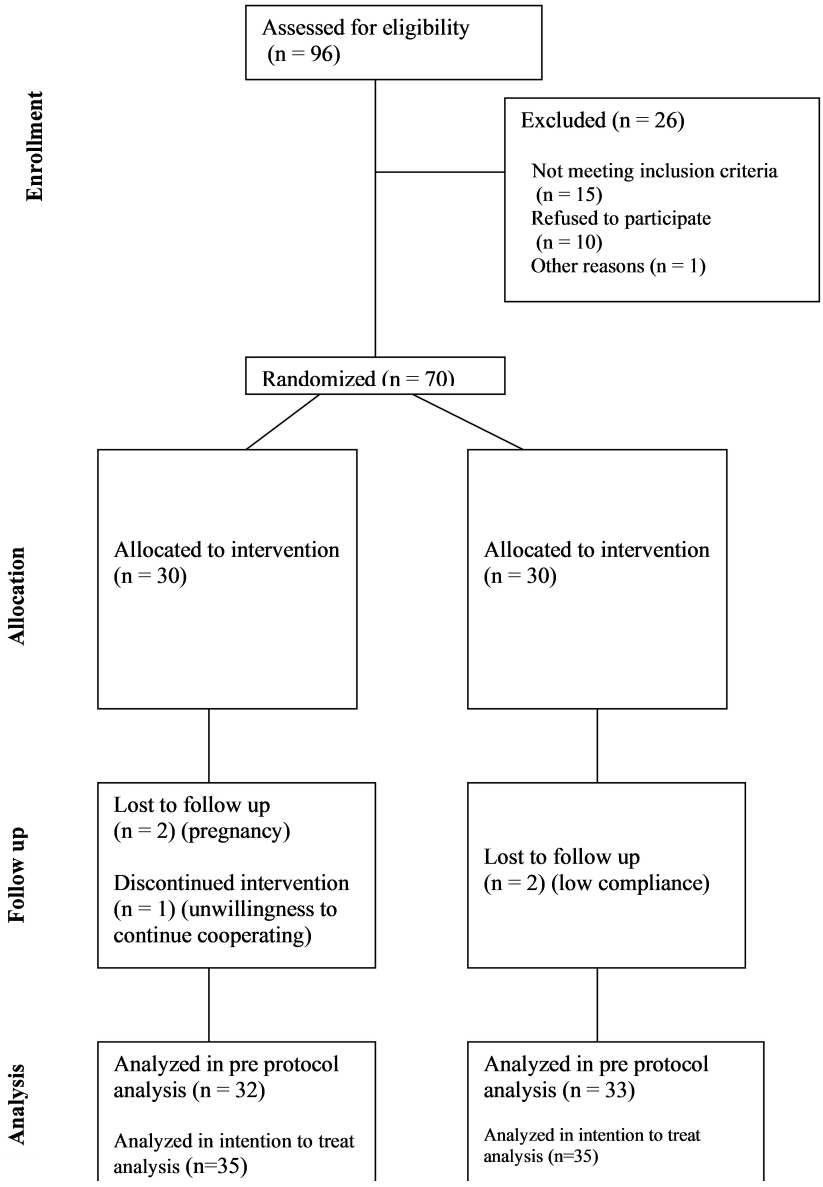


FIGURE 1
CONSORT diagram showing the flow of participants through each stage of this randomized trial.

Participants' diets were recorded over a three-day period using a 24-hour recall method administered at baseline and after study completion. The 24-hour recall was conducted using a standardized form developed for this study, which has been provided as Supplementary Data. Participants provided detailed information about their food and beverage consumption over these periods, and the data were subsequently analyzed for macronutrient and micronutrient content using the Nutritionist IV software package (Version 3.5, Axxya Systems, Stafford, TX, USA). The software is designed to analyze dietary intake data, provide nutrient breakdowns, and assess compliance with dietary guidelines.

Physical activity levels were assessed using the Metabolic Equivalent of Task (MET), a standardized unit that quantifies the energy expenditure of various activities. MET values were calculated based on self-reported physical activity data, where one MET represents the energy cost of resting metabolic rate, and higher MET values indicate greater physical activity levels. This measurement allowed for a comparison of baseline and post-intervention activity levels between the study groups.

## Statistical analysis

Data analysis was conducted using SPSS software (Version 20). Descriptive statistics were calculated for all variables, and the Kolmogorov-Smirnov test was employed to assess data distribution. Normally distributed continuous data were expressed as mean plus or minus standard deviation, while skewed data were presented as median with interquartile range. To assess differences between the groups at the study's outset, numerical variables were analyzed using Student's t-test, while categorical variables were compared with the chi-square test. In line with CONSORT recommendations for non-inferiority studies, both intention-to-treat (ITT) and per-protocol (PP) analyses were performed to evaluate the primary and secondary

TABLE 1 Baseline characteristics of the study participants, including demographic, anthropometric, and biochemical parameters at the beginning of the trial.

| Characteristics | All (n=70) | Flaxseed (n=35) | Control (n=35) | p value |
|---|---|---|---|---|
| Age (years) | 27.43 ± 7.05 | 27.13 ± 7.12 | 27.69 ± 6.90 | 0.82 |
| Weight (kg) | 77.49 ± 16.23 | 76.90 ± 15.40 | 78.35 ± 16.55 | 0.67 |
| BMI (kg/m$^2$) | 29.12 ± 6.87 | 28.77 ± 6.24 | 29.76 ± 7.05 | 0.71 |
| HC (cm) | 103.21 (95.23-117.31) | 102.75 (93.45-116.24) | 104.55 (95.83-119.20) | 0.65 |
| WC (cm) | 90.32 (79.43-105.37) | 88.23 (78.41-103.8) | 93.26 (80.15-108.29) | 0.23 |
| FSH (μU/mL) | 9.75 ± 12.27 | 9.72 ± 11.95 | 9.88 ± 12.45 | 0.87 |
| LH (μU/mL) | 8.65 ± 5.93 | 8.74 ± 5.62 | 8.57 ± 5.49 | 0.77 |
| LH/FSH ratio | 1.39 ± 0.76 | 1.35 ± 0.64 | 1.42 ± 0.79 | 0.23 |
| Estradiol (pg/mL) | 58.37 ± 24.93 | 57.23 ± 20.65 | 60.74 ± 25.82 | 0.29 |
| DHEAS (μg/mL) | 1.93 ± 1.12 | 1.86 ± 1.10 | 1.95 ± 1.15 | 0.36 |
| Androstenedione (ng/mL) | 3.67 ± 1.85 | 3.61 ± 1.62 | 3.70 ± 1.94 | 0.46 |
| AMH (ng/mL) | 8.12 ± 5.17 | 7.89 ± 4.93 | 8.36 ± 5.42 | 0.29 |
| FBS (mg/dl) | 91.43 ± 9.15 | 93.79 ± 9.76 | 89.50 ± 8.75 | 0.58 |
| TG (mg/dl) | 148.54 ± 25.56 | 157.62 ± 30.19 | 143.87 ± 24.98 | 0.36 |
| TC (mg/dl) | 162.34 ± 34.67 | 170.12 ± 37.54 | 156.23 ± 32.65 | 0.28 |
| LDL (mg/dl) | 86.14 ± 33.39 | 89.26 ± 34.57 | 82.68 ± 31.22 | 0.68 |
| HDL (mg/dl) | 37.26 ± 12.17 | 39.12 ± 13.85 | 34.76 ± 12.05 | 0.42 |
| Fasting insulin (mU/L) | 11.24 ± 4.39 | 11.55 ± 4.55 | 11.15 ± 4.18 | 0.78 |
| METs (min/week) at study baseline | 2495.67 ± 234.67 | 2478.22 ± 230.61 | 2510.37 ± 239.82 | 0.53 |
| Menstrual irregularity (%) | 88.4 | 89.2 | 87.9 | 0.72 |
| Oligomenorrhea (%) | 70.4 | 72.6 | 67.8 | 0.53 |
| Hypermenorrhea (%) | 2.5 | 1.7 | 3.5 | 0.36 |
| Amenorrhea (%) | 15.5 | 14.9 | 16.6 | 0.63 |

Data are shown as means with standard deviation, medians and interquartile range, or as percentages, as appropriate. Comparisons of baseline characteristics between the Flaxseed and the control group were performed using unpaired Student's t test, Mann–Whitney U test, X2 test, or Fisher's exact test, as appropriate. BMI, Body mass index; HC, Hip circumference; FBS, fasting blood sugar; HDL, High density lipoprotein; LDL, low-density lipoprotein, TC, total cholesterol; TG, triglyceride, WC, Waist circumference; AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; DHEAS, dehydroepiandrosterone sulfate; MET, Metabolic Equivalent of Task.

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outcomes (29). Analysis of covariance (ANCOVA) was employed to assess differences in outcome variables between the flaxseed and control groups at study conclusion, with baseline values included as covariates to control for potential confounding effects. For all analyses, a p-value below 0.05 was deemed statistically significant.

## Results

Among the 96 screened patients, 70 were allocated to the flaxseed and control groups, and 65 completed the study (Figure 1). The intention-to-treat (ITT) analysis included all 70 randomized participants, whereas the per-protocol (PP) analysis considered 33 participants in the flaxseed group and 32 in the control group. In the PP analysis, two participants were excluded due to low compliance with flaxseed consumption (<90%), two due to pregnancy, and one participant withdrew from the study. Table 1 summarized the baseline demographic and clinical characteristics of participants.

At baseline, the mean age of participants was 27.43 ± 7.05 years, with no significant differences between groups in terms of age, weight, or BMI. Similarly, biochemical parameters, including fasting blood sugar, triglycerides, total cholesterol, LDL, HDL, and fasting insulin levels, were comparable between groups. The mean intake of calories, macronutrients and fatty acids are summarized in Table 2. Dietary intake analysis indicated no significant differences in total energy, macronutrient, and fatty acid intake between groups at the beginning of the study, and these remained stable throughout the intervention.

After 12 weeks, FSH levels showed a significant increase in the flaxseed group compared to the control group (mean treatment effect: 0.87, 95% CI 0.086 to 1.75, P = 0.027). Additionally, the LH/FSH ratio significantly decreased following flaxseed supplementation (mean treatment effect: -0.341, 95% CI -0.63 to -0.08, P= 0.031). No significant differences were observed in AMH, LH, estradiol, DHEAS, or androstenedione changes between the two study groups (Table 3).

Anthropometric measurements, including weight and BMI, remained unchanged between groups from baseline to study completion. Similarly, there were no significant differences in lipid profiles or fasting glucose levels at the end of the study. Analysis of dietary compliance confirmed that participants adhered to the study protocol, with no significant variations in reported dietary intake across the intervention period.

Throughout the study, no adverse events or side effects were reported. The intervention was well-tolerated, and no participants discontinued due to side effects related to flaxseed consumption.

## Discussion

Currently, there is limited research investigating the effects of flaxseed supplementation on reproductive endocrine profiles in individuals diagnosed with PCOS. This study sought to address this gap and demonstrated that a 12-week regimen of flaxseed supplementation resulted in notable improvements, particularly in FSH levels and the LH/FSH ratio.

The present study has demonstrated a significant elevation in FSH levels associated with flaxseed consumption. Recent studies indicate that patients with PCOS exhibit either normal or diminished levels of FSH within the hypothalamic-pituitary-ovarian axis (8, 30), which may contribute to impaired follicular development.

The consistently low yet stable levels of FSH in PCOS patients perpetuate the development of new follicles that fail to achieve full maturation and undergo atresia without ovulating. This disrupted folliculogenesis contributes to the characteristic endocrine abnormalities and clinical manifestations observed in PCOS (30, 31). Notably, research in animal models suggests that flaxseed consumption enhances FSH receptor expression in ovarian follicles, potentially contributing to improved follicular function (32). Furthermore, as previously noted, flaxseed serves as an abundant source of omega-3 polyunsaturated fatty acids. Recent research indicates that omega-3 may promote the expression of CYP51 in granulosa cells via the PI3K/Akt signaling pathway, subsequently facilitating the conversion of lanosterol into cholesterol, which is a substrate for estrogen (33).

TABLE 2 Nutritional parameters at baseline and end of the study in both study groups.

| Variable | Intervention group (n=33) | Control group (n=32) | $P_1$-value |
|---|---|---|---|
| Energy (kcal) | | | |
| Baseline | 1875.35 ± 376.43 | 1753.24 ± 338.42 | 0.36 |
| End | 1743.44 ± 324.59 | 1678.45 ± 310.86 | 0.29 |
| $P_2$-value | 0.22 | 0.47 | |
| Carbohydrate (g) | | | |
| Baseline | 283.39± 71.44 | 264.37 ± 66.41 | 0.25 |
| End | 259.66 ± 67.19 | 260.32 ± 57.42 | 0.78 |
| $P_2$-value | 0.21 | 0.72 | |
| Protein (g) | | | |
| Baseline | 88.25 ± 27.19 | 76.59 ± 20.47 | 0.38 |
| End | 78.44 ± 25.83 | 69.85 ± 22.56 | 0.44 |
| $P_2$-value | 0.39 | 0.45 | |
| Fat (g) | | | |
| Baseline | 66.74 ± 23.19 | 59.78 ± 19.52 | 0.52 |
| End | 57.37 ± 18.12 | 57.36 ± 16.80 | 0.92 |
| $P_2$-value | 0.48 | 0.74 | |
| PUFA (g) | | | |
| Baseline | 17.32± 10.20 | 15.77 ± 8.44 | 0.49 |
| End | 19.77 ± 9.47 | 16.51 ± 6.90 | 0.37 |
| $P_2$-value | 0.57 | 0.68 | |
| MUFA (g) | | | |
| Baseline | 25.38 ± 8.79 | 23.28 ± 7.36 | 0.55 |
| End | 22.73 ± 7.32 | 20.17 ± 5.75 | 0.61 |
| $P_2$-value | 0.61 | 0.47 | |
| SFA (g) | | | |
| Baseline | 18.47 ± 10.36 | 16.63 ± 7.85 | 0.49 |
| End | 16.18 ± 7.76 | 15.93 ± 7.69 | 0.75 |
| $P_2$-value | 0.53 | 0.71 | |

Data are shown as means with standard deviation, P1: between-group comparison of the variables; independent sample t-test. P2: within-group comparison of the variables; paired sample t-test. SFA, saturated fatty acid; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acids.

A significant reduction in the LH/FSH ratio was also observed, suggesting a potential role of flaxseed in normalizing gonadotropin secretion. The decline in the LH/FSH ratio may be attributed to a reduction in LH levels or an increase in FSH levels. Previous studies have suggested that flaxseed oil supplementation can modulate LH levels and enhance sex hormone balance in PCOS patients (23, 33). For example, Musazadeh et al. (21) reported a significant reduction in FSH levels following flaxseed supplementation for more than 12 weeks, whereas our study observed an increase in FSH after 12 weeks. This divergence may also be due to differences in participant characteristics, baseline FSH levels, or the type of flaxseed product used (21). Similarly, Wang et al. (23) found that flaxseed oil, which is rich in α-linolenic acid but lacks lignans, resulted in FSH reduction, highlighting the potential role of specific flaxseed components in modulating hormonal responses (23). Again, the discrepancy with our results could be linked to variations in dietary background, metabolic status, or genetic factors affecting hormonal regulation in different populations. Studies have shown that in patients with PCOS, the LH/FSH ratio is elevated due to heightened sensitivity of the pituitary gland to hypothalamic GnRH or alterations in GnRH secretion patterns (8, 34, 35). Previous studies have suggested that flaxseed oil supplementation can modulate LH levels and enhance sex hormone balance in PCOS patients (23). However, variations in study duration, dosage, and flaxseed formulations may account for inconsistencies across studies.

While our study did not observe significant changes in AMH, estradiol, DHEAS, or androstenedione, these findings align with some studies reporting no effect of phytoestrogen supplementation on these parameters (36–42). However, conflicting findings exist in the literature, potentially due to differences in supplementation duration, dosage, and population characteristics (43).

While our study focused on FSH and the LH/FSH ratio, we recognize that testosterone and insulin could significantly influence these parameters. Previous studies investigating flaxseed supplementation have reported conflicting results for testosterone and insulin levels. For instance, Emmamat et al. observed a reduction in testosterone levels with flaxseed intervention (44), whereas other studies reported no significant changes in androgen or insulin levels (21). Including these outcomes could provide additional insights into the observed hormonal changes and their underlying mechanisms.

One key strength of our study is the implementation of a non-invasive and economically viable dietary intervention aimed at improving reproductive endocrine profiles in PCOS patients. This study is among the first randomized controlled trials to evaluate the effects of flaxseed supplementation on FSH levels and the LH/FSH ratio in women with PCOS. The 12-week duration of the intervention and the use of milled flaxseed, which is rich in phytoestrogens and omega-3 fatty acids, further enhance the significance of the study.

However, several limitations should be acknowledged. First, the open-labeled study design introduces the potential for bias, despite laboratory operators being blinded to treatment allocation. Second, the relatively small sample size and monocentric nature limit generalizability. Third, testosterone and insulin levels were not assessed, although these hormones could play a crucial role in modulating gonadotropin dynamics. Future research should incorporate these parameters to provide a more comprehensive understanding of flaxseed's effects on PCOS.

TABLE 3 Variations of the primary and secondary outcomes during the period of study in the flaxseed and control groups[1].

| | ITT (n=70) | | | | PP(n=65) | | | |
|---|---|---|---|---|---|---|---|---|
| | Placebo Mean (SD) | Flaxseed Mean (SD) | Point Estimate (95% CI) | P_value | Placebo Mean (SD) | Flaxseed mean (SD) | Point Estimate (95% CI) | P_value |
| **Primary endpoint** | | | | | | | | |
| FSH (μU/mL) | -0.71(2.05) | 0.14(2.52) | 0.87 (0.086-1.75) | 0.027 | -0.74(2.11) | 0.15(2.52) | 0.91 (0.087-1.84) | 0.02 |
| **Secondary endpoints** | | | | | | | | |
| LH (μU/mL) | -0.15(2.66) | 0.75(3.16) | -0.21 (-0.523-0.134) | 0.37 | -0.16(2.67) | 77(3.19) | -0.22 (-0.525-0.135) | 0.35 |
| AMH (ng/mL) | -0.1(5.26) | -0.67(4.34) | 0.091 (-0.075, 0.243) | 0.27 | -0.12(6.12) | -0.70(5.79) | 0.096 (-0.076-0.247) | 0.24 |
| Estradiol (pg/mL) | 5.65(19.23) | 2.15(15.34) | -0.093 (-0.326, 0.164) | 0.49 | 6.19(20.35) | 2.43(16.18) | -0.094 (-0.329,0.170) | 0.41 |
| LH/FSH ratio | 0.39(0.9) | -0.9(1.3) | -0.341 (-0.630, -0.080) | 0.031 | 0.43(1.18) | -1.03(1.3) | -0.346 (-0.633,0.083) | 0.027 |
| DHEAS (μg/mL) | 0.36(2.67) | 0.028(2.12) | -0.014 (-0.135, 0.13) | 0.84 | 0.37(2.67) | 0.03(2.15) | -0.016 (-0.137,0.136) | 0.81 |
| Androstenedione (ng/mL) | 0.56(3.17) | -0.09(1.76) | -0.009 (-0.128,0.125) | 0.96 | 0.57(2.96) | -0.094(1.82) | -0.01 (-0.130,0.127) | 0.86 |

CI, confidence interval; ITT, intention-to-treat; PP, per protocol; AMH, anti-Müllerian hormone; FSH, Follicle-Stimulating Hormone; LH, Luteinizing hormone; DHEAS, Dehydroepiandrosterone. 1. Means are changes before and after the control or flaxseed period for the primary and secondary endpoints for the ITT analysis, point estimates, and 2-sided 95% CIs.

## Conclusion

Our findings suggest that flaxseed supplementation, rich in omega-3 PUFAs and phytoestrogenic lignans, may serve as a beneficial dietary intervention for managing hormonal imbalances in PCOS patients. The observed increases in FSH levels and reductions in the LH/FSH ratio indicate a potential role for flaxseed in improving follicular development and ovulatory function. Further randomized controlled trials with larger sample sizes and extended follow-up periods are warranted to confirm these findings and explore the long-term impact of flaxseed supplementation on reproductive outcomes in PCOS patients.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The study was approved by the local ethics review board at Ahvaz Jundishapur University of Medical Sciences in Iran (IR.AJUMS.REC.1396.619). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

## Author contributions

ZN: Formal Analysis, Funding acquisition, Investigation, Writing – review & editing. FS: Investigation, Methodology, Validation, Writing – review & editing. SG: Formal Analysis, Investigation, Methodology, Writing – review & editing. MF: Conceptualization, Methodology, Writing – original draft. MR: Investigation, Methodology, Writing – original draft, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2025.1531762/full#supplementary-material

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# Metabolomics profiling identifies diagnostic metabolic signatures for pregnancy loss: a cross-sectional study from northwestern China

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**Objective:** To identify potential diagnostic metabolic biomarkers for pregnancy loss (PL) by performing untargeted metabolomics analysis.

**Methods:** The present study performed untargeted metabolomics analysis on plasma samples from PL patients (n=70) and control subjects (n=122) using liquid chromatography–mass spectrometry (LC–MS). Metabolic profiles were evaluated using orthogonal partial least squares discriminant analysis (OPLS-DA), and pathway enrichment analysis was conducted via the KEGG database. LASSO regression was employed to identify significant metabolites, and their diagnostic performance was evaluated through receiver operating characteristic (ROC) curves. Pearson correlation analysis was used to explore the relationships between differentially abundant metabolites and clinical parameters.

**Results:** In total, 359 metabolites were identified, 57 of which were significantly altered between the control and PL group through OPLS-DA. Differential metabolites were significantly enriched in caffeine metabolism, tryptophan metabolism, and riboflavin metabolism pathways. Key metabolites, such as testosterone glucuronide, 6-hydroxymelatonin, and (S)-leucic acid, exhibited strong diagnostic potential, with AUC values of 0.991, 0.936 and 0.952, respectively, and the combined AUC was 0.993. Furthermore, Pearson correlation analysis revealed a significant negative correlation between the waist–to–hip ratio (WHR) and the abundance of testosterone glucuronide ($r = -0.291$, $p = 0.0146$), and a significant positive correlation between WHR and (S)-leucic acid ($r = 0.248$, $p = 0.0381$) in the PL group.

**Conclusion:** We identified a panel of plasma metabolites with significant diagnostic potential for PL. These biomarkers may facilitate early, noninvasive diagnosis and offer insights into metabolic dysregulation associated with pregnancy loss.

KEYWORDS

**pregnancy loss, untargeted metabolomics, LASSO regression, metabolic signature, diagnosis**

# Introduction

Pregnancy loss (PL) refers to the natural end of a pregnancy before the fetus becomes viable, including all losses from conception until 24 weeks of gestation, affecting approximately 12–15% of recognized pregnancies globally (1, 2). Owing to increased environmental pollution and increasing life stress, the clinical incidence of PL has significantly increased in recent years (3, 4). Despite extensive research, the underlying causes of PL remain poorly understood and are often attributed to a complex interplay of genetic, immunological, and environmental factors (5). The absence of reliable metabolites complicates the early diagnosis and management of PL, leading to profound psychological and physiological impacts on affected women and their families (6).

In the context of PL, metabolomics can reveal critical metabolic alterations associated with this condition (7). Previous research has shown the effectiveness of metabolomics in identifying metabolic signatures for numerous conditions, such as cancer, cardiovascular diseases, autoimmune disease and metabolic disorders (8–11). For example, Lili Zhang et al. investigated serum metabolic profiles in women with recurrent abortion due to antiphospholipid syndrome, and they reported significant disruptions in purine, amino acid, and tyrosine metabolism (12). Another study utilized untargeted GC–MS and targeted liquid chromatography–mass spectrometry (LC–MS) to identify metabolic disturbances associated with recurrent spontaneous abortion. Through this approach, lactic acid and 5-methoxytryptamine were identified as significantly different metabolites between the two groups, and their plasma concentrations were further validated using targeted LC–MS (13). Despite these advances, comprehensive metabolomics analyses specifically targeting PL remain limited.

The integration of metabolomics with well-established analytical techniques, such as LC–MS, enables the detection of subtle metabolic alterations associated with PL. Moreover, the application of machine learning algorithms to metabolomics data has further improved the ability to identify and validate potential metabolites (14). One such machine learning algorithm, least absolute shrinkage and selection operator (LASSO) regression, is particularly effective in managing high-dimensional data characteristic of complex datasets (15, 16). LASSO regression stands out for its ability to perform both variable selection and regularization simultaneously, which helps prevent overfitting while managing large datasets (17). This is especially beneficial in metabolomics, where the number of metabolites often far exceeds the number of samples. By shrinking the coefficients of less relevant variables to zero, LASSO effectively refines the model, focusing on the most significant metabolites (18). This enhances the robustness of the model and increases the reproducibility of identifying the most important metabolites.

The present study aimed to leverage untargeted metabolomics combined with LASSO regression to identify a panel of diagnostic metabolites for PL. By comparing the metabolic profiles of plasma samples from women with a history of PL to those from women who have had normal pregnancies without PL, the present study sought to uncover metabolic signatures that can aid in the early detection and better understanding of PL. In addition, analyses that incorporated clinical parameters, such as age, BMI, and waist–to–hip ratio (WHR), which are potential confounding factors in PL, were performed to explore the relationships between the identified metabolites and these variables. The present findings may provide valuable insights for developing noninvasive diagnostic metabolites for PL.

# Methods

## Study design and participants

The present study enrolled 192 participants, comprising 122 in the control group and 70 in the PL group. The participants were recruited from Lanzhou University Second Hospital between February 2023 and September 2023. The inclusion criteria for the PL group included women aged 18 to 42 years who had experienced at least one PL in the past six months, in accordance with the ESHRE diagnostic criteria (1). The exclusion criteria for the PL group were as follows: presence of endocrine disorders, infections, or immunological diseases; or a history of the most recent pregnancy ending in an ectopic pregnancy, hydatidiform mole, or congenital defects. The inclusion criteria for the control group were women with no history of PL and at least one successful full-term pregnancy. The exclusion criteria for the control group included the presence of endocrine disorders, infections, or immunological diseases. The present study was approved by the Institutional Review Board of Lanzhou University Second Hospital (ethical approval number: 2023A-553). All patients provided written informed consent.

## Sample collection and preparation

Fasting blood samples were collected from participants using EDTA tubes and centrifuged at 1,500×g for 10 minutes at 4°C to obtain plasma, which was then stored at -80°C. For metabolomics analysis, 100 µL of plasma was placed in Eppendorf tubes, mixed with 80% prechilled methanol, and vortexed thoroughly. The samples were incubated on ice for 5 minutes, followed by centrifugation at 15,000×g for 20 minutes at 4°C. A portion of the supernatant was diluted with LC–MS-grade water to reach a 53% methanol concentration. This mixture was transferred to fresh Eppendorf tubes and centrifuged again at 15,000×g for 20 minutes at 4°C. The final supernatant was then injected into the LC–MS/MS system for analysis via an Orbitrap Q Exactive™ HF–X mass spectrometer (19, 20).

## UHPLC–MS analysis

UHPLC–MS/MS analysis was performed using a Vanquish UHPLC system coupled with an Orbitrap Q Exactive™ HF-X mass spectrometer (Thermo Fisher, Germany) at Novogene Co., Ltd. Samples were injected onto a Hypersil Gold column (100 × 2.1

mm, 1.9 μm) and separated over a 12-minute linear gradient at 0.2 mL/min. The eluents for positive polarity mode consisted of 0.1% formic acid in water (eluent A) and methanol (eluent B), whereas those for negative polarity mode included 5 mM ammonium acetate (pH 9.0, eluent A) and methanol (eluent B). The solvent gradient was programmed as follows: initial 2% B for 1.5 minutes, increased to 85% B over 3 minutes, increased to 100% B over the next 10 minutes, decreased to 2% B over 0.1 minutes, and maintained at 2% B for 12 minutes. The mass spectrometer was operated in both positive and negative modes with a spray voltage of 3.5 kV, capillary temperature of 320°C, sheath gas flow rate of 35 psi, auxiliary gas flow rate of 10 L/min, S-lens RF level of 60, and auxiliary gas heater temperature of 350°C. High-energy collision dissociation (HCD) was used as the fragmentation method, with an isolation window scanning the *m/z* range of 100–1,500. Collision energy was applied in three steps, namely, 20 V, 40 V, and 60 V. The quality control (QC) samples were interspersed among the study samples during analysis. The QC samples were used to monitor and assess the data quality by evaluating the correlation between QC injections, ensuring the reliability and consistency of the analytical results. All samples were analyzed in a single batch during the analytical run.

## Data processing and metabolite identification

The raw data files from UHPLC–MS/MS were processed using Compound Discoverer 3.3 for peak alignment, peak picking, and metabolite quantitation, following the protocols established in previous studies (21, 22). First, retention times and mass-to-charge ratios (*m/z*) were aligned across samples with a mass tolerance of 5 ppm, a signal intensity tolerance of 30%, and a defined intensity threshold. Metabolite identification was performed by matching accurate *m/z* values, adduct ions, and MS/MS fragmentation patterns against the mzCloud (https://www.mzcloud.org/), mzVault, and MassList databases. In databases containing fragmentation data, the acquired MS spectra were compared with reference fragment ions and collision energies, ensuring higher confidence annotations. Next, peak intensities were normalized to reduce run-to-run variations and enable consistent comparative analyses. Quality control (QC) samples were injected to monitor instrument stability, and only features with a coefficient of variation (CV) < 30% in the QC samples were retained. Finally, the normalized peak areas of the confidently identified metabolites were used for subsequent statistical analyses.

## Data analysis

The identified metabolites were annotated using the KEGG and HMDB databases (https://www.metaboanalyst.ca/). Since our research focus is not on exogenous metabolites, we have excluded them from further analysis. A detailed list of these excluded exogenous metabolites is provided in Supplementary Table 1. To visualize metabolic differences and identify potential metabolic signatures, orthogonal partial least squares discriminant analysis (OPLS-DA) was performed using the 'ropls' package in R software. Statistical significance was assessed using the t test, with differentially abundant metabolites selected on the basis of a variable importance in projection (VIP) score > 1 and a p value < 0.05. Heatmaps and volcano plots were generated using the 'pheatmap' and 'ggplot2' packages in R. Pathway analysis was conducted using the MetaboAnalyst platform (version 6.0), a widely used web-based tool for metabolomics data interpretation. The list of significantly altered metabolites was uploaded using HMDB (Human Metabolome Database) IDs, and these metabolites were assigned on the basis of MS/MS spectral matching and database searches. The KEGG database was used for pathway enrichment analysis. Enrichment analysis was performed using the hypergeometric test to identify overrepresented pathways compared with a reference metabolome; pathways with a p value < 0.05 were considered statistically significant. To assess the biological importance of metabolites within pathways, the relative-betweenness centrality algorithm was applied during pathway topology analysis. The results were visualized via a pathway impact plot, which integrates enrichment and topology analyses to highlight the most significantly impacted pathways.

## LASSO regression for metabolites selection and ROC curve analysis

LASSO regression was employed via the 'glmnet' package in R to identify significant metabolites associated with PL (23). The data were divided into a training set (60% of the data) and a validation set (40% of the data). Within the training set, 10-fold cross-validation was applied using the 'caret' package in R to optimize the model and avoid overfitting (24). The selected features from the LASSO model were then validated using the validation set. ROC curve, sensitivity, specificity, and confusion matrix analyses were conducted using the 'pROC' package in R. Additionally, ROC curve analysis on clinical data, including age, BMI, and WHR, was conducted to compare their predictive performance with that of the differentially metabolite abundance-based model.

## Correlation analysis

Pearson correlation analysis was performed using the 'Hmisc' package in R to investigate the relationships between differentially abundant metabolites and clinical parameters, such as age, BMI, and WHR. Heatmaps were created with the 'ComplexHeatmap' package, and significant correlations were visualized using scatter plots generated with the 'ggplot2' package, offering insights into the metabolic alterations linked to clinical features in the control and PL group. Additionally, a permutation test with 200 iterations was employed to assess the statistical significance of the correlation coefficient differences between the control group and PL group.

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# Results

## Baseline clinical characteristics

Table 1 presents the baseline clinical characteristics of the study participants, which included 122 women in the control group and 70 women in the PL group. The median age in the PL group was significantly lower than that in the control group (30 years vs. 33 years, $p < 0.001$). The PL group had a higher median BMI (22.6 kg/m$^2$ [Q1-Q3: 21.5-24.7]) than the control group (21.8 kg/m$^2$ [Q1-Q3: 20.2-23.4], $p = 0.006$). Furthermore, the PL group presented a significantly greater WHR (0.9 [Q1-Q3: 0.8-0.9]) than the control group (0.8 [Q1-Q3: 0.8-0.8], $p < 0.001$). In terms of race, the majority of participants in both groups were Han Chinese, with no significant difference observed between the two groups ($p = 0.726$). However, education levels significantly differed between the two groups. A significantly greater proportion of women in the control group had a university education or above (91% vs. 67.1%, $p < 0.001$), whereas the PL group had a greater percentage of participants with a high school education or below (32.9% vs. 9%, $p < 0.001$). With respect to the number of pregnancy losses, none of the women in the control group experienced a loss, whereas the PL group included participants with varying numbers of losses, including 17.1% with one loss, 50% with two losses, and 32.9% with three or more losses ($p < 0.001$).

## OPLS-DA model and permutation test

Figure 1 illustrates the OPLS-DA model and its evaluation through permutation tests. The OPLS-DA plot (Figure 1A) revealed a separation between the control and PL group, indicating distinct metabolic profiles. The x-axis (t1) explained 7.0% of the variance between the two groups, whereas the y-axis (to1) explained 8.0% of the variance within each group. A permutation test with 200 iterations (Figure 1B) was used to evaluate the OPLS-DA model. The histogram shows the frequency distribution of the permuted $R^2Y$ and $Q^2$ values, with an $R^2Y$ value of 0.932 and an $Q^2$ value of 0.899, which were both significantly higher than those obtained from the permuted models ($p = 0.005$), confirming the reliability and predictive performance of the model.

## Differential metabolite analysis

Figure 2 shows an in-depth analysis of the differentially abundant metabolites between the control and PL group. In total, 57 differentially abundant metabolites were identified on the basis of VIP> 1 and p values<0.05 (Supplementary Table 2). The heatmap shown in Figure 2A displays the differentially abundant metabolites, revealing distinct clustering patterns between the two groups. In addition, the volcano plot shown in Supplementary Figure S1A illustrates the differentially abundant metabolites using log2-fold changes and -log10 p values. Testosterone glucuronide, 6-hydroxymelatonin, and (S)-leucic acid demonstrated statistical significance and fold changes, with testosterone glucuronide exhibiting the greatest significance and fold change. Among the 57 significantly altered metabolites, 54 were successfully matched to known metabolic pathways in the KEGG database using the MetaboAnalyst platform. The pathway impact plot revealed several significantly impacted pathways, including caffeine metabolism, tryptophan metabolism, and riboflavin metabolism (Figure 2B). The bubble plot shows these pathways, with larger and more vividly colored bubbles indicating pathways with greater impact and significance.

TABLE 1 Clinical characteristics of PL patients and controls.

| Variables | Total (n = 192) | Control (n = 122) | PL (n = 70) | p |
|---|---|---|---|---|
| Age (year) | 32 (29.8, 36) | 33 (30.2, 37) | 30 (28, 32.8) | < 0.001 |
| BMI (kg/m2) | 22 (20.6, 23.5) | 21.8 (20.2, 23.4) | 22.6 (21.5, 24.7) | 0.006 |
| WHR | 0.8 (0.8, 0.9) | 0.8 (0.8, 0.8) | 0.9 (0.8, 0.9) | < 0.001 |
| Race, n (%) | | | | 0.726 |
| Han Chinese | 183 (95.3) | 117 (95.9) | 66 (94.3) | |
| Other ethnicities | 9 (4.7) | 5 (4.1) | 4 (5.7) | |
| Education, n (%) | | | | < 0.001 |
| High school or below | 34 (17.7) | 11 (9) | 23 (32.9) | |
| University or above | 158 (82.3) | 111 (91) | 47 (67.1) | |
| Number of PL, n (%) | | | | < 0.001 |
| 0 | 122 (63.5) | 122 (100) | 0 (0) | |
| 1 | 12 (6.2) | 0 (0) | 12 (17.1) | |
| 2 | 35 (18.2) | 0 (0) | 35 (50) | |
| ≥3 | 23 (12) | 0 (0) | 23 (32.9) | |

BMI, Body mass index; WHR, Waist-Hip Ratio; PL, Pregnancy loss. p<0.05 was considered statistically significant.

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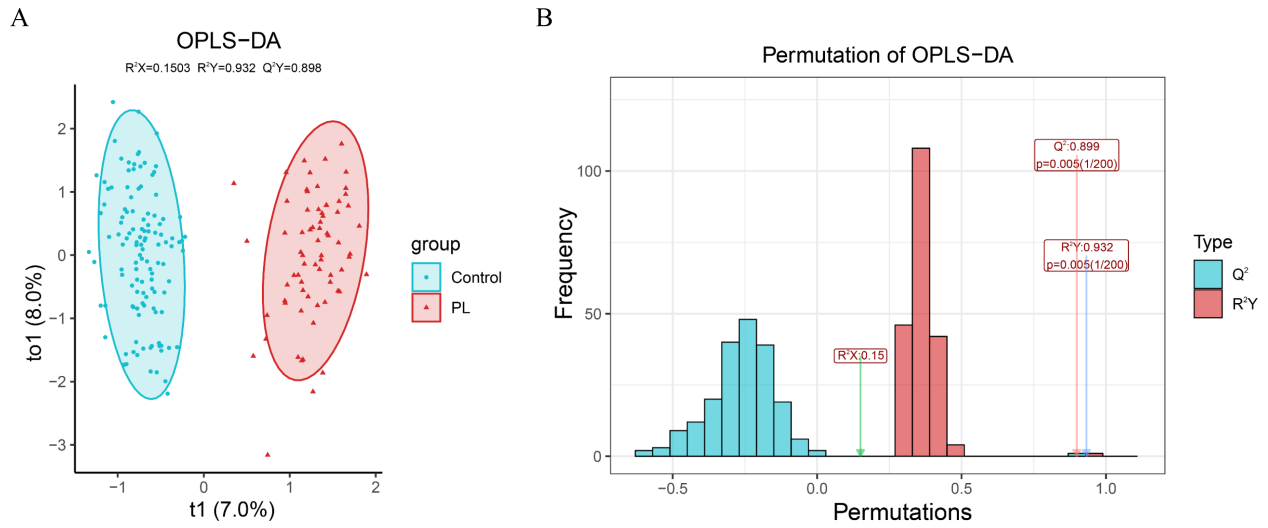


FIGURE 1
OPLS-DA model and permutation test evaluation. **(A)** OPLS-DA score plot showing the separation between the PL group and the control group based on their metabolic profiles. **(B)** Permutation test results with 200 iterations to evaluate the robustness of the OPLS-DA model.

## Screening of differential metabolites

Figure 3 shows the results of LASSO regression and the subsequent analysis of selected metabolites. In Figure 3A, the coefficients of the metabolites are plotted against the log of the regularization parameter (λ), demonstrating the selection process for the most important metabolites in distinguishing between the control and PL group. Figure 3B shows the three most important metabolites selected by the LASSO model, namely, testosterone glucuronide, 6-hydroxymelatonin and (S)-leucic acid.

## Abundance and diagnostic performance of key metabolites

Figure 4A compares the abundance levels of these metabolites between the control and PL group. Significant differences in abundance were observed for all three metabolites, with p values indicating significant differences ($p<0.0001$). These findings suggested that these metabolites play crucial roles in the metabolic alterations associated with PL. An ROC curve of the LASSO model revealed that the combined AUC value was 0.993,

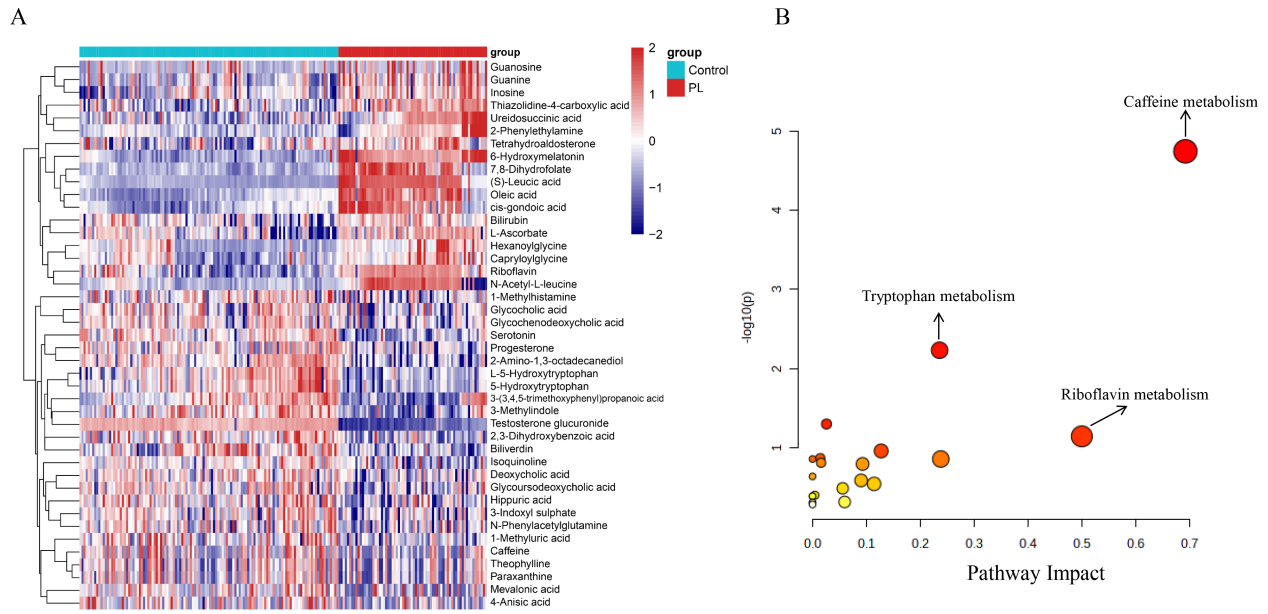


FIGURE 2
Differential metabolites and pathway enrichment analysis. **(A)** Heatmap of the top 43 differential metabolites identified between the control and PL group, showing distinct clustering patterns. **(B)** Pathway enrichment analysis bubble plot. The x-axis represents the pathway impact, and the y-axis represents the -log10 of the p-value.

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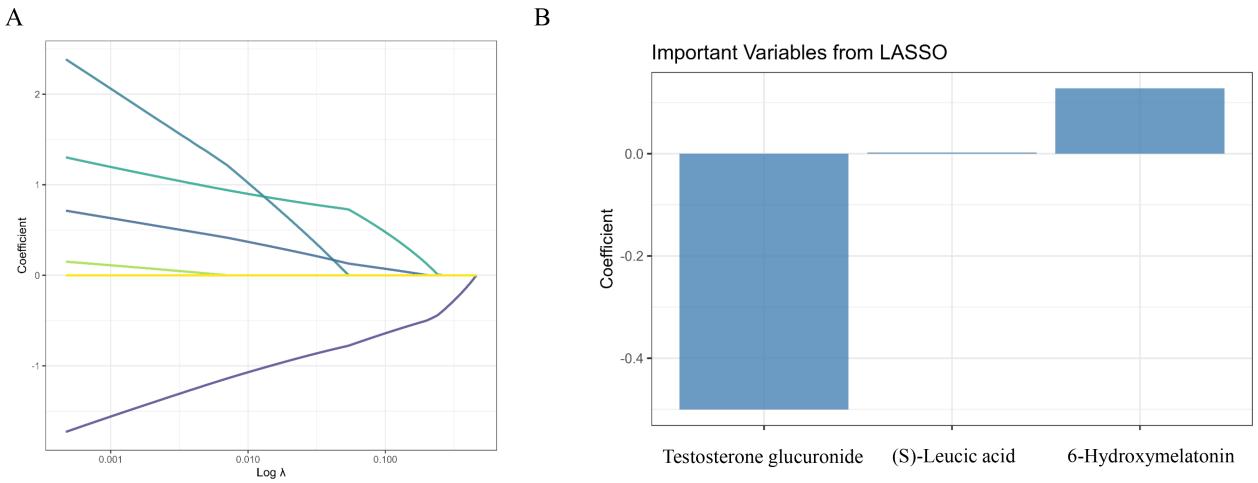


FIGURE 3
Screening of differential metabolites using LASSO regression. **(A)** LASSO regression cross-validation plot identifying the optimal λ to minimize deviance. **(B)** Bar plot of the regression coefficients for the three most important metabolites selected by the LASSO model.

indicating high predictive accuracy for the selected metabolites in the validation set (Figure 4B). The performance of the optimal metabolite model was evaluated using a confusion matrix (Supplementary Figure S1B) and classification performance indicators (Supplementary Figure S1C). The confusion matrix revealed that the model achieved a sensitivity of 1.0000 and a specificity of 0.9643, indicating high diagnostic accuracy. In addition, the model had an accuracy of 0.9868. The positive predictive value (PPV) was 0.9796, and the negative predictive value (NPV) was 1.0000, reflecting the robust ability of the model to distinguish between the control and PL group.

## Comparative diagnostic performance of the key metabolites and clinical parameters

The diagnostic performance of the clinical parameters and metabolites was evaluated via receiver operating characteristic (ROC) curve analysis. Among the clinical parameters, WHR had the highest AUC (0.724), followed by age (AUC = 0.696) and BMI (AUC = 0.435). The combined clinical model achieved an AUC value of 0.775 (Figure 5A). Furthermore, the combined model, which integrated both key metabolites and clinical parameters, achieved an AUC value of 1.000 (Figure 5B).

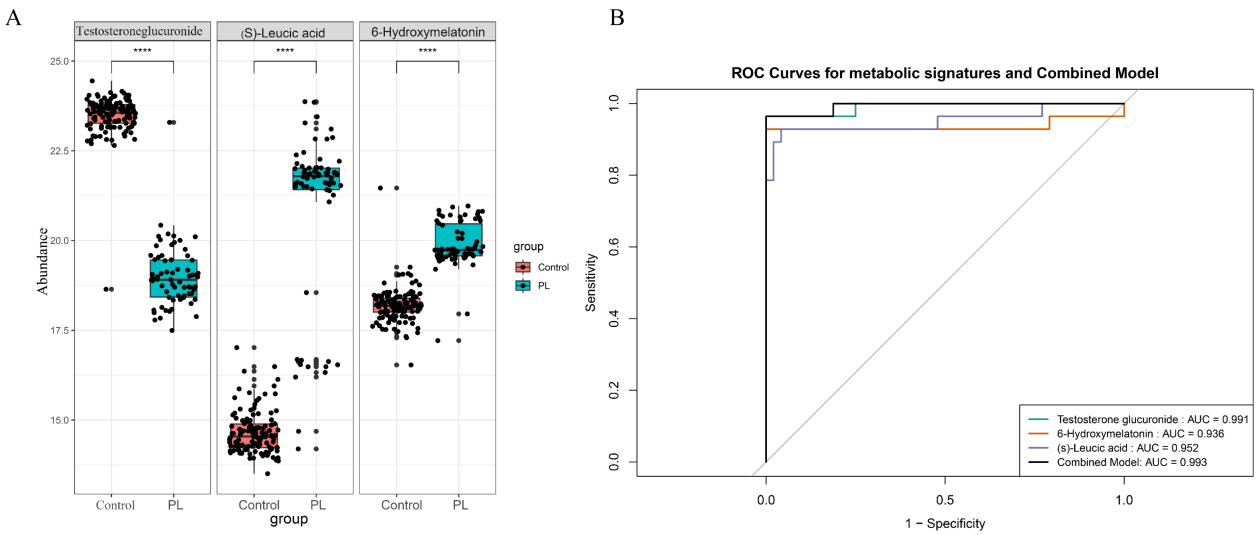


FIGURE 4
The most important metabolites and their diagnostic performance. **(A)** Box plots comparing the abundance levels of the three significant metabolites between the control and PL group (****p < 0.0001). **(B)** ROC curves demonstrating the diagnostic performance of a panel of metabolites in the test dataset.

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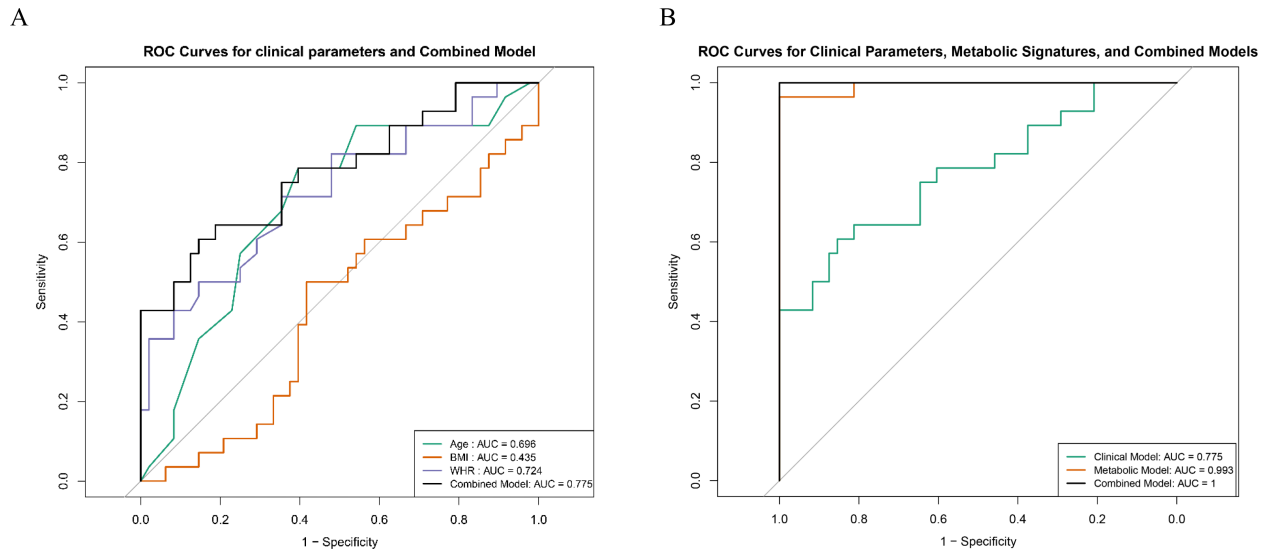


FIGURE 5
Comparative diagnostic performance of key metabolites and clinical indicators. **(A)** ROC curves illustrating the diagnostic performance of individual clinical parameters (age, BMI, and WHR). **(B)** ROC curves comparing the diagnostic performance of combined key metabolites, clinical parameters, and the integrated model.

## Correlation analysis between differentially abundant metabolites and clinical parameters

Figure 6 shows the Pearson correlations between differentially abundant metabolites and clinical parameters within the PL group. The heatmap shown in Figure 6A depicts the Pearson correlation coefficients between core differentially abundant metabolites and clinical phenotypes. The scatter plot shown in Figure 6B further illustrates the negative correlation between WHR and testosterone glucuronide, which indicated that higher WHR values were associated with lower levels of testosterone glucuronide ($r = -0.291$, $p = 0.0146$), whereas (S)-leucic acid was significantly positively correlated with WHR ($r = 0.248$, $p = 0.0381$). Additionally, the correlation of WHR with the levels of testosterone glucuronide or (S)-leucic acid were evaluated in the control group. The scatter plot shown in Supplementary Figure S1D indicates that both correlations were nonsignificant in the control group (WHR vs. testosterone glucuronide: $r = -0.0294$, $p = 0.748$; WHR vs. (S)-leucic acid: $r = 0.126$, $p = 0.168$), suggesting no evident associations (Supplementary Figures S1D, E). Moreover, a permutation test comparing the correlation coefficients between the control and PL group revealed a statistically significant difference ($p = 0.04$) in the correlation between WHR and testosterone glucuronide, whereas the correlation between WHR and (S)-leucic acid did not differ significantly ($p = 0.28$).

# Discussion

In the present study, we identified differentially abundant metabolites between the control and PL group, which were primarily enriched in the caffeine metabolism, tryptophan metabolism, and riboflavin metabolism pathways. Caffeine metabolism emerged as a key pathway in the present analysis. Caffeine is widely consumed, and its metabolism involves several enzymes, such as cytochrome P450, which are integral to oxidative stress responses and liver function (25–27). Disruptions in caffeine metabolism may reflect dysregulated detoxification processes or oxidative stress, both of which are critical for maintaining pregnancy. Studies have suggested that high caffeine intake during pregnancy may increase the risk of pregnancy complications, including PL (28). Therefore, the significant alterations in caffeine metabolism observed in PL patients may reflect either direct caffeine exposure or dysregulated detoxification processes that impact pregnancy outcomes.

Amino acids play fundamental roles in various biological processes, including protein synthesis, immune regulation, and neuroendocrine function (29, 30). Our findings revealed significant alterations in amino acid metabolism pathways, with tryptophan metabolism emerging as a key pathway in PL patients. Tryptophan, an essential aromatic amino acid, plays a critical role in protein synthesis, immune regulation, and neuroendocrine function (31). The metabolism of tryptophan primarily proceeds through three major pathways, namely, the kynurenine (KYN) pathway, the serotonin (5-HT) pathway, and the indole pathway (32). The KYN pathway, which dominates tryptophan metabolism, is mediated by several enzymes, including tryptophan-2,3-dioxygenase (TDO) and indoleamine-2,3-dioxygenase (IDO). These enzymes are critical for modulating immune responses (33, 34). Dysregulated IDO activity has been associated with decreased kynurenine levels in PL patients, which may impair immune tolerance and increase the risk of fetal rejection. Additionally, tryptophan serves as a precursor for serotonin synthesis, a key

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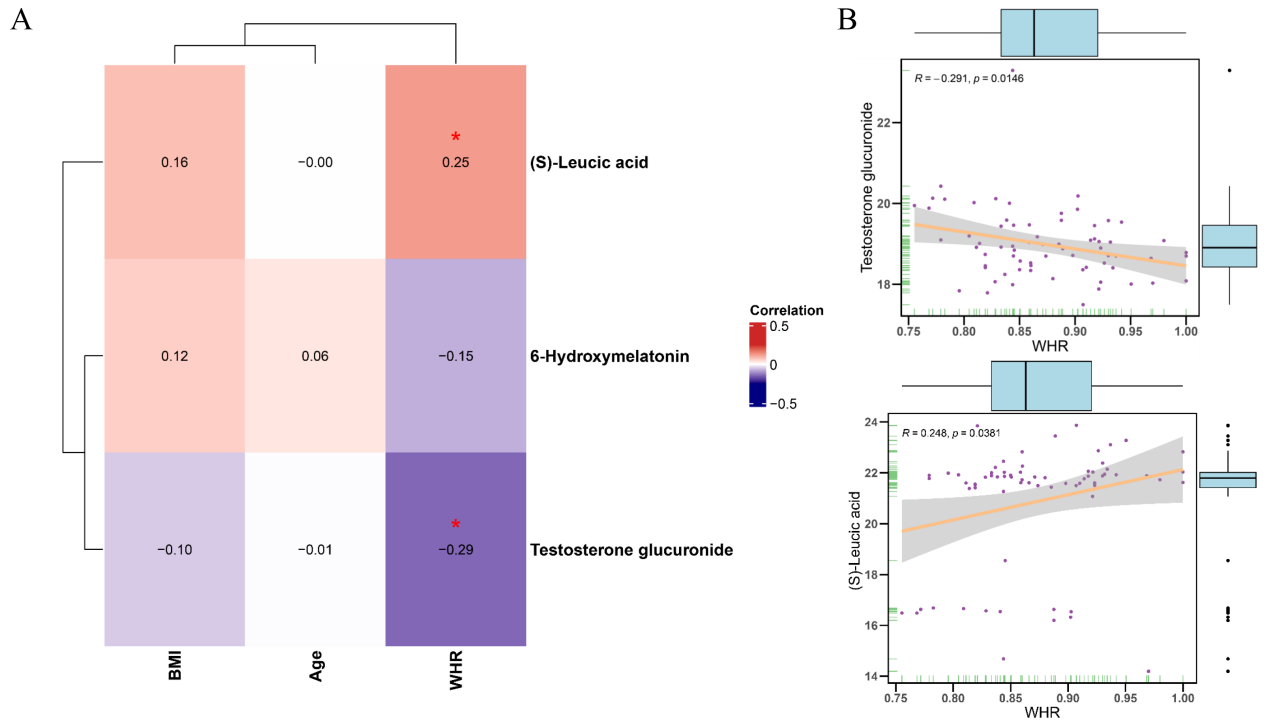


FIGURE 6
Correlation analysis between differential metabolites and clinical parameters in PL group. **(A)** Heatmap showing the Pearson correlation coefficients between core metabolites and clinical phenotypes parameters (age, BMI, WHR) in the PL group. **(B)** Scatter plots illustrating the significant correlations between WHR and testosterone glucuronide levels (negative correlation) and (S)-Leucic acid levels (positive correlation).

neurotransmitter involved in regulating inflammation and maternal–fetal communication (35). Reduced tryptophan availability in PL patients may compromise serotonin production, exacerbating systemic inflammation and negatively affecting pregnancy outcomes (36). The present findings aligned with these observations, further supporting the critical role of tryptophan metabolism in pregnancy maintenance.

In addition, the present study revealed enrichment in the riboflavin metabolism pathway, highlighting its potential involvement in PL. Riboflavin, also known as vitamin B2, is a precursor for the synthesis of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), which act as essential cofactors for numerous redox enzymes involved in energy metabolism, oxidative stress regulation, and detoxification processes (37). Riboflavin metabolism plays a critical role in redox reactions, energy production, and cellular homeostasis (38, 39). Deficiency in riboflavin has been shown to impair mitochondrial function, resulting in disrupted ATP synthesis and increased production of reactive oxygen species (ROS) (40, 41). This imbalance may contribute to oxidative stress, a condition linked to adverse pregnancy outcomes, such as preeclampsia, intrauterine growth restriction, and spontaneous abortion (42, 43). In the present study, the significant alterations in riboflavin metabolism observed in PL patients may indicate disrupted mitochondrial energy production and heightened oxidative stress, both of which may compromise pregnancy maintenance.

LASSO algorithms were further employed to refine the selection of significant metabolites. The present analysis identified testosterone glucuronide, 6-hydroxymelatonin and (S)-leucic acid as key metabolites with strong diagnostic potential. These metabolites demonstrated high area under the curve (AUC) values in the ROC curve analysis, indicating their utility as noninvasive metabolites for PL and surpassing the diagnostic accuracy of clinical indicators alone. Testosterone glucuronide is a conjugated form of testosterone involved in androgen metabolism (44). In the context of PL, altered levels of testosterone glucuronide may reflect disruptions in hormonal regulation. 6-Hydroxymelatonin is a metabolite of melatonin, a hormone known for regulating sleep–wake cycles and reproductive functions (45). Changes in melatonin metabolism have been linked to various reproductive disorders, suggesting that alterations in melatonin metabolism in PL patients may indicate disruptions in circadian rhythms and reproductive health. (S)-Leucic acid is a metabolite of leucine, a branched-chain amino acid, and it has been studied for its anabolic effects (46), particularly in promoting muscle protein synthesis. However, the role of (S)-leucic acid in reproductive health and pregnancy remains largely unexplored. The present findings suggested that (S)-leucic acid may be involved in metabolic pathways that are critical for maintaining a healthy pregnancy.

Correlation analysis revealed a significant negative association between waist–to–hip ratio (WHR) and testosterone glucuronide in the PL group, implying that an elevated WHR may coincide with lower levels of testosterone glucuronide. Such disruptions could impair maternal–fetal communication and contribute to adverse

pregnancy outcomes. Of note, this relationship was not observed in the control group, highlighting the specificity of metabolic alterations in PL patients. Although (S)-leucic acid was also positively correlated with WHR in the PL group, the difference in correlation coefficients compared with those in the control group was not statistically significant, possibly reflecting the limited sample size. Nevertheless, the apparent involvement of (S)-leucic acid in metabolic pathways relevant to pregnancy warrants further study to clarify its mechanistic role in PL and to assess its potential diagnostic value.

In conclusion, the present findings suggest that caffeine metabolism, tryptophan metabolism and riboflavin metabolism pathway may play important roles in the pathophysiology of PL, Moreover, testosterone glucuronide, 6-hydroxymelatonin, and (S)-leucic acid showed potential as noninvasive diagnostic metabolites. Further validation in larger cohorts is necessary to confirm these findings and to better understand the role of metabolic alterations in the pathophysiology of PL.

### Limitation

Despite the promising findings, this study has several limitations. Firstly, the sample size is relatively small, which may limit the generalizability of the results and prevent detailed stratification analyses, such as differentiating metabolic variations among PL cases with different recurrence histories. Secondly, the study design is cross-sectional, which prevents us from establishing causal relationships between the identified metabolic alterations and PL. Although we applied OPLS-DA and LASSO regression to prioritize key metabolites and minimize irrelevant variation and multicollinearity, these methods cannot fully account for confounding factors such as age, BMI, and WHR. We employed a permutation test to further address this issue, but future studies incorporating matched or statistically adjusted designs are warranted. Additionally, we did not collect detailed dietary information or account for variations in education level, which may influence metabolic profiles. Finally, the study focuses solely on plasma metabolites and includes limited clinical data. Future studies should aim to integrate multi-omics approaches and more comprehensive clinical datasets to provide a deeper understanding of the molecular mechanisms underlying PL.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

## Ethics statement

The studies involving humans were approved by the Institutional Review Board of Lanzhou University Second Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

## Author contributions

ND: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Software, Writing – original draft. XY: Formal analysis, Investigation, Project administration, Software, Supervision, Validation, Writing – original draft. RW: Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Writing – review & editing. FW: Data curation, Funding acquisition, Resources, Visualization, Writing – review & editing, Conceptualization, Validation.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2025.1518043/full#supplementary-material

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# Pregnancy outcomes in freeze-all versus fresh embryo transfer cycles of women with adenomyosis and endometriosis: a systemic review and meta-analysis

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**Background:** Endometriosis (EMS) and adenomyosis have adverse effects on women's fertility. *In vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are effective treatments for these diseases. Research has shown that different embryo transfer strategies in IVF/ICSI can influence gestational outcomes. This systematic review and meta-analysis aimed to evaluate the impact of freeze-all embryo transfer (FET) versus fresh embryo transfer (ET) strategies in IVF/ICSI cycles for infertile women with EMS and adenomyosis.

**Method:** A comprehensive search was conducted across PubMed, EMBASE, MEDLINE, Web of Science, Google Scholar, and Chinese databases to identify studies examining different embryo transfer strategies in IVF/ICSI cycles among patients with EMS and adenomyosis. The outcomes analyzed included rates of implantation, clinical pregnancy, miscarriage, and live birth. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated using random-effects or fixed-effects models.

**Results:** In patients with EMS, the results demonstrated that the FET strategy yielded higher clinical pregnancy (OR: 1.25; 95% CI: 1.11, 1.40), live birth rates (OR: 1.31; 95% CI: 1.15, 1.49), and implantation rates (OR: 1.27; 95% CI: 1.05, 1.54) compared to the fresh ET strategy. The miscarriage rate (OR: 0.89; 95% CI: 0.52, 1.52) and the ectopic pregnancy rate (OR: 0.51; 95% CI: 0.24, 1.07) were comparable between groups. For the group of women with adenomyosis, the IVF/ICSI outcomes were comparable between the FET and fresh ET strategies.

**Conclusion:** In IVF/ICSI, the FET strategy has been associated with more favorable reproductive outcomes compared to the fresh ET strategy in women

with EMS. Whereas in women with adenomyosis, pregnancy outcomes were comparable between the FET and fresh ET groups.

**Systematic Review Registration:** https://www.crd.york.ac.uk/PROSPERO/view/CRD42024563268, identifier CRD42024563268.

KEYWORDS

**endometriosis, adenomyosis, assist reproductive technology, freeze-all, embryo transfer, pregnancy outcomes**

# Introduction

Endometriosis (EMS) and adenomyosis are interrelated chronic diseases, both originating from ectopically located intracavitary endometrium. EMS is characterized by the presence of endometrial stroma and glands outside the uterine cavity, while adenomyosis is defined by the infiltration of endometrial tissue within the myometrium (1). Women with EMS and adenomyosis often suffer from subfertility and infertility (2). Up to 35–50% of infertile women are affected by EMS, while the prevalence of adenomyosis in infertile women is reported to be approximately 7.5-24.4% (3). The pathological processes may involve inflammation and fibrosis, immune modulation, altered steroid hormone metabolism, increased oxidative stress, and intrauterine abnormalities. These factors potentially interfere with folliculogenesis, sperm function, embryo transport, and endometrial receptivity (4, 5).

*In vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) and embryo transfer is a valid option for infertile women with EMS and adenomyosis (6, 7). The current clinical policy of transferring includes both fresh embryo transfer (ET) and freeze-all embryo transfer (FET). The FET strategy, initially designed to mitigate ovarian hyper-stimulation syndrome, facilitates embryo cryopreservation for subsequent suitable cycles. In IVF/ICSI, the key factors to conception are embryo quality, embryo-endometrium interaction, and endometrial receptivity (8). Recently, heightened attention has been drawn to the elevated sex steroid levels resulting from hyper-stimulation during controlled ovarian hyperstimulation (COS). It may exacerbate endometrial receptivity issues, reducing the likelihood of successful conception. As a results, there has been a proposal that FET approach can separate the COS process from the embryo transfer, thereby serving to circumvent the potential adverse impacts of COS on the endometrium (9). One randomized Controlled Trials (RCTs) involving patients with ovulatory women demonstrated favorable outcomes in either pregnancy outcomes or the incidence of ovarian hyper-stimulation syndrome for the FET group (10). With the advancement of vitrification techniques, FET approach has been applied to patients with EMS and adenomyosis. Bourdon et al. investigated 270 infertile women with EMS undergoing IVF/ICSI. Their results indicated that the FET strategy yielded higher cumulative clinical pregnancy and live birth rates compared to the fresh ET strategy (11). Similarly, a retrospective cohort analysis found that the FET strategy in women with adenomyosis was associated with significantly higher odds of live birth compared to fresh ET (12). However, other studies reported comparable pregnancy outcomes among women with EMS or adenomyosis, regardless of whether FET or fresh ET was used (13–15). Additionally, Roque et al. conducted a systematic review and meta-analysis, demonstrating that FET significantly improved live birth rates compared to fresh ET, particularly in hyper-responders and preimplantation genetic testing for aneuploidy cycles (16). However, the lack of distinction between study populations limited its clinical applicability. To evaluate embryo transfer strategies in infertile patients with endometriosis and adenomyosis, we conducted a systematic review and meta-analysis.

# Materials and methods

## Protocol and registration

This systematic review and meta-analysis were conducted following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (17). The protocol of this meta-analysis was registered in PROSPERO (CRD42024563268).

## Study design

We conducted a systematic review and meta-analysis compared the pregnancy outcomes after different embryo transfer strategies in patients with EMS and adenomyosis. In all cases, EMS and adenomyosis were diagnosed by biopsy through surgery or medical imaging evidence like transvaginal ultrasound (TVUS) or magnetic resonance imaging (18). In TVUS, ovarian endometrioma appeared as a persistent unilocular or multilocular cyst with homogeneous low-level echogenicity of the cyst fluid and absent or moderate cyst wall vascularization. Deeply infiltrating endometriosis in the TVUS appeared as thick tissue blocks,

nodular formations, or irregularly shaped hypoechoic, commonly affecting the uterosacral ligament, pouch of Douglas, and/or vagina. In addition, adenomyosis showed myometrial cystic areas, hyperechoic islands, linear striations, and buds or irregular/infiltrated endometrial-myometrial junction zones (19–21).

First, we conducted a comparative analysis across the entire study population, including individuals with EMS or adenomyosis. Subsequently, we performed a subgroup analysis based on the included studies. One analysis comprised nine studies focusing on EMS, while the other included two studies on adenomyosis. We separately summarized the gestational outcomes for patients with EMS or adenomyosis.

## Search strategy

A systematic search for relevant papers was carried out in PubMed, EMBASE, MEDLINE, Web of Science, Google Scholar, China National Knowledge Infrastructure, Wanfang Data Knowledge Service Platform, and China Biomedical Literature Database. English search keywords included "endometriosis", "adenomyosis", "*in vitro* fertilization/IVF-ET", "Intracytoplasmic sperm injection/ICSI", "freeze-all/frozen embryo transfer", "Fresh Embryo Transfer", and "pregnancy outcomes", as showed in support information. No restrictions were placed on the language or publication date of the studies. Additionally, we examined the reference lists of the eligible studies and review articles to identify any additional relevant articles.

## Inclusion and exclusion criteria

Studies were included in this meta-analysis if they matched the following inclusion criteria (1): subjects of study were women diagnosed with either EMS or adenomyosis. (2) study focused on the pregnancy outcomes of different embryo transfer types in IVF/ICSI cycles. The exposure group consisted of women undergoing FET, while the control group comprised those undergoing fresh ET. (3) the outcomes include at least one of the following: implantation rate, miscarriage rate, clinical pregnancy rate, ectopic pregnancy rate, or live births rate. (4) randomized controlled trials (RCTs) or cohort studies. exclusion criteria: (1) study involved with donor or recipient oocyte treatments. (2) Review articles, abstracts, letters, conference papers, and case reports were excluded. (3) Necessary data was not available. (4) studies included animal experiments.

## Study selection

All titles and abstracts were independently reviewed by two investigators (Yixian Han and Lukanxuan Wu). Studies that potentially met the inclusion criteria were further assessed through full-text review. Any discrepancies were resolved by consulting a third author(Chang Liu). The kappa statistic was used to evaluate inter-examiner agreement in study selection (22). Both researchers manually extracted data from the included studies using specially designed data collection forms. The following data were collected: lead author, publication year, country, study period, study design, intervention, age, body mass index, duration of infertility, diagnosis, EMS phenotype or stage, stimulation protocol, FET protocol, oocyte retrieved, and pregnancy outcomes.

## Risk of bias assessment

The quality of the included studies was evaluated using the Newcastle Ottawa scale (NOS) for assessing observational studies based on selection, comparability, and outcome domains. The total score of this scale was 9 points (0–3: poor quality, 4–6: fair quality,7–9: good quality) (23).

## Outcome measure

The primary outcomes were clinical pregnancy and live birth rate, and the secondary outcomes were miscarriage, implantation rate, and ectopic pregnancy rate. The implantation rate was defined as the proportion of transferred embryos that were successfully implanted. Clinical pregnancy was confirmed by the presence of at least one gestational sac on ultrasound, including ectopic pregnancy. Ectopic pregnancy was defined as the detection of a gestational sac outside the uterine cavity using TVUS, or clinically diagnosed when no gestational sac is observed within the uterine cavity but serum hCG levels continue to rise. A miscarriage was defined as the loss of gestation before 28 weeks of pregnancy. Live birth was described as a live birth event after 28 weeks.

## Statistical analysis

Statistical analysis was performed with Review Manager 5.4 software. All the outcomes in our analysis were binary. Dichotomous data was analyzed using the Mantel–Hansel odds ratio and the CIs between groups. The $I^2$ statistic and Cochran's Q-statistic were used to assess methodologic and clinical heterogeneity across studies. High heterogeneity was defined as $I^2 \geq 50\%$ or $p < 0.10$, in which case a random-effects model was applied; otherwise, a fixed-effects model was used. Sensitivity analysis and subgroup analysis were conducted when $I^2$ or Cochran's Q-statistic detected significant heterogeneity. Funnel plot analysis was used to assess the potential publication bias. All statistical tests were two-sided, and $p$ values $< 0.05$ were considered statistically significant. The GRADE approach was employed to evaluate the quality of each outcome (24). This assessment considered factors such as risk of bias, inconsistency, indirectness, imprecision, and publication bias.

Based on the GRADE criteria, the evidence was categorized into four levels: high, moderate, low, and very low quality.

# Results

## Study selection

In the initial screening, 2,465 citations were identified across the databases. After removing duplicates, 1,513 articles were evaluated based on their titles and abstracts. Subsequently, 43 articles were retrieved for full-text assessment. After a thorough examination, 5 articles were excluded due to unsuitable study types, 24 for lack of relevance, and 3 for not reporting relevant outcomes. Ultimately, 11 articles that met our eligibility criteria were included in the final analysis. A high level of concordance was observed among the reviewers in terms of data screening and integration (kappa = 0.81). Of the included studies, 4 were published in Chinese and 7 in English. The flow chart of the study selection is demonstrated in Figure 1.

## Characteristics of included studies

A total of 5650 patients were enrolled, with 3127 undergoing FET and 2523 undergoing fresh ET in the IVF/ICSI cycles. Six studies originated from China (15, 25–29), two from France (11, 12). and the remaining three from Canada, England, and Turkey, respectively (13, 14, 30). Of the eleven studies, two primarily addressed adenomyosis (12, 15), while the remaining nine focused on various types of EMS (8, 11, 13, 14, 25, 27–30). Among the EMS studies, three exclusively included patients with ovarian endometriomas, rASRM stage I-II EMS, or rASRM stage III-IV EMS, respectively (25, 26, 30). In four studies, the diagnosis of EMS was made via laparoscopy or laparotomy, while in five studies, it was determined using either laparoscopy or imaging

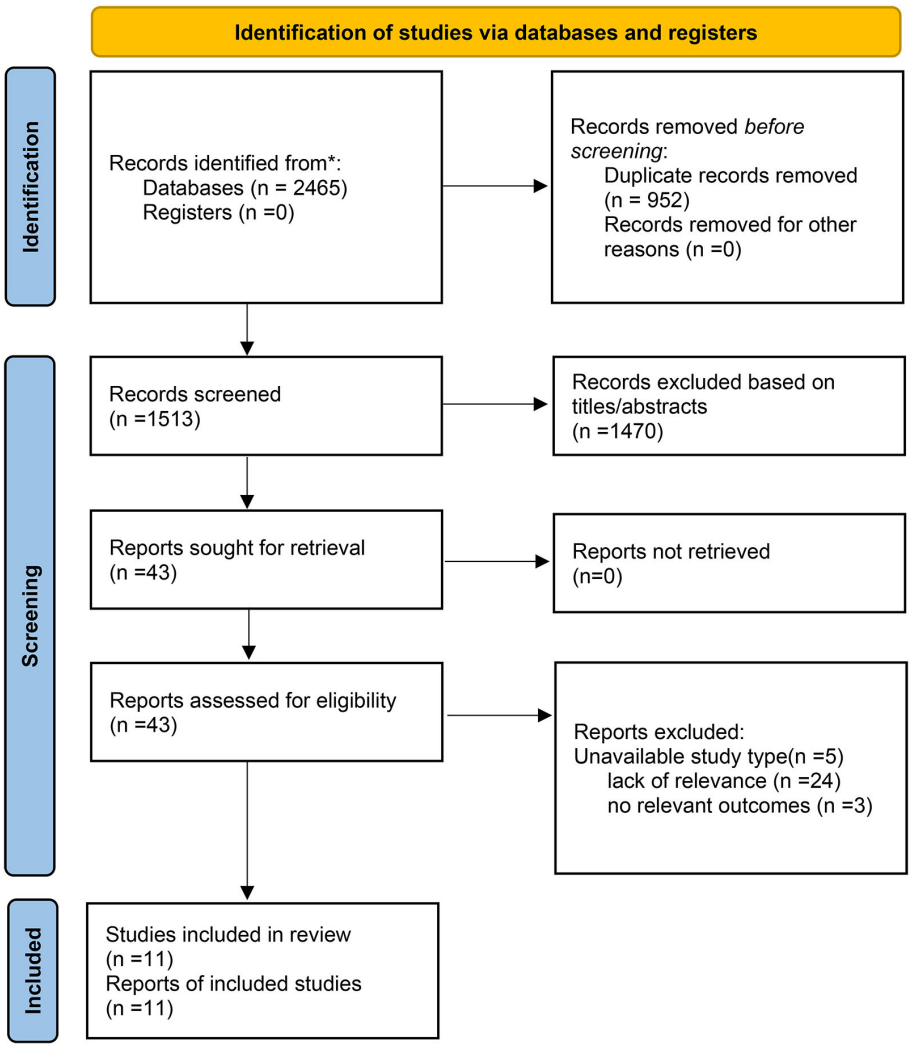


FIGURE 1
Search strategy.

examinations such as TVUS and magnetic resonance imaging. Adenomyosis was diagnosed by TVUS. Among the included studies, all involved clinical pregnancy rate, five involved embryo implantation rate (25, 26, 28–30), five involved ectopic pregnancy rate (25, 26, 28–30), ten involved live birth rate (11–13, 15, 25–30), and ten involved miscarriage rate (11, 13–15, 25–30). However, the definitions of miscarriage rate varied, with pregnancy loss ranging from 6 to 28 weeks, potentially introducing confounding factors. Therefore, we adopted the most common definition of miscarriage rate, specifically as a pregnancy loss occurring before 28 weeks after clinical pregnancy confirmation, and excluded four studies with differing definitions of miscarriage rate (13, 14, 26, 30). Table 1 presents the characteristics of each study included and Table 2 outlines the main results from each study included in the analysis.

## Meta analysis

### Clinical pregnancy rate

There were 11 studies investigating the difference in clinical pregnancy rate between FET and fresh ET groups. Compared to fresh ET, FET group improved clinical pregnancy rate (0R: 1.22; 95% CI: 1.09, 1.37; $p$ <0.01; $I^2$ = 48%) (Figure 2A). In the subgroup analysis, the results of women with EMS were consistent with the overall analysis, observing a higher clinical pregnancy rate in FET group (0R: 1.25; 95% CI: 1.11, 1.40; $p$ <0.01; $I^2$ = 38%) (Figure 2B). However, among women with adenomyosis, the clinical pregnancy rate did not significantly differ between the FET and fresh ET groups (0R: 0.92; 95% CI: 0.40, 2.13; $p$ =0.85; $I^2$ = 79%) (Figure 2C).

### Live birth rate

Of the included studies, 10 reports investigated the live birth rate between different embryo transfer groups. A significant increase in live birth rates was observed in women undergoing FET cycles compared to those undergoing fresh ET cycles (OR: 1.29; 95% CI: 1.14, 1.45; p <0.01; $I^2$ = 38%) (Figure 3A). Subgroup analysis consistently showed a higher live birth rate in the FET group among women with EMS (OR: 1.31; 95% CI: 1.15, 1.49; $p$ <0.01; $I^2$ = 32%) (Figure 3B). However, for women with adenomyosis, the live birth rate was similar between groups (OR: 0.98; 95% CI:0.44, 2.16; p =0.95; $I^2$ = 69%) (Figure 3C).

### Miscarriage rate

Five studies provided data on miscarriage rates between the FET and fresh ET groups. The results indicated no statistical difference between groups (OR: 0.92; 95% CI: 0.58, 1.46; $p$ =0.73; $I^2$ = 55%) (Figure 4A). This finding was consistent in the subgroup analysis focusing on patients with EMS (OR: 0.89; 95% CI: 0.52, 1.52; p =0.67; $I^2$ = 64%) (Figure 4B). Moreover, the sensitivity analysis of EMS did not alter the conclusion after eliminating any study. Similarly, among women with adenomyosis, the risk of miscarriage was comparable between the FET and fresh ET groups (OR: 1.06; 95% CI: 0.38, 2.96; p =0.92) (Figure 4C).

### Implantation rate

Of the 11 studies included, 5 described the association between different embryo transfer types and implantation rates in patients with EMS. The combined analysis demonstrated that FET group exhibited a higher implantation rate compared to fresh ET group (OR: 1.27; 95% CI: 1.05, 1.54; $p$ =0.01; $I^2$ = 62%) (Supplementary Figure S2). A sensitivity analysis was conducted to address heterogeneity, confirming the findings with consistent results after excluding the study by Wang et al. (25) (OR: 1.32; 95% CI: 1.18, 1.49; $p$ <0.01; $I^2$ = 38%). Data regarding implantation rate specifically in women with adenomyosis were lacking.

### Ectopic pregnancy rate

In this meta-analysis, 5 studies were included to investigate the ectopic pregnancy rate between FET and fresh ET groups in women with EMS. The finding indicated a similar ectopic pregnancy rate between groups (OR: 0.51; 95% CI:0.24, 1.07; $p$ =0.08; $I^2$ = 0%) (Supplementary Figure S3). There were no available data to investigate the relationship between ectopic pregnancy rate and different embryo transfer types in women with adenomyosis.

## Publication bias assessment of the included studies

Funnel plots for each of the meta-analyses, as shown in Supplementary Figure S1, appeared to be symmetrical, except for the ectopic pregnancy rate, which suggested a potential, though minimal, publication bias likely due to the non-publication of smaller studies.

## Quality assessment of the included studies

The NOS used for assessing the quality of studies in this meta-analysis is presented in Table 3. The included studies received scores ranging from 7 to 9, indicating they were classified as high-quality studies with a low risk of bias.

## GRADE assessment

The quality of evidence for each outcome was evaluated using the GRADE approach as demonstrated in Supplementary Figure S4. The findings revealed that the clinical pregnancy and live birth rates, as well as the ectopic pregnancy rate, following different embryo transfer strategies for patients with EMS and adenomyosis, or EMS alone, were assessed as having moderate certainty of evidence. In contrast, all other outcomes were rated as low or very low certainty of evidence.

# Discussion

The objective of this study was to compare pregnancy outcomes between FET and fresh ET cycles in infertile patients with EMS and adenomyosis. The results of the meta-analysis involved 11 studies with a total of 5,650 patients, of which nine focused on

TABLE 1 Baseline characteristics.

| Study | Country | Study period | Study design | Type of disease | Intervention | Age (years) | BMI (kg/m$^2$) | Infertility years | Diagnosis | EMS Pheno-type or stage | Stimulation protocol | FET protocol | Oocyte retrieved | Outcomes |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tan2021 (14) | Canada | 2014-2019 | retrospective cohort study | EMS | FET(n=389) | 35.9 ± 0.3 | 22.2 ± 0.1 | 2.8 ± 0.3 | surgery | ASRM Stage I-IV | GnRH antagonist | HRT | 7.4 ± 0.3 | CPR, MR |
| | | | | | Fresh ET(n=339) | 35.5 ± 0.2 | 22.4 ± 0.2 | 2.9 ± 0.3 | | | | | 8.2 ± 0.8 | |
| Asoglu2020 (30) | Turkey | 2014-2018 | retrospective cohort study | EMS | FET(n=180) | 33.1 ± 4.2 | 22.7 ± 2.4 | 2.9 ± 2.0 | TVUS | Endometrioma | GnRH antagonist/long GnRH agonist | HRT | 8.5 ± 3.5 | IR, CPR, MR, LBR, EPR |
| | | | | | Fresh ET(n=135) | 33.3 ± 3.4 | 22.9 ± 3.8 | 3.2 ± 2.0 | | | | | 8.5 ± 3.2 | |
| Wu2019 (26) | China | 2006-2017 | retrospective cohort study | EMS | FET(n=506) | 33.0 (30.0, 35.0) | 20.7 (19.2, 22.4) | 3.0 (2.0, 5.0) | Laparoscopy | ASRM Stage III-IV | NOS | NOS | 7.0 (4.0, 12.0) | IR, CPR, MR, LBR, EPR |
| | | | | | Fresh ET(n=225) | 32.0 (29.0, 35.0) | 20.6 (19.1, 22.3) | 4.0 (2.0, 6.0) | | | | | 8.0 (5.0, 13.0) | |
| Mohamed2011 (13) | England | 2000-2008 | retrospective cohort study | EMS | FET(n=148) | NOS | NOS | NOS | Laparoscopy | NOS | long GnRH agonist | GnRH-a+HRT | NOS | CPR, MR, LBR |
| | | | | | Fresh ET(n=267) | | | | | | | | | |
| Bourdon2018 (11) | France | 2012-2014 | prospective cohort study | EMS | FET(n=135) | 34.3 ± 4.1 | 22.8 ± 3.6 | 4.7 ± 2.7 | TVUS/ MRI/ surgery | SUP, OMA, DIE | HRT | HRT | 9.9 ± 7.0 | CPR, MR, LBR |
| | | | | | Fresh ET(n=135) | 34.3 ± 3.9 | 22.3 ± 3.3 | 4.4 ± 2.3 | | | | | 7.4 ± 4.3 | |
| Wang2018 (25) | China | 2010-2017 | retrospective cohort study | EMS | FET(n=419) | 30.4 ± 3.9 | 22.1 ± 2.3 | 4.0 ± 2.2 | Laparoscopy | ASRM Stage I-II | long GnRH agonist | GnRH-a+HRT | 15.1 ± 8.9 | IR, CPR, MR, LBR, EPR |
| | | | | | Fresh ET(n=102) | 31.2 ± 3.8 | 21.7 ± 1.9 | 4.1 ± 3.0 | | | | | 13.2 ± 8.0 | |
| Yue2022 (27) | China | 2014-2018 | retrospective cohort study | EMS | FET(n=231) | 31.3 ± 3.9 | NOS | NOS | TVUS/ Laparoscopy | NOS | GnRH antagonist/long or short GnRH agonist | NC/ OI/HRT | 13.2 ± 6.6 | CPR, MR, LBR |
| | | | | | Fresh ET(n=231) | 31.4 ± 3.9 | | | | | | | 8.3 ± 4.4 | |
| Zhang2024 (29) | China | 2011-2022 | retrospective cohort study | EMS | FET(n=516) | 31.6 ± 3.6 | 20.9 ± 2.5 | 4.5 ± 2.8 | TVUS/ Laparoscopy | NOS | NOS | NC/ OI/HRT | NOS | IR, CPR, MR, LBR, EPR |
| | | | | | Fresh ET(n=645) | 31.4 ± 3.5 | 21.0 ± 2.6 | 4.8 ± 2.6 | | | | | | |

*(Continued)*





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TABLE 1 Continued

| Study | Country | Study period | Study design | Type of disease | Intervention | Age (years) | BMI (kg/m$^2$) | Infertility years | Diagnosis | EMS Phenotype or stage | Stimulation protocol | FET protocol | Oocyte retrieved | Outcomes |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dai2024 (28) | China | 2017-2021 | retrospective cohort study | EMS | FET(n=310) | 31.8 ± 4.4 | 23.3 ± 3.1 | 4.9 ± 3.7 | TVUS/surgery | ASRM Stage I-IV | ultralong/long GnRH agonist | GnRH-a +HRT/HRT | NOS | IR, CPR, MR, LBR, EPR |
| | | | | | Fresh ET(n=242) | 30.5 ± 4.1 | 23.1 ± 3.3 | 4.7 ± 3.0 | | | | | | |
| Zhang2023 (15) | China | 2018-2021 | retrospective cohort study | AD | FET(n=98) | 35.1 ± 4.7 | 22.9 ± 3.8 | 3.7 ± 2.3 | TVUS | NOS | GnRH antagonist GnRH agonist/PPOS | NC/OI/HRT | 5.0 (2.0, 10.0) | CPR, MR, LBR |
| | | | | | Fresh ET(n=91) | 34.7 ± 3.9 | 23.8 ± 4.0 | 3.3 ± 2.1 | | | | | 7.0 (4.0, 11.0) | |
| Bourdon2024 (12) | France | 2018-2021 | retrospective cohort study | AD | FET(n=195) | 35.6 ± 3.4 | 23.9 ± 4.2 | 4.2 ± 2.7 | TVUS | NOS | GnRH antagonist/long or short GnRH agonist | NC/OI/HRT | 10.8 ± 6.7 | CPR, LBR |
| | | | | | Fresh ET(n=111) | 35.2 ± 3.5 | 23.5 ± 3.6 | 3.7 ± 2.5 | | | | | 7.3 ± 3.3 | |

ASRM, American Society of Reproductive Medicine; AD, adenomyosis; CPR, clinical pregnancy rate; DIE, deeply infiltrating endometriosis; EMS, endometriosis; FET, freeze-all embryo transfer; GnRH gonadotropin-releasing hormone; HRT, hormone replace treatment; IR, implantation rate; LBR, live birth rate. MR, miscarriage rate; MRI, magnetic resonance imaging; NC, natural cycle; NOS, not otherwise specified; OI, ovulation induction; OMA, ovarian endometrioma; PPOS, progestin-primed ovarian stimulation; SUP, superficial peritoneal endometriosis; TVUS, transvaginal ultrasound.

endometriosis, while the remaining studies examined adenomyosis. In endometriosis-associated infertility patients undergoing different embryo transfer strategies, FET was associated with increased implantation, clinical pregnancy, and live birth rates, while no significant differences were observed in miscarriage and ectopic pregnancy rates. Compared to previous meta-analysis (31), our findings highlight that FET protocols improve clinical pregnancy rates while maintaining comparable miscarriage rates. These differences may be attributed to the inclusion of a greater number of studies in our analysis. In women with adenomyosis, IVF/ICSI outcomes were similar between FET and fresh ET groups. However, given that only two studies focused on pregnancy outcomes in adenomyosis, these findings should be interpreted with caution.

Due to the elusive nature of their pathogenesis, both EMS and adenomyosis are often referred to as "enigmatic diseases" (32). The question of whether EMS and adenomyosis represent two phenotypes of the same disease or are distinct entities remains a topic of ongoing debate (1, 32, 33). Dysfunction of the myometrial junctional zone and aberrations in the eutopic and heterotopic endometria suggest that adenomyosis and EMS may share a common origin (34). However, a shared origin does not imply they are identical, as they differ significantly in their histological, and clinical manifestations, as well as in their associated risk factors (32). Regardless of whether they are considered the same disease, it is clear that both conditions have a detrimental impact on women's fertility.

EMS is associated with poor pregnancy outcomes during IVF/ICSI, the mechanisms are highly complex. In addition to alterations in pelvic anatomy and diminished ovarian reserve, changes in the immune microenvironment and reduced endometrial receptivity also play significant roles (35). Implantation is a pivotal aspect of assisted reproductive technology (ART). Supraphysiologic levels of estradiol and progesterone during COS could impair endometrial receptivity and lead to embryo-endometrium asynchrony, thereby reducing implantation rates during IVF/ICSI (36). Meanwhile, EMS, an estrogen-dependent disease, is characterized by inflammation, dysregulated differentiation of endometrial mesenchymal cells, and abnormal epigenetic marks in both the intracavitary endometrium and ectopic endometriotic tissue, all of which are associated with the imbalance between estrogen and progesterone (37). As a result, it is plausible to conjecture that the heightened steroid levels during COS and the intricate pathological characteristic of EMS itself might synergistically impede endometrial receptivity. The FET strategy, an alternative to fresh ET, allows for the separation of COS and embryo transfer. In this way, endometrial development can be controlled more precisely than in cycles of COS with gonadotropins (38). Additionally, embryos are transferred to an environment that has not been exposed to the supraphysiologic hormonal levels associated with COS. Consequently, it has been proposed that FET is advantageous for ART outcomes compared to fresh ET. Yue et al. included 462 patients with EMS to compare pregnancy outcomes between different embryo transfer methods. Their findings indicated that the cumulative clinical pregnancy rate was significantly higher in the FET group compared to the fresh ET group (27). Similarly, a

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TABLE 2 Pregnancy outcomes of FET group compared with fresh group.

| Study | Subjects (n) | Implantation rate (%) | | Clinical pregnancy rate (%) | | Miscarriage rate (%) | | Ectopic pregnancy rate (%) | | Live birth rate (%) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | FET group | Fresh ET | FET group | Fresh ET | FET group | Fresh ET | FET group | Fresh ET | FET group | Fresh ET |
| Tan2021 (14) | 728 | NOS | NOS | 40.9 (159/389) | 41.3 (140/339) | NOS | NOS | NOS | NOS | NOS | NOS |
| Asoglu2020 (30) | 315 | 64.4 (116/180) | 49.6 (67/135) | 63.3 (114/180) | 50.4 (68/135) | NOS | NOS | 0.9(1/114) | 4.4(3/68) | 56.1 (101/180) | 39.9 (55/135) |
| Wu2019 (26) | 731 | 34.4 (332/964) | 25.5 (125/491) | 51.8 (262/506) | 44.0 (99/225) | NOS | NOS | 0.3(1/262) | 1.0(1/99) | 45.3 (229/506) | 36.4 (82/225) |
| Mohamed2011 (13) | 415 | NOS | NOS | 18.2 (27/148) | 20.2 (54/267) | NOS | NOS | NOS | NOS | 16.9 (25/148) | 19.5 (52/267) |
| Bourdon2018 (11) | 270 | NOS | NOS | 37.0 (50/135) | 28.9 (39/135) | 16.0(8/50) | 38.5 (15/39) | NOS | NOS | 28.9 (39/135) | 15.6 (21/135) |
| Wang2018 (25) | 521 | 26.2 (289/1105) | 28.8 (64/222) | 43.0 (180/419) | 47.1 (48/102) | 6.7 (12/180) | 14.6 (7/48) | 3.9(7/180) | 4.2(2/48) | 35.6 (149/419) | 28.4 (29/102) |
| Yue2022 (27) | 462 | NOS | NOS | 57.1 (132/231) | 43.7 (101/231) | 18.2 (24/132) | 13.9 (14/101) | NOS | NOS | 46.8 (108/231) | 37.7 (87/231) |
| Zhang2024 (29) | 1161 | 46.3 (478/1032) | 42,2 (545/1290) | 66.7 (344/516) | 60.0 (387/645) | 13.7 (47/344) | 10.9 (42/387) | 1.5(5/344) | 2.8 (11/387) | 56.6 (292/516) | 51.8 (334/645) |
| Dai2024 (28) | 552 | 41.5 (231/556) | 36.9 (167/453) | 57.4 (178/310) | 52.9 (128/242) | 16.9 (30/178) | 13.3 (17/128) | 0.6(1/178) | 0.8 (1/128) | 47.4 (147/310) | (110/242) |
| Zhang2023 (15) | 189 | NOS | NOS | 33.7 (33/98) | 46.2 (42/91) | 27.3(9/33) | 26.2 (11/42) | NOS | NOS | 19.4 (19/98) | 27.5 (25/91) |
| Bourdon2024 (12) | 306 | NOS | NOS | 41.0 (80/195) | 33.3 (37/111) | NOS | NOS | NOS | NOS | 27.2 (53/195) | 20.7 (23/111) |

ET, embryo transfer; FET, freeze-all embryo transfer; NOS, not otherwise specified.

meta-analysis involving six cohort studies revealed that in patients with endometriosis-related infertility, the live birth rate following FET was significantly higher than that of fresh ET. Additionally, the miscarriage rate was statistically lower in the FET group (31). Our study, which incorporated more recent research, confirmed part of these findings, demonstrating consistent improvements in pregnancy outcomes with the FET strategy in patients with endometriosis. However, the miscarriage rate in our analysis was comparable between the FET and fresh ET groups. We considered this discrepancy may arise from variations in the definition of miscarriage rate across studies. To minimize potential confounding, we only included studies that adhered to the most commonly accepted definition of miscarriage, excluding those with differing criteria. In addition, Tan et al. investigated early pregnancy outcomes in fresh versus freeze-all ET for patients with endometriosis, and the results showed comparable outcomes between the two groups (14). We believe that the observed differences may associated with the type of embryos transferred. The study by Tan et al. exclusively included patients who received blastocyst transfers. Previous research has shown that there are significant differences between blastocysts and cleavage-stage embryos in terms of embryo development and synchronization with the endometrium, which could potentially impact pregnancy outcomes (39–41). Additionally, variations in controlled ovarian stimulation protocols and differences in the staging and classification of endometriosis may also influence pregnancy outcomes (8).

In women with adenomyosis, infertility may arise from local endometrial inflammation due to alterations in the eutopic endometrium. Immunologic changes, fibrosis, local hyperestrogenism, and dysperistalsis of the myometrium could be responsible for altered endometrial receptivity and implantation (42, 43). ART is an effective method to improve pregnancy outcomes in infertile adenomyosis-associated patients. In this meta-analysis, we summarized the IVF/ICSI pregnancy outcomes after different embryo transfer strategies. The results indicated that the pregnancy outcomes were comparable between groups. A retrospective cohort analysis involving 306 infertile adenomyosis-associated patients compared the IVF/ICSI outcomes after FET or fresh ET strategies. It also revealed that the rate of clinical pregnancy, miscarriage, and live birth were not significantly different between the two groups (15). However, Bourdon et al. revealed that the FET groups showed higher cumulative ongoing pregnancy rate and cumulative live birth rate versus fresh ET groups in infertile patients affected by adenomyosis (12). They considered that a freeze-all strategy could be beneficial by avoiding

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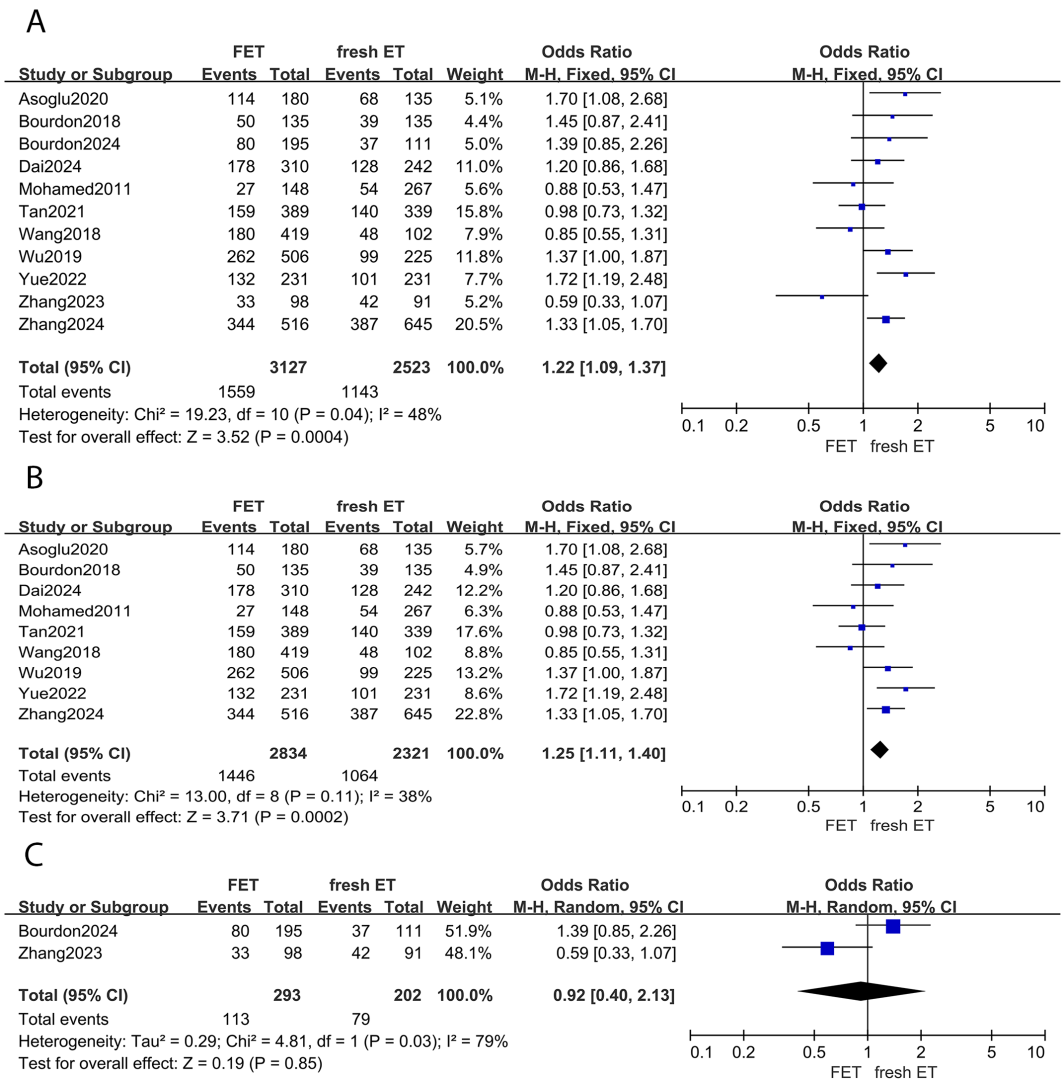


FIGURE 2
Forest plot for clinical pregnancy rate: **(A)** FET group versus fresh ET group in patients with endometriosis and adenomyosis; **(B)** FET group versus fresh ET group in patients with endometriosis; **(C)** FET group versus fresh ET group in patients with adenomyosis.

the negative effects of ovarian stimulation on an already impaired endometrium. In addition, FET strategy offers an opportunity for gonadotropin-releasing hormone agonist (GnRH-a) pretreatment before embryo transfer, which potentially improve uterine cavity morphology and create a more favorable endometrial environment (44). Due to the limitations of the study design and the small sample size, more rigorous and large-scale multicenter randomized controlled trials are needed to explore this topic.

Overall, it is essential to follow women not only during their journey to achieve pregnancy but throughout their entire reproductive lifespan. Patients with adenomyosis and EMS may experience long-term reproductive health challenges that extend beyond conception, necessitating ongoing monitoring and management to optimize their overall gynecological and reproductive well-being.

## Strengths and limitations

This review has several limitations and strengths that may have influenced the findings. The strengths of our study include the following: it is a large-scale meta-analysis based on 11 studies with a novel focus on patients with EMS and adenomyosis. We conducted a systematic literature search adhering to strict inclusion and exclusion criteria, ensuring a rigorous methodology. In addition to reporting the pregnancy outcomes of clinical pregnancy and live birth, we also addressed key reproductive outcomes, such as ectopic pregnancy, which is particularly significant in the context of assisted reproductive treatments. Despite the precautions taken, our study is subject to certain limitations. The quality of each of the included studies varies. Since there are some significant differences in baseline characteristics concerning the age of patients, type of

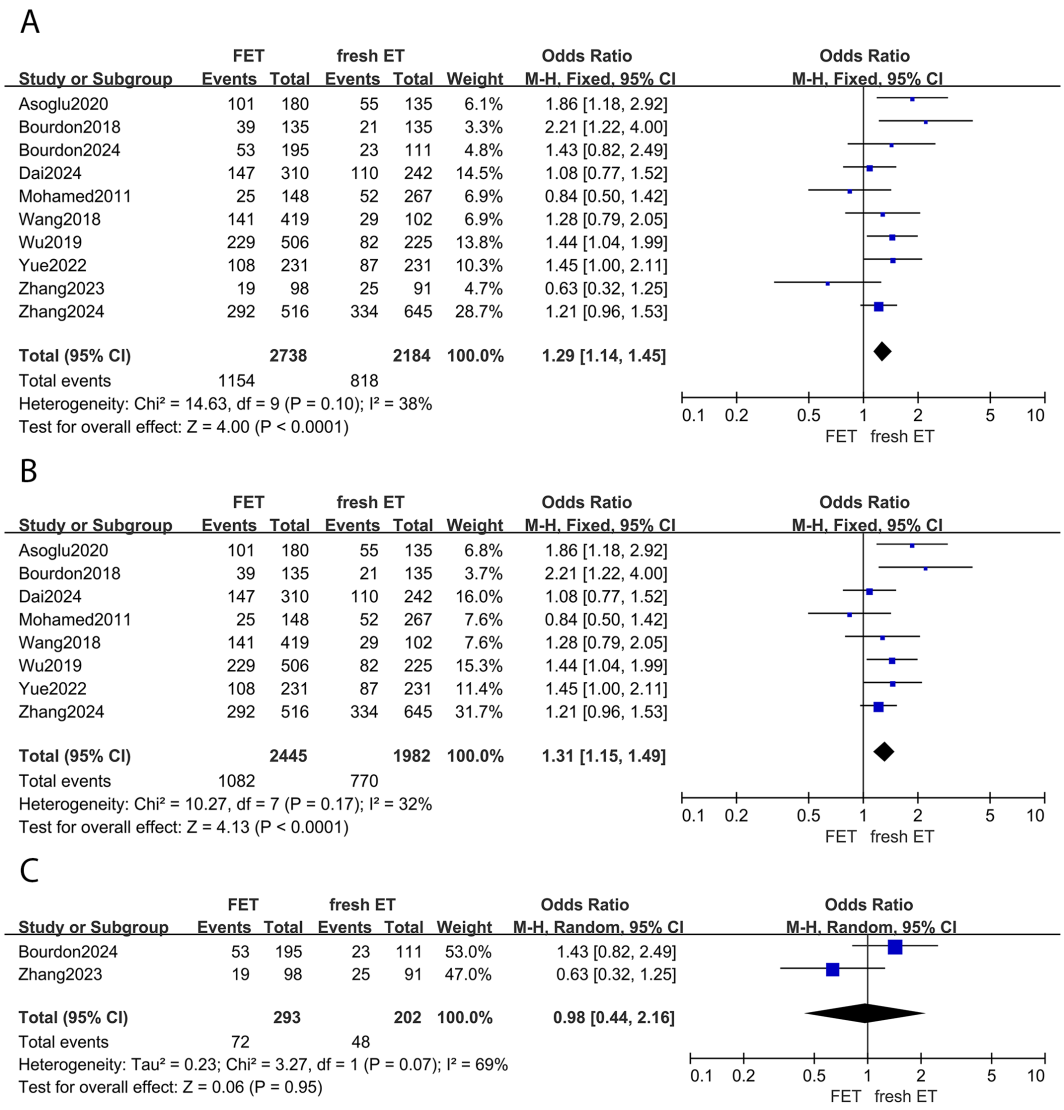


FIGURE 3
Forest plot for live birth rate: **(A)** FET group versus fresh ET group in patients with endometriosis and adenomyosis; **(B)** FET group versus fresh ET group in patients with endometriosis; **(C)** FET group versus fresh ET group in patients with adenomyosis.

infertility, ovarian reserve function and ovarian protocols, among others. it could introduce confounding factors into the results. Additionally, the included studies differed in their ascertainment of EMS and adenomyosis, without explicitly distinguishing between the two conditions. This lack of distinction may have led to the analysis of gestational outcomes in a mixed group. Furthermore, our study did not examine pregnancy complications or obstetric outcomes, which warrant further investigation.

In clinical practice, factors such as uterine environment, endometrial receptivity, and the patient's overall health should be carefully considered during IVF/ICSI cycles before embryo transfer. Based on current evidence, for women with EMS undergoing IVF/ICSI, clinicians might prioritize FET protocols over fresh embryo transfer. despite the potential for increased financial costs. Stimulation protocols should be adjusted to optimize oocyte retrieval and subsequent cryopreservation for FET cycles. For women with adenomyosis, pregnancy outcomes appear comparable between FET and fresh ET strategies. Clinicians should evaluate uterine abnormalities using imaging or hysteroscopy to guide decisions regarding endometrial preparation and the appropriate type of embryo transfer.

Heterogeneity exists across the studies due to substantial variations in study design, participant characteristics, and sample size. Although subgroup analyses for EMS and adenomyosis were performed to mitigate some of the heterogeneity, differences in the types of observational studies and patient characteristics, particularly variations in disease severity among EMS or adenomyosis patients, contribute to variability in baseline data, potentially impacting the outcomes. Consequently, randomized controlled trials focusing on different subtypes and severity levels of EMS and adenomyosis are warranted to better evaluate the efficacy and applicability of various embryo transfer strategies.

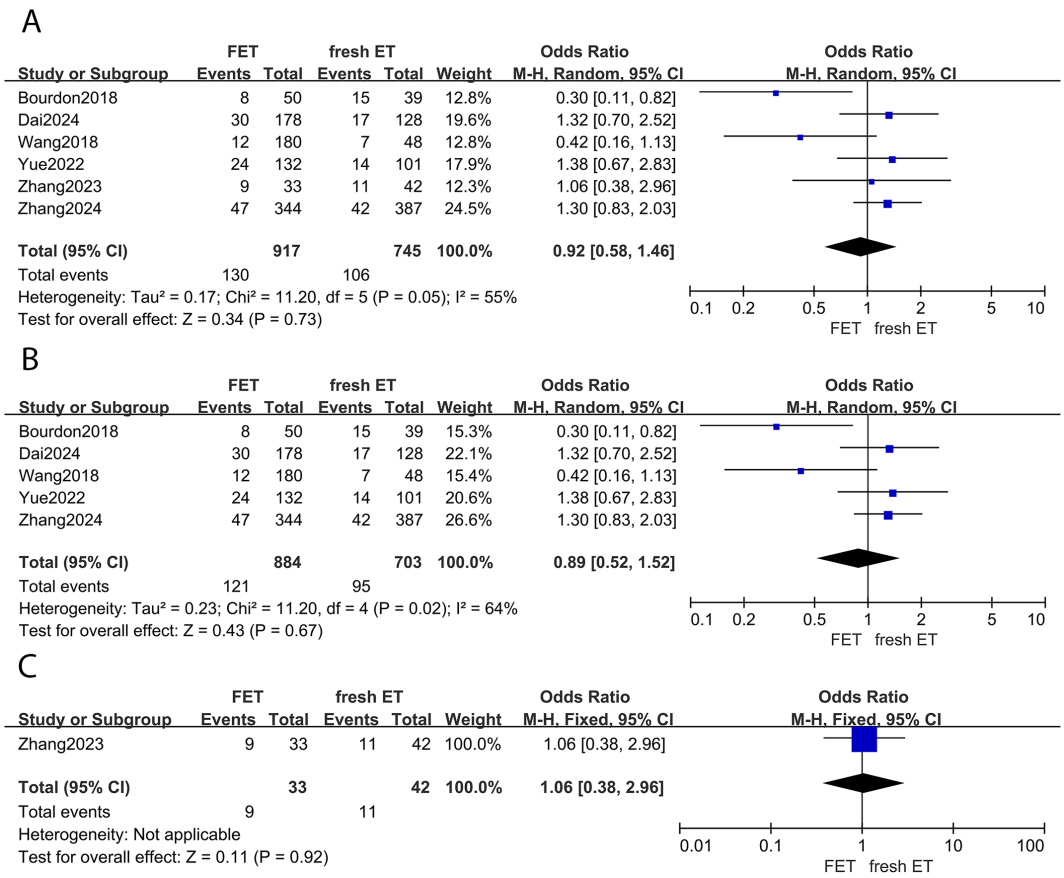


FIGURE 4
Forest plot for miscarriage rate: **(A)** FET group versus fresh ET group in patients with endometriosis and adenomyosis; **(B)** FET group versus fresh ET group in patients with endometriosis; **(C)** FET group versus fresh ET group in patients with adenomyosis.

TABLE 3 Quality assessment of cohort studies using the NOS.

| Study | Selection | | | | Comparability | Outcome | Follow-up long enough | Adequacy of follow up |
|---|---|---|---|---|---|---|---|---|
| | Representativeness of exposed cohort | Selection of non-exposed cohort | Ascertainment of exposure | Demonstration that outcome of interest was not present at start of study | Comparability of cohorts on the basis of the design or analysis | Assessment of outcome | | |
| Tan2021 (14) | – | * | * | * | * | * | * | * |
| Asoglu2020 (30) | – | * | * | * | ** | * | * | * |
| Wu2019 (26) | * | * | * | * | ** | * | * | * |
| Mohamed2011 (13) | – | * | * | * | – | * | * | * |
| Bourdon2018 (11) | * | * | * | * | * | * | * | * |
| Wang2018 (25) | – | * | * | * | * | * | * | * |
| Yue2022 (27) | * | * | * | * | ** | * | * | * |
| Zhang2024 (29) | * | * | * | * | * | * | * | * |

*(Continued)*

TABLE 3 Continued

| Study | Selection | | | | Comparability | Outcome | Follow-up long enough | Adequacy of follow up |
|---|---|---|---|---|---|---|---|---|
| | Representativeness of exposed cohort | Selection of non-exposed cohort | Ascertainment of exposure | Demonstration that outcome of interest was not present at start of study | Comparability of cohorts on the basis of the design or analysis | Assessment of outcome | | |
| Zhang2023 (15) | * | * | * | * | * | * | * | * |
| Bourdon2024 (12) | – | * | * | * | ** | * | * | * |
| Dai2024 (28) | * | * | * | * | * | * | * | * |

NOS, Newcastle–Ottawa Scale. * means one score, ** means two score.

## Conclusion

In IVF/ICSI, the FET strategy has been associated with more favorable reproductive outcomes compared to the fresh ET strategy in women with EMS, whereas in women with adenomyosis, pregnancy outcomes were comparable between the FET and fresh ET groups. However, the current evidence remains limited. More investigations are underscored to confirm our findings.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author/s.

## Author contributions

YH: Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. CL: Funding acquisition, Methodology, Writing – review & editing. DL: Funding acquisition, Software, Visualization, Writing – review & editing. LW: Writing – review & editing. WH: Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2025.1507252/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Funnel plot.

SUPPLEMENTARY FIGURE 2
Forest plot for implantation rate in the FET group versus fresh ET group in patients with endometriosis.

SUPPLEMENTARY FIGURE 3
Forest plot for ectopic pregnancy rate in the FET group versus fresh ET group in patients with endometriosis.

SUPPLEMENTARY FIGURE 4
Summary of findings (GRADE).

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# Review of Mendelian randomization studies on common male-specific diseases

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Although numerous Mendelian randomization studies on risk factors have been conducted in male medicine, a systematic synthesis of these findings is still lacking. This review searched relevant literature in PubMed and the Web of Science published before May 2024; systematically summarized the progress in the application of Mendelian randomization in male infertility, erectile dysfunction, prostate cancer, and prostatitis; summarized and classified the risk factors affecting men's health, such as the gut microbiota, modifiable risk factors and related diseases; and presented some problems and solutions that were presented in these studies. This information offers valuable insights into the etiology and pathogenesis of male-specific diseases.

KEYWORDS

**Mendelian randomization, male infertility, prostate cancer, erectile dysfunction, prostatitis**

## 1 Introduction

Numerous medical statistics show that the incidence of male-specific diseases is increasing, and men's health problems need urgent attention (1). Currently, the knowledge of risk factors associated with male-specific diseases needs to be further deepened. Mendelian randomization (MR) employs genetic variants highly correlated with exposure factors as instrumental variables (IVs) to ascertain the causal link between exposure and study outcomes. MR effectively reduces the impact of reverse causality and confounding factors. It also addresses the limitations of traditional medical statistics and epidemiological studies, offering a stronger foundation for identifying causal links between risk factors and disease risk (2). In recent years, many risk factor MR studies have been conducted in the field of male medicine, but there is a lack of systematic collation and summarization. In addition, a summary of the problems in the published literature is lacking. This article provides a systematic review of previous MR studies on risk factors for

male-specific diseases, with the aim of providing ideas for the etiologic study and scientific prevention of male-specific diseases.

# 2 Methods

## 2.1 Fundamentals

MR serves as a methodological tool in scientific inquiry aimed at elucidating causal connections between exposure factors and outcomes. It operates by leveraging genetic variants that are strongly associated with exposure factors as IVs. Unlike conventional observational epidemiological studies, MR draws on the principles of Mendelian inheritance. This approach can be likened to a naturally occurring randomized controlled trial (RCT), albeit conducted within the framework of genetic inheritance. This method reduces the impact of confounding factors found in observational studies and offers strong evidence. MR studies need to follow 3 core assumptions (2, 3): (1) the assumption of association, meaning the instrumental variable is strongly linked to the exposure factor; (2) the assumption of independence, meaning the instrumental variable is not related to confounding factors; (3) the assumption of exclusivity, meaning the instrumental variable affects the outcome only through the exposure factor The description of MR method is shown in Figure 1.

## 2.2 Common types of MR methods

The study of MR can be divided into single-sample Mendelian randomization, two-sample Mendelian randomization (TSMR), multivariate Mendelian randomization (MVMR), two-step Mendelian randomization, bidirectional Mendelian randomization, etc.

Single-sample Mendelian randomization means that the association between genetic variations and exposure, as well as the correlation between genetic variations and outcomes, is obtained in the same sample. In this research method, a correlation exists between the regression coefficients of the numerator and denominator due to confounders between exposure and outcome, and weak instrumental bias can lead to overestimation of the exposure–outcome association (4).

Two-sample Mendelian randomization (TSMR) refers to obtaining two types of data, namely, relationships between genetic variations and exposure as well as relationships between genetic variations and outcomes, from two nonoverlapping datasets. This method mitigates the effects of weak instrumental bias and has greatly expanded the application scope of MR studies (5).

Multivariate Mendelian randomization (MVMR) considers the causal effects of multiple exposures on one or more outcome variables. It enables the simultaneous evaluation of various causal pathways and helps to resolve confounding among these factors (6).

Two-step Mendelian Randomization investigates potential mediating mechanisms linking risk factors to outcomes (7).

Bidirectional Mendelian Randomization is employed to validate causal directionality when the direction of causal association between a risk factor and outcome remains ambiguous (8).

## 2.3 Common statistical methods

Commonly used MR statistical analyses include inverse variance weighting (IVW), Weighted median, MR-Egger regression, MR-PRESSO, etc (9).

Inverse variance weighting (IVW) is the standard method used to aggregate MR data, which integrates summary data from multiple genetic variants, weighting individual causal effect estimates by inverse variances to provide consistent and efficient causal inference under valid IVs without linkage disequilibrium (10). The weighted median approach calculates a weighted median estimate of causal effects derived from multiple IVs, with weights assigned based on the inverse of each estimate's sampling variance to prioritize precision. This method operates under the assumption that valid instruments collectively contribute over 50% of the total weighting scheme, ensuring robustness even in the presence of invalid IVs (11).

The MR-Egger method employs weighted regression to analyze the influence of associations between IVs and exposures on the associations between IVs and outcomes. This method incorporates an intercept term to quantify the average direct effect of IVs on the outcome, with its core assumption being the absence of correlated horizontal pleiotropy. To enhance the model's adaptability, the method can be extended by including a random effects term, which is used to analyze the over-dispersion of causal effects across different IVs, thereby modeling pleiotropic variation (12).MR-PRESSO conducts a global assessment to detect potential outliers within an IVW framework, followed by a localized analysis to pinpoint specific outliers. The method further quantifies their impact through a distortion test evaluating systematic bias in causal effect estimates (13).

## 2.4 Disease selection strategy

Reference to the International Classification of Diseases (ICD-11) published by the World Health Organization (WHO) and authoritative urological literature (Campbell-Walsh-Wein

**Abbreviations:** MR, Mendelian randomization; RCT, randomized controlled trial; TSMR, two-sample Mendelian randomization; IVW, inverse variance weighting; IVs, instrumental variables; MVMR, multivariable Mendelian randomization; GRS, genetic risk scores; MI, male infertility; BMI, body mass index; SNP, single nucleotide polymorphisms; HGF, hepatocyte growth factor; T2DM, type 2 diabetes mellitus; 25OHD, 25 hydroxyvitamin D; VD, vitamin D; PCa, prostate cancer; LTL, leukocyte telomere length; BCR, biochemical recurrence; MSP, microseminoprotein-beta; GWAS, genome-wide association studies; SD, standard deviation; LDL, low-density lipoprotein; ED, erectile dysfunction; CVD, cardiovascular disease; IS, ischemic stroke; HF, heart failure; IBD, inflammatory bowel disease.

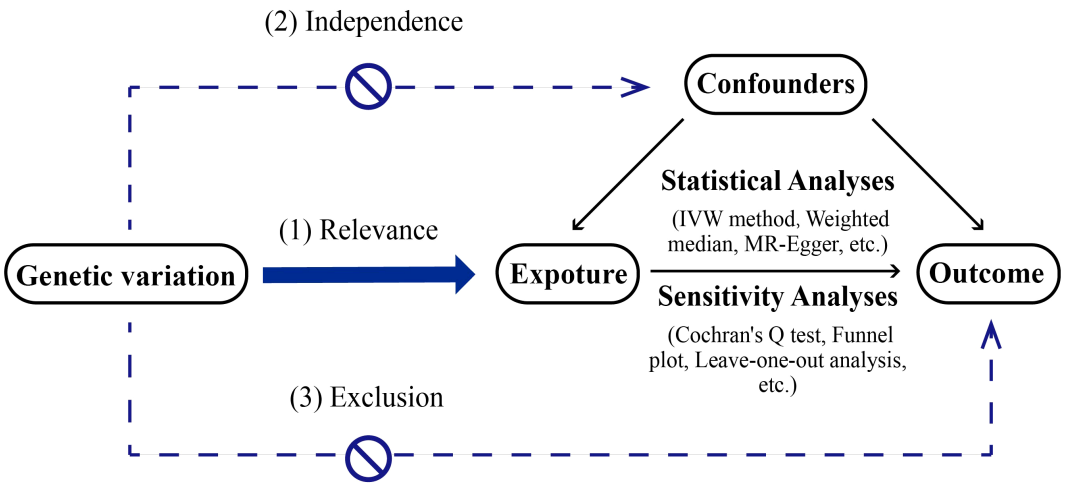


FIGURE 1
Diagram of mendelian randomization method.

Urology, volume 6) (14, 15), male diseases can be classified into the following categories: 1. Sexual health-related disorders: Including male infertility (MI), erectile dysfunction (ED), ejaculatory dysfunction (e.g., premature ejaculation [PE]), and sexually transmitted infections, etc. 2. Neoplasms of male genital organs: Such as prostate cancer (PCa), testicular tumors, and penile tumors. 3. Prostate diseases: Encompassing prostatitis, other prostatic disorders, and benign prostatic hyperplasia. 4. Structural abnormalities and congenital disorders of genitalia: Including hydrocele, testicular torsion, phimosis, and cryptorchidism, etc. 5. Inflammatory and infectious diseases: Such as orchitis and genital herpes. The selected 4 diseases in this article hold priority within the aforementioned disease categories:

1. For sexual health-related disorders, MI exhibits a high incidence rate. Globally, approximately 8-12% of couples suffer from infertility (16), with 50% of fertility issues attributable to male factors (17). Additionally, ED and PE are prevalent male sexual disorders in the general population. Population-based research indicates that 5% to 20% of males experience clinically significant ED (18), while PE occurs in about 30% of men aged 40–80 years (19). Despite PE being more prevalent than ED under the category of ejaculatory dysfunction, significant disparities in global incidence statistics and low healthcare-seeking rates among patients have resulted in far fewer research resources on PE compared to ED. Moreover, MR methodology-related studies on PE remain nearly absent. Therefore, this article selects ED as a research focus.
2. Among male genital organ tumors, PCa accounts for 29% of male cancers (20), ranking as the most common cancer among males in developed countries today (21). Given its representativeness in male genital tumors and abundant research data, PCa is emphasized in this article.
3. In prostate diseases, prostatitis is the most common urinary system disorder in males under 50 years old (15), while benign prostatic hyperplasia (BPH) represents the most prevalent benign tumor in elderly males (22). Comparatively, against the backdrop of population aging, research on BPH primarily stems from public health urgency, whereas prostatitis requires intensified mechanistic exploration due to its younger onset trends and chronic disease management challenges. MR methodology is particularly suitable for investigating the pathogenesis of prostatitis. Between these two conditions, this article prioritizes prostatitis as the study subject.
4. For structural/genital abnormalities and congenital diseases (e.g., hydrocele, testicular torsion, phimosis, and cryptorchidism), their relatively low incidence precludes their selection in this study. Among inflammatory and infectious diseases, prostatitis is chosen as a representative condition.

In summary, considering the article's scope limitations, this review focuses on 4 diseases characterized by high incidence rates, broad societal impact, significant impairment of male patients' quality of life, complex etiological mechanisms, and substantial existing literature.

## 2.5 Risk factor selection and classification strategy

This study employed a three-tiered criteria for risk factor selection: First, integration of authoritative guidelines and consensus statements, including the World Health Organization (WHO) framework for noncommunicable disease risks, the European Association of Urology (EAU) Guidelines on Sexual and Reproductive Health, and etiological evidence from classical urological literature (15). Second, prioritization of factors with high evidence strength validated by large-scale cohort studies (e.g., smoking, sedentary behavior) to ensure conclusion reliability.

Third, focus on clinically actionable risk indicators, particularly lifestyle-related factors (e.g., dietary patterns, exercise habits), as these can be modulated through public health policies or individual behavioral adjustments. This strategy balances scientific rigor with practical translational value, providing multidimensional evidence for male reproductive health management.

Based on these criteria, the risk factors included in this study are categorized into 6 types: Gut microbiota, circulatory substance (cytokines), related diseases, modifiable risk factors, drug targets, other risk factors (limited studies or irrelevant factors).

## 2.6 Search strategy and selection criteria of references

Original studies were identified by searching relevant articles in the PubMed and Web of Science databases through May 2024.The following terms were used to search: "mendelian randomization" or "genetic instrumental variable" or "genetic instrument", "male infertility" or "male sterility", "prostate cancer" or "prostatic carcinoma" or "prostatic cancer", "erectile dysfunction" or "impotence", "prostatitis" or "prostate inflammation", etc. Inclusion criteria: (1) Mendelian Randomization (MR) study design; (2) Genetic variants or Genetic Risk Scores (GRS) were used as Instrumental Variables (IVs) to analyze the relationship between exposure and outcome. Articles were excluded for the following reasons: reviews, non-original articles, non-human studies, study protocols, letters, conference abstracts, and articles for which the full text is not available. We finally included 122 articles and categorized them according to disease type, as shown in Tables 1–4.

# 3 Results

## 3.1 Male infertility

Infertility is defined as the inability to achieve pregnancy following 12 months of regular unprotected intercourse, 50% of infertility cases are attributable to male factors (23). The MR studies included in this article investigate the causal relationships between MI and risk factors such as gut microbiota, cytokines, related diseases, modifiable risk factors, and other factors.

### 3.1.1 Gut microbiota

Previous studies have demonstrated the association between gut microbes and MI but have not elucidated a causal relationship (24). The seven studies utilized various methods, including IVW, MR-Egger and maximum likelihood ratios, to evaluate the causal connection between the gut microbiota and MI risk.

The MR analyses indicated that certain microbes, including Anaerotruncus (25–28), Allisonella (27, 29), Barnesiella, Intestinibacter and Lactococcus (27) are positively associated with MI risk. In contrast, Bacteroidaceae (26, 28–30), Bacteroides (25–30), Romboutsia (27, 29) and Ruminococcaceae (Ruminococcaceae, genus NK4A2140group, genus UCG011) (26–28, 30, 31) are protective against the development of MI.

Moreover, Li TZ et al. identified the family Enterobacteriaceae and the order Enterobacteriales as being linked to a low risk of MI (29). An MR study by Xi YJ et al. indicated that Eubacterium venereum and Eubacterium rectale have protective effects on MI, whereas Eubacterium oxidoreducens contribute to MI risk (31). Using TSMR analysis, Ma S-C et al. reported that Bacteroideae, Bacteriaceae, Pasteurella, Clostridium rectalis are associated with MI (30).

### 3.1.2 Cytokines

Zhang L et al. used MR methods such as IVW, MR-Egger and weighted median analyses to analyze the genetic association between cytokines and the risk of MI and concluded that the cytokines hepatocyte growth factor (HGF), IL-2ra, and RANTES potentially increase MI risk (32). Zou H et al. found that HGF reduced the risk of MI, and monocyte chemotactic protein 3 increased the risk of MI (25).

### 3.1.3 Related diseases

Using MR analysis, Zhu XB et al. reported that in type 2 diabetes mellitus (T2DM) can cause ED and MI a European population (33, 34). Two MR studies showed no significant association between COVID-19 and MI (35, 36). Wang X et al. proposed that ulcerative colitis may increase the risk of MI (37). Chen X et al. 's results found that mood disorders and attention deficit hyperactivity disorder were positively correlated with MI, whereas obsessive-compulsive disorder was negatively associated with MI (38).

### 3.1.4 Modifiable risk factors

Body mass index (BMI), body fat percentage, alcohol consumption and smoking are modifiable lifestyle factors linked to various health outcomes. Wentao et al. employed TSMR analyses to investigate the causal impacts of 22 diverse risk factors on MI and female infertility. Their findings indicated that BMI, body fat percentage, and alcohol consumption contribute to the risk of MI (39, 40). Greater smoking intensity was not strongly associated with MI according to MR analysis (41). The study of Chen X et al. found that coffee intake and cooked vegetable intakes increased the risk of MI (42). These insights underscore that the multifaceted interplay between lifestyle factors and health outcomes, moderate alcohol consumption, maintaining a healthy body weight and body fat, and practicing good lifestyle habits may help reduce MI risk and improve the quality of fertility.

### 3.1.5 Other factors

Yuan et al. observed that for every unit increase in genetically predicted 25 hydroxyvitamin D (25OHD) levels, there was a corresponding decrease in the risk of MI (43). This finding underscores the potential importance of vitamin D (VD) in mitigating the risk of MI. Therefore, the clinical use of VD supplements that increase serum 25OHD levels may have

TABLE 1 Application of Mendelian randomization in male infertility.

| Disease | Reference no. | Exposure | SNPs, n | OR (95%CI) | P-value | Sample size | | Correlation | Population | MR method |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Cases | Control | | | |
| **Male infertility** | PMID: 36235694 | BMI | 35 | 1.24 (1.09-1.40) | 0.001 | 680 | 72,799 | Risk factor | European | IVW |
| | | Body fat percentage | 499 | 1.73 (1.13-2.64) | 0.011 | | | Risk factor | | |
| | | Alcohol consumption | 50 | 6.58 (1.20-36.14) | 0.03 | | | Risk factor | | |
| | PMID: 34668019 | BMI | 896 | 1.26 (1.08-1.48) | 0.003 | 26,252 | | Causal role | | IVW |
| | PMID: 35562204 | Smoking | 378 | 1.10 (0.78-1.56) | 0.179 | 3,275 | 23,821 | No robust evidence | European | IVW |
| | PMID: 38715795 | Coffe intake | 40 | 3.70 (1.03-13.21) | 0.044 | 680 | 72,799 | Positive correlation | European | |
| | | Cooked vegetable intakes | 17 | 54.79 (2.90-1030.55) | 0.008 | | | Positive correlation | | |
| | PMID: 38711980 | Anaerotruncus | 13 | 1.81 (1.18-2.77) | 0.006 | 1,271 | 119,297 | Increased the risk | European | IVW |
| | | Bacteroides | 8 | 0.57 (0.33-0.96) | 0.036 | | | Decreased the risk | | |
| | | HGF | 7 | 0.50 (0.35-0.71) | <0.001 | | | Reduced the risk | | |
| | | MCP-3 | 5 | 1.29 (1.09-1.53) | 0.004 | | | Increased the risk | | |
| | PMID: 38489097 | Bacteroidaceae | 8 | 0.54 (0.31-0.96) | 0.035 | 1,271 | 119,297 | Decreased the risk | European | IVW |
| | | Bacteroides | 8 | 0.54 (0.31-0.96) | 0.035 | | | Decreased the risk | | |
| | | RuminococcaceaeNK4A214group | 13 | 0.56(0.36-0.89) | 0.014 | | | Decreased the risk | | |
| | | RuminococcaceaeUCG011 | 8 | 0.76 (0.59-0.99) | 0.042 | | | Decreased the risk | | |
| | | Anaerotruncus | 13 | 1.96 (1.13-3.40) | 0.016 | | | Increased the risk | | |
| | PMID: 37822739 | Anaerotruncus | 13 | 1.96 (1.13-3.40) | 0.016 | 1,128 | 110,070 | Positively associated | European | IVW |
| | | Bacteroides | 9 | 0.58 (0.34-0.99) | 0.048 | | | Negatively associated | | |
| | | Ruminococcaceae (NK4A214group) | 13 | 0.57 (0.36-0.89) | 0.014 | | | Negatively associated | | |

*(Continued)*





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TABLE 1 Continued

| Disease | Reference no. | Exposure | SNPs, n | OR (95%CI) | P-value | Sample size | | Correlation | Population | MR method |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Cases | Control | | | |
| | | Ruminococcaceae (UCG011) | 8 | 0.76 (0.59-0.99) | 0.042 | | | Negatively associated | | |
| | | Romboutsia | 13 | 0.67 (0.43-1.03) | 0.067 | | | Negatively correlated | | |
| | | Lactococcus | 9 | 1.29 (0.97-1.72) | 0.085 | | | Positively associated | | |
| | | Allisonella | 8 | 1.28 (0.96-1.71) | 0.091 | | | Positive | | |
| | | Anaerotruncus | 13 | 1.96 (1.13-3.40) | 0.016 | | | Positive | | |
| | | Intestinibacter | 15 | 1.51 (0.96-2.39) | 0.074 | | | Positive | | |
| | | Anaerofilum | 10 | 0.70 (0.46-1.07) | 0.097 | | | Negative | | |
| | | Barnesiella | 14 | 1.41 (0.86-2.31) | 0.175 | | | Positive | | |
| | PMID: 37764164 | Bacteroidaceae | 8 | 0.57 (0.33-0.96) | 0.036 | 1,271 | 119,297 | Positive | European | IVW |
| | | Bacteroides | 8 | 0.57 (0.33-0.96) | 0.036 | | | Positive | | |
| | | Ruminococcaceae NK4A214 group | 13 | 0.61 (0.39-0.97) | 0.037 | | | Positive | | |
| | | Anaerotruncus | 13 | 1.81 (1.14-2.87) | 0.011 | | | Causal association | | |
| | PMID: 37454180 | Allisonella | 8 | 1.32 (1.02-1.72) | 0.03 | 994 | 100,050 | Positive | European | IVW |
| | | Bacteroidaceae | 7 | 0.44 (0.23-0.83) | 0.01 | | | Negative | | |
| | | Bacteroides | 7 | 0.44 (0.23-0.83) | 0.01 | | | Negative | | |
| | | Enterobacteriales | 7 | 0.47 (0.23-0.95) | 0.03 | | | Negative | | |
| | | Romboutsia | 14 | 0.64 (0.42-0.96) | 0.03 | | | Negative | | |
| | | Enterobacteriaceae | 7 | 0.47 (0.23-0.95) | 0.03 | | | Negative | | |
| | PMID: 38619404 | Pasteurellales | 17 | 0.67 (0.47-0.94) | 0.022 | 680 | 72,799 | Decreased the risk | European | IVW |
| | | Bacteroidaceae | 12 | 0.49 (0.27-0.90) | 0.022 | | | Decreased the risk | | |
| | | Pasteurellale | 17 | 0.67 (0.47-0.94) | 0.022 | | | Decreased the risk | | |
| | | Bacteroide | 12 | 0.49 (0.27-0.91) | 0.022 | | | | | |

*(Continued)*





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TABLE 1 Continued

| Disease | Reference no. | Exposure | SNPs, n | OR (95%CI) | P-value | Sample size | | Correlation | Population | MR method |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Cases | Control | | | |
| | | | | | | | | Decreased the risk | | |
| | | Eubacterium rectale group | 12 | 0.45 (0.26-0.78) | 0.004 | | | Decreased the risk | | |
| | | RuminococcaceaeNK4A214group | 16 | 0.55 (0.32-0.95) | 0.033 | | | Decreased the risk | | |
| | PMID: 37605651 | Eubacterium oxidoreducens | 5 | 2.05 (1.20-3.49) | 0.008 | 680 | 72,799 | Risk factor | European | IVW |
| | | Lactococcus | 9 | 1.45 (1.01-2.06) | 0.042 | | | Risk factor | | |
| | | Eubacterium ventriosum | 9 | 0.44 (0.22-0.87) | 0.018 | | | Protective factor | | |
| | | Eubacterium rectale | 8 | 0.31 (0.15-0.64) | 0.002 | | | Protective factor | | |
| | | Ruminococcaceae NK4A214 | 13 | 0.54 (0.29-0.99) | 0.045 | | | Protective factor | | |
| | PMID: 36593707 | 25OHD | 99 | 0.62 (0.44-0.89) | 0.01 | 825 | 85,722 | Protective factor | European | IVW |
| | PMID: 38479056 | HGF | 6 | 3.77 (1.80-7.91) | 0.0004 | 1,271 | 11,9297 | Positively associated | European | IVW |
| | | IL-2ra | 17 | 1.29 (1.11-1.49) | 0.001 | | | Positively associated | | |
| | | RANTES | 1 | 2.59 (1.37-4.91) | 0.003 | | | Positively associated | | Wald ratio |
| | | SCF | 5 | 0.40 (0.18-0.88) | 0.023 | | | Positively associated | | |
| | PMID: 38152129 | T2DM | 58 | 0.77 (0.60-0.98) | 0.034 | 680 | 72,799 | Significant causal relationship | European | IVW |
| | PMID: 38529400 | T2DM | 62 | 0.82 (0.70-0.97) | 0.017 | 680 | 72,799 | Substantial causal relationship | European | IVW |
| | DOI: 10.22514/jomh.2024.009 | COVID-19 | 12 | 0.86 (0.65-1.15) | 0.308 | 680 | 72,799 | No causal effect | European | IVW |
| | PMID: 38457599 | COVID-19 | 5 | 0.47 (0.16-1.41) | 0.178 | 680 | 72,799 | No clear causal relationship | European | IVW |
| | PMID: 38814907 | Ulcerative colitis | 86 | 1.13 (1.00-1.26) | 0.046 | 680 | 72,799 | Positive | European | IVW |

*(Continued)*





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TABLE 1 Continued

| Disease | Reference no. | Exposure | SNPs, n | OR (95%CI) | P-value | Sample size | | Correlation | Population | MR method |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Cases | Control | | | |
| | PMID: 38699446 | Mood disorders | 35 | 1.45 (1.01-2.08) | 0.044 | 680 | 72,799 | Positive | European | IVW |
| | | Attention deficit hyperactivity disorder | 30 | 0.83 (0.34-2.02) | 0.686 | | | Positive | | |
| | | Obsessive-compulsive disorder | 13 | 0.93 (0.68-1.26) | 0.637 | | | Negative | | |
| | PMID: 38456015 | Chronotype | 147 | 0.88 (0.42-1.83) | 0.725 | 680 | 72,799 | No evidence | European | IVW |
| | | Sleep duration | 64 | 0.99 (0.26-3.77) | 0.994 | | | No evidence | | |
| | | Insomnia | 38 | 0.34 (0.05-2.49) | 0.29 | | | No evidence | | |
| | | Snoring | 39 | 0.53 (0.03-9.40) | 0.667 | | | No evidence | | |
| | | Dozing | 30 | 3.62 (0.19-70.21) | 0.395 | | | No evidence | | |
| | | Daytime nap | 93 | 2.64 (0.67-10.40) | 0.164 | | | No evidence | | |
| | | Oversleeping | 31 | 1.55 (0.25-9.58) | 0.635 | | | No evidence | | |
| | | Undersleeping | 25 | 4.28 (0.44-43.22) | 0.206 | | | No evidence | | |
| | PMID: 38512957 | LTL | 135 | 1.27 (0.84-1.92) | 0.261 | 680 | 72,799 | No causal associations | European | IVW |
| | PMID: 34778177 | Educational Attainment | 1,271 | 0.79 (0.52-1.20) | 0.269 | 680 | 72,799 | Not related | European | IVW |





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TABLE 2 Application of Mendelian randomization in erectile dysfunction.

| Disease | Reference no. | Exposure | SNPs, n | OR | P-value | Sample size | | Correlation | Population | MR method |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Cases | Control | | | |
| **Erectile dysfunction** | PMID: 36844727 | Coronary heart disease | 43 | 1.09 (1.01-1.18) | 0.022 | 6,175 | 217,630 | Increase the risks | European | IVW |
| | | Heart failure | 9 | 1.36 (1.07-1.74) | 0.013 | | | Increase the risks | | |
| | | Ischemic heart disease | 31 | 3.22 (0.64-16.22) | 0.156 | | | No causal association | | |
| | | Atrial fibrillation | 139 | 1.03 (0.97-1.08) | 0.312 | | | No causal association | | |
| | PMID: 36891666 | IS | 9 | 1.34 (1.08-1.21) | 0.007 | 6,175 | 223,805 | Causally associated | European | IVW |
| | | HF | 9 | 1.36 (1.07-1.74) | 0.013 | | | Causally associated | | |
| | | Coronary heart disease | 43 | 1.15 (1.09-1.18) | 0.022 | | | Causally associated | | |
| | PMID: 37363097 | Hypertension | 154 | 1.10 (1.02-1.20) | 0.017 | 6,175 | 217,630 | Increases the risk | European | IVW |
| | PMID: 37025676 | Hypertension | 67 | 3.83 (2.30-6.38) | 0.0085 | 6,175 | 223,805 | Positive causal link | European | IVW |
| | DOI: 10.22514/jomh.2023.084 | High blood pressure | 149 | 1.66 (1.13-2.45) | 0.001 | 6,175 | 223,805 | Increased odds | European | IVW |
| | PMID: 37782322 | Coronary artery disease | 88 | 1.09 (1.02-1.16) | 0.013 | 6,175 | 217,630 | Causally associated | European | IVW |
| | | Coronary heart disease | 42 | 1.07 (1.01-1.13) | 0.017 | | | Causally associated | | |
| | | Myocardial infection | 87 | 1.09 (1.02-1.17) | 0.011 | | | Causally associated | | |
| | | Atrial fibrillation | 216 | 1.06 (1.00-1.12) | 0.04 | | | Causally associated | | |
| | PMID: 37833702 | HF | 30 | 1.17 (0.99-1.39) | 0.074 | 6,175 | 217,630 | No significant causal relationship | European | IVW |
| | | Coronary heart disease | 61 | 1.08 (0.99-1.17) | 0.068 | | | No significant causal relationship | | |
| | PMID: 36891666 | IS | 9 | 1.34 (1.08-1.66) | 0.007 | 6,175 | 223,805 | Causally associated | European | IVW |
| | | HF | 9 | 1.36 (1.07-1.74) | 0.013 | | | Causally associated | | |
| | | Coronary heart disease | 43 | 1.09 (1.01-1.18) | 0.022 | | | Causally associated | | |
| | PMID: 34842357 | T2DM | 137 | 1.15 (1.05-1.25) | 0.001 | 6,175 | 223,805 | Direct causal effect | European | IVW |
| | PMID: 30583798 | T2DM | 103 | 1.11 (1.05-1.17) | 3.50E-04 | 6,175 | 223,805 | Causally implicated | European | IVW |
| | PMID: 36313469 | Depression | 73 | 1.68 (1.38-2.05) | < 0.001 | 6,175 | 223,805 | Increases the incidence | European | IVW |

*(Continued)*





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TABLE 2 Continued

| Disease | Reference no. | Exposure | SNPs, n | OR | P-value | Sample size | | Correlation | Population | MR method |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Cases | Control | | | |
| | PMID: 36997981 | Major depression | 37 | 1.53 (1.19-1.96) | 0.001 | 6,175 | 223,805 | Causally related | European | IVW |
| | | Bipolar disorder | 34 | 0.95 (0.87-1.04) | 0.36 | | | No causal impact | | |
| | PMID: 37541893 | Major depressive disorder | 44 | 1.32 (1.08-1.62) | < 0.001 | 6,175 | 217,630 | Higher risk | European | IVW |
| | PMID: 35819009 | COVID-19 | 6 | 1.24 (1.04-1.46) | < 0.05 | 6,175 | 217,630 | Elevated risk | European | IVW |
| | PMID: 38085233 | COVID-19 | 91 | 1.07 (1.01-1.13) | < 0.06 | 6,175 | 217,630 | Correlated | European | IVW |
| | PMID: 37415973 | COVID-19 | 7 | 1.09 (1.03-1.16) | 0.004 | 6,175 | 217,630 | Increased risk | European | IVW |
| | PMID: 38264202 | Ulcerative colitis | 37 | 0.96 (0.91-1.01) | 0.08 | 6,175 | 223,805 | No evidence | European | IVW |
| | | Crohn's disease | 54 | 1.04 (0.99-1.08) | 0.091 | | | No evidence | | |
| | PMID: 38784037 | IBD | 62 | 1.11 (1.02-1.21) | 0.019 | 1,154 | 94,024 | Increased risk | European | IVW |
| | | Ulcerative colitis | 35 | 1.02 (0.92-1.14) | 0.679 | | | No significant evidence | | |
| | | Crohn's disease | 51 | 1.09 (1.02-1.17) | 0.014 | | | Increased risk | | |
| | PMID: 38272986 | IBD | 62 | 1.11 (1.02-1.21) | 0.019 | 1,154 | 94,024 | Increased the incidence | European | IVW |
| | | Crohn's disease | 52 | 1.09 (1.02-1.16) | 0.016 | | | Increased the incidence | | |
| | | Ulcerative colitis | 36 | 1.02 (0.92-1.13) | 0.743 | | | No causal effect | | |
| | PMID: 37928685 | Lachnospiraceae | 17 | 1.27 (1.05-1.52) | 0.012 | 6,175 | 217,630 | Risk factor | European | IVW |
| | | Senegalimassilia | 5 | 1.32 (1.06-1.64) | 0.012 | | | Risk factor | | |
| | | Lachnospiraceae NC2004 group | 8 | 1.20 (1.01-1.41) | 0.03 | | | Risk factor | | |
| | | Tyzzerella3 | 13 | 1.14 (1.02-1.27) | 0.024 | | | Risk factor | | |
| | | Oscillibacter | 13 | 1.20 (1.04-1.39) | 0.016 | | | Risk factor | | |
| | | Ruminococcaceae UCG013 | 12 | 0.77 (0.62-0.97) | 0.023 | | | Protective effect | | |
| | PMID: 38311371 | Lachnospiraceae | 27 | 1.27 (1.05-1.52) | 0.01 | 6,175 | 223,805 | Higher risk | European | IVW |
| | | LachnospiraceaeNC2004 group | 10 | 1.17 (1.01-1.37) | 0.04 | | | Higher risk | | |
| | | Oscillibacter | 17 | 1.17 (1.02-1.35) | 0.03 | | | Increase the risk | | |
| | | Senegalimassilia | 8 | 1.32 (1.06-1.64) | 0.01 | | | Increase the risk | | |

*(Continued)*





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TABLE 2 Continued

| Disease | Reference no. | Exposure | SNPs, n | OR | P-value | Sample size | | Correlation | Population | MR method |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Cases | Control | | | |
| | | Tyzzerella3 | 14 | 1.14 (1.02-1.27) | 0.02 | | | Increase the risk | | |
| | | RuminococcaceaeUCG013 | 14 | 0.77 (0.61-0.96) | 0.02 | | | Protective effect | | |
| | PMID: 38390206 | LDL Receptor agonists | 42 | 0.76 (0.56-0.95) | 0.005 | 6,175 | 223,805 | Reduced risk | European | IVW |
| | | Lipoprotein Lipase agonists | 56 | 0.91 (0.78-1.04) | 0.138 | | | Reduced risk | | |
| | | Apolipoprotein C-III inhibitors | 37 | 0.90 (0.77-1.02) | 0.087 | | | Reduced risk | | |
| | | Apolipoprotein B-100 inhibitors | 29 | 1.03 (0.75-1.32) | 0.816 | | | Elevated risk | | |
| | PMID: 38741592 | Atorvastatin use | | 23.91 | 0.02 | 6,175 | 217,630 | Increased risk | European | IVW |
| | PMID: 38260164 | Aspirin use | 9 | 20.896 (2.077-210.2) | 0.01 | 6,175 | 217,630 | Predisposing factor | European | IVW |
| | PMID: 38827362 | Fibroblast growth factor 5 | 503 | 1.05 (1.01-1.11) | 0.0307 | 2,205 | 164,104 | Increased risk | European | IVW |
| | | IL-22 receptor subunit alpha-1 | 21 | 1.28 (1.01-1.62) | 0.0406 | | | Increased risk | | |
| | | Protein S100-A12 | 23 | 1.22 (1.02-1.47) | 0.0314 | | | Increased risk | | |
| | | TNF-related activation-induced cytokine | 43 | 0.88 (0.78-0.99) | 0.048 | | | Decreased risk | | |
| | PMID: 38680495 | Interferon-inducible protein-10 | 9 | 1.27 (1.01-1.60) | 0.043 | 2,205 | 164,104 | Elevate the risk | European | IVW |
| | | Interleukin-1 receptor antagonist | 8 | 0.77 (0.60-0.98) | 0.037 | | | Reduce the risk | | |
| | PMID: 37236543 | BMI | 834 | 1.23 (1.11-1.37) | <0.001 | 6,175 | 217,630 | Increased risk | European | IVW |
| | | Waist circumference | 278 | 1.30 (1.13-1.49) | <0.002 | | | Increased risk | | |
| | | Trunk fat mass | 632 | 1.13 (1.01-1.36) | 0.035 | | | Increased risk | | |
| | | Whole body fat mass | 630 | 1.18 (1.06-1.37) | 0.003 | | | Increased risk | | |
| | PMID: 37082877 | BMI | | 1.84 (1.05-1.36) | 0.006 | 6,175 | 223,805 | Increased risk | European | IVW |
| | PMID: 35692403 | Snoring | 19 | 3.45 (1.68-7.09) | <0.001 | 6,175 | 217,630 | Increased risk | European | IVW |
| | PMID: 38505341 | Ever smoked | 16 | 5.89 (1.60-21.94) | 0.01 | 6,175 | 217,630 | Increased risk | European | IVW |
| | | Alcohol consumption | 38 | 1.50 (1.05-2.14) | 0.03 | | | Increased risk | | |
| | | BMI | 444 | 1.18 (1.06-1.31) | 0.003 | | | Increased risk | | |
| | | Earlier age at first intercours | 260 | 0.66 (0.55-0.78) | 2.50E-06 | | | Reduced risk | | |

*(Continued)*





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TABLE 2 Continued

| Disease | Reference no. | Exposure | SNPs, n | OR | P-value | Sample size | | Correlation | Population | MR method |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Cases | Control | | | |
| | PMID: 35946227 | Insomnia | 196 | 1.15 (1.07-1.23) | <0.001 | 6,175 | 217,630 | Increased the risk | European | IVW |
| | PMID: 38131625 | Insomnia | 33 | 3.44 (1.59-7.44) | 0.001 | 6,175 | 217,630 | Higher risk | European | IVW |
| | PMID: 33548002 | TSH | 60 | -0.00 (-0.001, 0.001) | 0.914 | 166,988 | | No evidence | European | IVW |
| | PMID: 37581767 | Periodontal disease | 6 | 1.07 (0.96-1.20) | 0.22 | 6,175 | 217,630 | No evidence | European | IVW |

TABLE 3 Application of Mendelian randomization in prostate cancer.

| Disease | Reference no. | Exposure | SNPs, n | OR (95%CI) | P-value | Sample Size | | Correlation | Population | MR method |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Cases | Control | | | |
| **Prostate cancer** | PMID: 31802111 | Physical activity | 2 | 0.49 (0.33-0.72) | 3.00E-04 | 79,148 | 61,106 | Inversely associated | European | IVW |
| | | Serum iron levels | 5 | 0.92 (0.86-0.98) | 0.007 | | | Inversely associated | | |
| | | BMI | 535 | 0.90 (0.84-0.97) | 0.003 | | | Inversely associated | | |
| | | Circulating monounsaturated fat | 5 | 1.11 (1.02-1.20) | 0.02 | | | Circulating monounsaturated fat | | |
| | PMID: 26387087 | Increased height | 179 | 0.99 (0.96-1.00) | 0.23 | 20,848 | 20,214 | Not associated | European | GRS+ Logistic regression |
| | | BMI | 32 | 0.98 (0.97-1.01) | 0.07 | | | Decreased risk | | |
| | PMID: 37305903 | Unfavourable adiposity | 27 | 0.85 (0.61-1.19) | 0.35 | 85,554 | 91,972 | No strong evidence | European | IVW |
| | | Favourable adiposity | 34 | 0.80 (0.53-1.23) | 0.32 | | | No strong evidence | | |
| | | BMI | 506 | 0.97 (0.88-1.08) | 0.59 | | | No strong evidence | | |
| | PMID: 32701947 | Smoking | 361 | 0.90 (0.80-1.02) | 0.104 | 79,148 | 61,106 | Nonsignificant inverse association | European | IVW |
| | PMID: 37237487 | Smoking | 108 | 1.95 (1.09-3.50) | 0.027 | 79,148 | 61,106 | Risk factor | European | IVW |
| | PMID: 33193711 | Shorter LTL | 17 | 0.94 (0.91-0.98) | 0.005 | 27,641 | 307,395 | Decreased risk | European | IVW |
| | PMID: 31981976 | Shorter LTL | 10 | HR:1.73 (1.08-2.78) | 0.021 | 1,889 | | High Gleason scores, worse prognosis | European | IVW |

*(Continued)*





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TABLE 3 Continued

| Disease | Reference no. | Exposure | SNPs, n | OR (95%CI) | P-value | Sample Size | | Correlation | Population | MR method |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Cases | Control | | | |
| | PMID: 37352282 | Longer LTL | 134 | 1.37 (1.25-1.50) | 2.84E-11 | 79,148 | 61,106 | Increased risk | European | IVW |
| | PMID: 31089709 | MSP | 1 | 0.65 (0.51-0.84) | 0.001 | 1,871 | 1,871 | Inversely associated | European | IVW |
| | PMID: 38594418 | Zinc | 3 | 1.06 (1.01-1.12) | 0.026 | 79,194 | 61,112 | Weak causal effect | European | IVW |
| | PMID: 36923697 | Zinc | 2 | 1.06 (1.00-1.12) | 0.04 | 79,148 | 61,106 | Increased risk | European | IVW |
| | PMID: 34617559 | Phosphorus | 125 | 1.19 (1.09-1.31) | 1.82E-04 | 79,148 | 61,106 | Increased risk | European | IVW |
| | PMID: 36561528 | Iron | 3 | 0.91 (0.84-0.99) | 0.035 | 79,148 | 61,106 | Decreased risk | | IVW |
| | PMID: 35085228 | lipoprotein A | 10 | 1.07 (0.91-1.25) | 0.431 | 79,166 | 61,106 | Increased risk | European | IVW |
| | PMID: 26992435 | LDL | 11 | 1.50 (0.92-2.46) | 0.11 | 22,249 | 22,133 | Weak evidence | European | GRS+ Logistic regression |
| | PMID: 36595504 | PCSK9 | 28 | 0.85 (0.76-0.96) | 0.009 | 79,194 | 61,112 | Lower risk | European | IVW |
| | PMID: 35151363 | PCSK9 | 8 | 0.81 (0.73-0.90) | 4.52E-05 | 79,148 | 61,106 | Reduced risk | European | IVW |
| | PMID: 36316671 | Triglyceride | 48 | 1.002 (1.000-1.004) | 0.016 | 3436 | 459,574 | Increased risk | European | IVW |
| | PMID: 35296245 | Aspartate | 4 | 1.04 (1.00-1.08) | 0.034 | 79,148 | 61,106 | Positively associated | European | IVW |
| | PMID: 36330075 | Alanine | 16 | 1.16 (1.01-1.33) | 0.037 | 79,148 | 61,106 | Increased risk | European | IVW |
| | | Aminotransferase | 237 | 0.43 (0.27-0.68) | 3.28E-04 | | | Inversely associated | | |
| | DOI: 10.21203/rs.3.rs-2815251/v1 | Mean corpuscular volume | 378 | 0.95 (0.90-0.98) | 0.004 | 79,148 | 61,106 | Decreased risk | European | IVW |
| | | Mean corpuscular hemoglobin | 366 | 0.94 (0.91-0.99) | 0.019 | | | Decreased risk | | |
| | | Mean corpuscular hemoglobin concentration | 102 | 0.89 (0.81-0.98) | 0.023 | | | Decreased risk | | |
| | PMID: 35012533 | Macrophage inflammatory protein 1a | 35 | 1.06 (1.03-1.10) | 5.62E-04 | 79,148 | 61,106 | Positive association | European | IVW |
| | | Vascular endothelial growth factor | 21 | 0.86 (0.79-0.93) | 2.28E-04 | | | Inverse association | | |
| | PMID: 36733309 | IL-6 | 2 | 1.12 (1.07-1.17) | 6.61E-07 | 79,148 | 61,106 | Increased risk | European | IVW |
| | | IL-1ra | 4 | 0.92 (0.89-0.96) | 1.58E-05 | | | Reduced risk | | |
| | PMID: 36482455 | Bioavailable testosterone | 52 | 1.17 (1.09-1.26) | 2.51E-05 | 79,148 | 61,106 | Increased risk | European | IVW |
| | PMID: 35579976 | Testosterone | 67 | 1.23 (1.08-1.40) | 0.002 | 79,148 | 61,106 | Positive | European | IVW |

*(Continued)*





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TABLE 3 Continued

| Disease | Reference no. | Exposure | SNPs, n | OR (95%CI) | P-value | Sample Size | | Correlation | Population | MR method |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Cases | Control | | | |
| | PMID: 38867724 | Proinsulin | 48 | 0.94 (0.89-0.999) | 0.048 | 79,148 | 61,106 | Negative factor | European | IVW |
| | PMID: 38911377 | Sodium-glucose Cotransporter 2 Inhibition | 6 | 1.17 (0.59-1.74) | <0.001 | 79,194 | 61,112 | Increased risk | European | IVW |
| | PMID: 38701318 | HMGCR | 12 | 1.62 (1.23-2.12) | 0.0005 | 211,227 | | Elevated risk | European | IVW |
| | PMID: 38517045 | Genetically proxied metformin effects | 13 | 1.55 (1.23-1.96) | 0.003 | 79,148 | 61,106 | Increased risk | European | IVW |
| | PMID: 35151363 | Genetically proxied inhibition of PCSK9 | 11 | 0.81 (0.73-0.90) | 4.52E-05 | 79,148 | 61,106 | Negatively associated | European | IVW |
| | PMID: 38487860 | Drugs | 105 | 0.94 (0.91-0.97) | 7.00E-04 | 79,148 | 61,106 | Reduced risk | European | IVW |
| | PMID: 37735436 | KDELC2 | 1 | 0.89 (0.86-0.93) | 1.89E-08 | 79,148 | 61,106 | Negatively associated | European | IVW |
| | PMID: 33032658 | Prevotella | 1 | -0.758 (-1.354, -0.162) | 0.013 | 495 | 640 | Decrease | East Asia | IVW |
| | PMID: 36880394 | Class Alphaproteobacteria | 7 | 0.84 (0.75-0.93) | 0.001 | 79,148 | 61,106 | Negatively associated | European | IVW |
| | PMID: 38369514 | Odoribacter | 7 | 1.17 (1.05-1.31) | 0.005 | 79,148 | 61,106 | Higher risk | European | IVW |
| | | Dorea | 9 | 1.13 (1.02-1.25) | 0.025 | | | Higher risk | | |
| | | Christensenellaceae R7 | 9 | 1.12 (1.01-1.25) | 0.032 | | | Higher risk | | |
| | | Eubacterium fissicatena | 9 | 1.08 (1.02-1.13) | 0.006 | | | Higher risk | | |
| | | Ruminococcus gauvreaui | 12 | 1.10 (1.01-1.19) | 0.032 | | | Higher risk | | |
| | | Eubacterium nodatum | 11 | 1.06 (1.02-1.11) | 0.007 | | | Higher risk | | |
| | | Lachnospiraceae | 17 | 1.08 (1.00-1.16) | 0.046 | | | Higher risk | | |
| | | Flavonifractor | 5 | 0.84 (0.75-0.94) | 0.003 | | | Lower risk | | |
| | | Adlercreutzia | 8 | 0.89 (0.82-0.97) | 0.005 | | | Lower risk | | |
| | | Roseburia | 14 | 0.90 (0.83-0.98) | 0.019 | | | Lower risk | | |
| | | Ruminococcaceae UCG004 | 9 | 0.91 (0.84-0.99) | 0.027 | | | Lower risk | | |
| | | Coprobacter | 11 | 0.92 (0.87-0.98) | 0.008 | | | Lower risk | | |
| | | Allisonella | 6 | 0.93 (0.89-0.99) | 0.014 | | | Lower risk | | |
| | | Holdemania | 15 | 0.93 (0.88-0.99) | 0.014 | | | Lower risk | | |
| | | Rhodospirillaceae | 15 | 0.94 (0.89-1.00) | 0.037 | | | Lower risk | | |

*(Continued)*





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TABLE 3 Continued

| Disease | Reference no. | Exposure | SNPs, n | OR (95%CI) | P-value | Sample Size | | Correlation | Population | MR method |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Cases | Control | | | |
| | | Rhodospirillales | 14 | 0.91 (0.86-0.97) | 0.003 | | | Lower risk | | |
| | | Alphaproteobacteria | 7 | 0.84 (0.76-0.92) | <0.001 | | | Lower risk | | |
| | PMID: 37697271 | Allisonella | 1 | 0.89 (0.81-0.99) | 0.038 | 79,148 | 61,106 | Decreased risk | European | IVW |
| | PMID: 38029073 | Akkermansia muciniphila | 5 | 0.79 (0.67-0.94) | 0.009 | 6,311 | 88,902 | Negatively associated | European | IVW |
| | | Bacteroides salyersiae | 6 | 0.90 (0.83-0.99) | 0.022 | | | Negatively associated | | |
| | | Eubacterium biforme | 4 | 1.16 (1.01-1.34) | 0.035 | | | Positively associated | | |
| | PMID: 37274339 | Genetically predicted hyperthyroidism | 13 | 0.86 (0.79-0.93) | 4.00E-04 | 6,321 | 354,873 | Declining risk | European | IVW |
| | PMID: 37213031 | Systemic lupus erythematosus | 48 | 0.98 (0.97-0.99) | 0.003 | 79,148 | 61,106 | Decreased risk | European | IVW |
| | PMID: 38783043 | Systemic lupus erythematosus | 4 | 0.94 (0.91-0.97) | 2.14E-04 | 79148 | 61106 | Lower risk | European | IVW |
| | | Hyperthyroidism | 25 | 0.02 (0.0016-0.2539) | 0.003 | | | Lower risk | | |
| | | Rheumatoid arthritis | 129 | 1.03 (1.02-1.05) | 2.13E-05 | | | Developing | | |
| | PMID: 38703296 | Obstructive sleep apnea | 5 | 0.87 (0.79-0.95) | 0.002 | 79,148 | 61,106 | Negatively associated | European | IVW |
| | PMID: 38741062 | Pernicious anemia | 17 | -0.022 (-0.035 , -0.006) | 0.007 | 6,311 | 74,685 | Reverse causal relationship | European | IVW |
| | PMID: 38403547 | Erysipelas | 28 | 1.05 (1.01-1.08) | 0.005 | 6,311 | 74,685 | Significant association | European | IVW |
| | PMID: 35303584 | Schizophrenia | 75 | 1.033 (0.998-1.069) | 0.065 | 79,148 | 61,106 | Not support | European | IVW |
| | PMID: 33027558 | Depression | 44 | 0.72 (0.35-1.47) | 0.364 | 79,148 | 61,106 | No strong evidence | European | IVW |
| | PMID: 31908803 | Fasting glucose | 21 | 0.93 (0.73-1.17) | $I^2$: 0.46 | 79,148 | 61,106 | No association | European | IVW |
| | | HbA1c | 34 | 0.90 (0.58-1.40) | $I^2$: 0.58 | | | No association | | |
| | | Type 2 diabetes | 159 | 1.02 (0.97-1.07) | $I^2$: 0.69 | | | No association | | |
| | PMID: 32349989 | Type 2 diabetes | 399 | 0.97 (0.93-1.01) | 0.108 | 7,872 | 359,711 | No association | European | IVW |
| | PMID: 27598322 | Adult height | 168 | 1.03 (0.92-1.15) | 0.642 | 14,160 | 12,724 | No association | European | IVW |
| | PMID: 35906597 | Circulating vitamin E | 3 | 0.85 (0.59-1.23) | 0.388 | 79,148 | 61,106 | No association | European | IVW |
| | PMID: 34325683 | Circulating vitamin C | 11 | 0.90 (0.74-1.09) | 0.29 | 79,148 | 61,106 | No association | European | IVW |

*(Continued)*





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TABLE 3 Continued

| Disease | Reference no. | Exposure | SNPs, n | OR (95%CI) | P-value | Sample Size | | Correlation | Population | MR method |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Cases | Control | | | |
| | PMID: 33420236 | Circulating vitamin D | 138 | -0.02 (-0.09, -0.05) | 0.57 | 79,148 | 61,106 | No association | European | IVW |
| | PMID: 34504857 | Homocysteine | 15 | 1.01 (0.93-1.11) | 0.774 | 79,148 | 61,106 | No association | European | IVW |
| | PMID: 35494045 | Tryptophan | 18 | -0.92 (-2.04, 0.20) | 0.11 | 79,148 | 61,106 | Not significantly associate | European | IVW |
| | PMID: 37178364 | Systolic blood pressure | 278 | 0.96 (0.92-1.01) | 0.11 | 79,148 | 61,106 | No strong evidence | European | IVW |
| | | Blocking calcium channel receptors | 16 | 1.22 (1.06-1.42) | 0.01 | | | Increased risk | | |
| | PMID: 33805346 | Serum urea | 6 | 1.02 (0.94-1.11) | 0.703 | 79,148 | 61,106 | Null association | European | IVW |
| | PMID: 32006205 | Allergic diseases | 132 | 1.00 (0.94-1.05) | 0.93 | 79,148 | 61,106 | No evidence | European | IVW |
| | PMID: 33671849 | Circulating Bilirubin Levels | 115 | 1.00 (0.97-1.03) | 1 | 79,194 | 61,112 | No evidence | European | IVW |
| | PMID: 36204379 | Processed meat | 23 | 1.02 (0.69-1.49) | 0.94 | 79,148 | 61,106 | No evidence | European | IVW |
| | PMID: 33199044 | Arachidonic acid | 5 | 1.02 (1.00-1.04) | 0.114 | 79,148 | 61,106 | No evidence | European | IVW |
| | PMID: 33178578 | C-reactive protein | 58 | 1.06 (0.96-1.16) | 0.24 | 79,148 | 61,106 | No evidence | European | IVW |

TABLE 4 Application of Mendelian randomization in prostatitis.

| Disease | Reference no. | Exposure | SNPs, n | OR (95%CI) | P-value | Sample size | | Correlation | Population | MR method |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Cases | Control | | | |
| **Prostatitis** | PMID: 38273299 | Faecalibacterium | 10 | 1.59 (1.08-2.34) | 0.018 | 1,859 | 72,799 | Positive association | European | IVW |
| | | LachospiraceaeUCG004 | 14 | 1.64 (1.15-2.34) | 0.007 | | | Positive association | | |
| | | Sutterella | 12 | 1.58 (1.14-2.19) | 0.007 | | | Positive association | | |
| | | Gastranaerophilales | 9 | 1.47 (1.10-1.97) | 0.008 | | | Positive association | | |
| | | Methanobacteriaceae | 9 | 0.69 (0.56-0.86) | 0.001 | | | Decreased risk | | |
| | | Erysipelatoclostridium | 15 | 0.71 (0.55-0.93) | 0.036 | | | Decreased risk | | |
| | | Parasutterella | 14 | 0.74 (0.57-0.96) | 0.023 | | | Decreased risk | | |
| | | Slackia | 6 | 0.69 (0.49-0.96) | 0.03 | | | Decreased risk | | |

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TABLE 4 Continued

| Disease | Reference no. | Exposure | SNPs, n | OR (95%CI) | P-value | Sample size | | Correlation | Population | MR method |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Cases | Control | | | |
| | PMID: 38369514 | Sutterella | 12 | 1.31 (1.03-1.68) | 0.029 | 3,299 | 110,070 | Increased morbidity | European | IVW |
| | | Ruminococcaceae UCG010 | 6 | 1.37 (1.00-1.88) | 0.049 | | | Increased morbidity | | |
| | | Odoribacter | 7 | 1.44 (1.04-2.00) | 0.03 | | | Increased morbidity | | |
| | | Gastranaerophilales | 9 | 1.35 (1.12-1.64) | 0.002 | | | Increased morbidity | | |
| | | NB1n | 12 | 1.19 (1.02-1.38) | 0.026 | | | Increased morbidity | | |
| | | Melainabacteria | 10 | 1.27 (1.06-1.53) | 0.01 | | | Increased morbidity | | |
| | | Cyanobacteria | 8 | 1.27 (1.02-1.58) | 0.031 | | | Increased morbidity | | |
| | | Erysipelatoclostridium | 15 | 0.82 (0.68-0.99) | 0.04 | | | Lower risk | | |
| | | Eubacterium eligens group | 6 | 0.69 (0.48-0.99) | 0.047 | | | Lower risk | | |
| | | Methanobacteriaceae | 9 | 0.81 (0.69-0.95) | 0.008 | | | Lower risk | | |
| | | Methanobacteriales | 9 | 0.81 (0.69-0.95) | 0.008 | | | Lower risk | | |
| | | Methanobacteria | 9 | 0.81 (0.69-0.95) | 0.008 | | | Lower risk | | |
| | PMID: 38573543 | Methanobacteria | 9 | 0.86 (0.74-0.99) | 0.04 | 3,760 | 119297 | Decreased risk | European | IVW |
| | | Methanobacteriales | 9 | 0.86 (0.74-0.99) | 0.04 | | | Decreased risk | | |
| | | Methanobacteriaceae | 9 | 0.86 (0.74-0.99) | 0.04 | | | Decreased risk | | |
| | | NB1n | 12 | 1.16 (1.01-1.34) | 0.037 | | | Decreased risk | | |
| | | Odoribactergenus Odoribacter | 7 | 1.43 (1.05-1.94) | 0.024 | | | Decreased risk | | |
| | | Sutterellagenus Sutterella | 12 | 1.33 (1.01-1.76) | 0.041 | | | Decreased risk | | |
| | PMID: 38680919 | Genus Sutterella | 12 | 1.37 (1.09-1.71) | 0.006 | 4,160 | 130,139 | Increased risk | European | IVW |
| | | Genus Holdemania | 15 | 1.21 (1.02-1.43) | 0.028 | | | Increased risk | | |
| | | Phylum Verrucomicrobia | 12 | 0.76 (0.58-0.98) | 0.033 | | | Negative association | | |
| | | Genus Parasutterella | 14 | 0.84 (0.70-1.00) | 0.045 | | | Negative association | | |
| | PMID: 36071874 | Complement C4 | 286 | 1.04 (0.44-2.47) | 0.039 | | | Causal relationship | East Asia | IVW |
| | PMID: 38816661 | HLA DR on Dendritic Cell | 7 | 0.92 (0.80-0.99) | 0.019 | 1,859 | 72,799 | Protective effect | European | IVW |
| | | HLA DR on plasmacytoid Dendritic Cell | 8 | 0.91 (0.86-0.97) | 0.006 | | | Protective effect | | |

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10.3389/fendo.2025.1541744

TABLE 4 Continued

| Disease | Reference no. | Exposure | SNPs, n | OR (95%CI) | P-value | Sample size | | Correlation | Population | MR method |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Cases | Control | | | |
| | | HLA DR on myeloid Dendritic Cell | 7 | 0.91 (0.84-0.98) | 0.018 | | | Protective effect | | |
| | PMID: 37143736 | Thyrotropin | 59 | 0.82 (0.70-0.97) | 0.018 | 1,859 | 72,799 | Significantly influenced | European | IVW |
| | | Overt hypothyroidism | 18 | 0.85 (0.73-0.99) | 0.046 | | | Significantly influenced | | |

implications for the prevention of MI in the general population. In addition, current MR studies have shown no or weak associations between MI and several risk factors, such as sleep traits (44), leukocyte telomere length (LTL) (45), and educational attainment (46).

The application of MR in MI is shown in Table 1.

## 3.2 Erectile dysfunction

ED, characterized by persistent difficulties in attaining or maintaining erections adequate for sexual intercourse, often stems from multifactorial etiologies and may signal underlying comorbidities requiring clinical assessment (19, 47). The MR studies included in this article investigate the causal relationships between ED and risk factors such as gut microbiota, cytokine, related diseases, drug targets and other factors.

### 3.2.1 Gut microbiota

The gut microbiota may cause ED due to changes in endocrine sex hormone levels, the metabolic state of the organism and neurotransmitters (48). Using TSMR studies, Xu R et al. reported that the abundance of the genus Ruminococcaceae UCG-013 exhibited an inverse association with the risk of developing ED. Conversely, the genus Tyzzerella3, genus Erysipelotrichaceae UCG-003, genus LachnospiraceaeNC2004group, genus Oscillibacter, genus Senegalimassilia, and family Lachnospiraceae demonstrated positive associations with an increased risk of ED (49, 50). However, further research is needed to elucidate the pathogenic mechanism of the intestinal microbiota in ED.

### 3.2.2 Cytokine

The IVW analysis of Kang Z et al. indicates that fibroblast growth factor 5, IL-22 receptor subunit alpha-1, and protein S100-A12 are associated with increased risk of ED, TNF-related activation-induced cytokine is associated with decreased risk (51). According to the study by Liu D et al., elevated levels of interferon-inducible protein-10 were found to significantly elevate the risk of ED, while higher levels of interleukin-1 receptor antagonist (IL-1RA) were observed to markedly reduce the risk of ED (52).

### 3.2.3 Related diseases

#### 3.2.3.1 Cardiovascular disease

Cardiovascular diseases include coronary heart disease (CHD), ischemic stroke (IS), myocardial infarction, heart failure (HF), ischemic heart disease, and atrial fibrillation, among others. Several studies have elucidated the causal relationship between CVD and ED using MR analyses. For example, genetically predicted CHD and HF increase the risk of ED (53). MR analysis by Miaoyong et al. revealed a causal link between genetic susceptibility to IS, HF, and CHD and ED. Additionally, bidirectional analyses indicated that a genetic predisposition to ED did not increase the risk of CVD (54). An MR study by Zhao C et al. indicated that hypertension increased the risk of ED (55–57). The causal connection between CVD and ED has been inconsistent

across multiple MR studies, and further research is needed to confirm these causal claims (54, 58, 59). These findings may inform ED prevention and intervention strategies for patients with CVD.

#### 3.2.3.2 Type 2 diabetes mellitus

ED and systemic health conditions such as metabolic syndrome (e.g., CVD and diabetes) may share many common risk factors (60). Bovijn J et al. used MR analysis to demonstrate that T2DM directly causes ED, independent of obesity and dyslipidemia (61, 62).

Furthermore, CVD, DM, and their comorbid conditions demonstrate frequent comorbidity with ED (63), likely mediated by shared pathological mechanisms such as endothelial dysfunction and chronic inflammatory cascades (64, 65). Therefore, these comorbidities should be carefully accounted for as potential confounders in MR analyses.

#### 3.2.3.3 Psychiatric disorders

The etiology of ED varies and can be organic, psychological or mixed (66). Consequently, ED is closely linked to neurological and mental health issues. Based on IVW analysis, Kai et al. suggested that psychiatric disorders, such as depression, significantly increase the incidence of ED, and genetically predicted depression plays a potential causal role in the development of ED (67–69).

#### 3.2.3.4 COVID-19

Multiple MR analyses have revealed a causal relationship between genetic susceptibility to COVID-19 and an increased risk of ED (70–72).

#### 3.2.3.5 Inflammatory bowel disease

MR analysis by Gao DW et al. did not reveal a causal connection between IBD and ED (73), but recent MR studies by Chen D et al. revealed that IBD can increase the risk of ED (74, 75).

### 3.2.4 Drug targets

Some drug targeting MR analysis showed that drugs such as LDL receptor, lipoprotein lipase agonists and apolipoprotein C-III inhibitors were associated with reduced ED risk, while apolipoprotein B-100 inhibitors (76), atorvastatin (77) and aspirin (78) were associated with increased ED risk.

### 3.2.5 Other factors

In addition to the above points, relevant MR studies have shown that numerous additional risk factors are associated with ED. For example, BMI, waist circumference, trunk fat mass, total body fat mass, poorer overall health scores, basal metabolic rate, stroke, smoking, snoring, insomnia, lipocalin and atorvastatin have been found to increase the risk of ED. A genetic predisposition to higher levels of sex hormone binding globulin reduces the risk of ED (79–84). In addition, there are many irrelevant factors, such as thyroid function (85), and periodontal disease (86) that are not associated with ED risk.

The application of MR in ED is shown in Table 2.

## 3.3 Prostate cancer

PCa remains the most prevalent malignancy in men (20), with mortality rates from metastatic PCa continuing to rise (87). Due to the complex mechanisms underlying the disease and the lack of a clearly defined optimal approach among diverse treatment options (88), exploring PCa-related risk factors is critical for refining clinical prevention and management strategies. The MR studies included in this article investigate the causal relationships between PCa and risk factors such as gut microbiota, circulatory substance, related diseases, modifiable risk factors, drug targets, leukocyte telomere length (LTL) and other factors.

### 3.3.1 Gut microbiota

A reverse MR analysis by Xu F et al. indicated that a greater risk of PCa was associated with a decrease in the abundance of Prevotella (89). Zixin W et al. confirmed that Alphaproteobacteria has a protective effect on PCa. MVMR analysis revealed that the protective effect of Alphaproteobacteria on PCa might be driven by BMI, smoking, and drinking behaviors (90, 91). Using the Wald ratio method, Mingdong W et al. reported that the abundance of Allisonella was negatively correlated with bladder cancer and PCa incidence (92). The IVW estimates of Xie Q et al. suggested that the relative abundance of Akkermansia muciniphila and Bacteroides salyersiae may decrease the odds of PCa, whereas that of Eubacterium biforme may increase the odds of PCa (93).

### 3.3.2 Circulatory substance

#### 3.3.2.1 Plasma microgranulin-beta

Plasma microseminoprotein-beta (MSP) is a protein secreted by prostate epithelial cells that may protect against the development of PCa. A nested case-control study using a two-sample inverse variance method to calculate MR estimates showed that plasma MSP concentrations were negatively related to PCa risk after adjusting for the concentration of total prostate-specific antigen. This study suggested that men with high levels of circulating MSP concentrations are at a lower risk of developing PCa and that MSP may play a causal protective role in PCa (94).

#### 3.3.2.2 Serum zinc, phosphorus and iron levels

The role of micronutrients in the development of urinary system tumors cannot be ignored. Using TSMR analysis, Marta et al. reported that an increase in serum zinc had a weak deleterious effect on PCa (95). Yi et al. conducted the TSMR study using pooled statistics from genome-wide association studies (GWAS) for four micronutrients and three major urologic cancer outcomes and demonstrated that each standard deviation (SD) increase in the serum zinc level increased the risk of PCa by 5.8% (96). The IVW analysis by Lin et al. indicated that for each SD increase in the serum phosphate concentration predicted by genetics, the risk of PCa increases by 19% (97). Using MR analysis, Jiacheng et al. reported that a genetically predicted increase in iron status was associated with a decrease in PCa risk and that iron has a protective effect on PCa risk. However, the mechanism by which micronutrients affect PCa needs further study (98, 99).

#### 3.3.2.3 Blood lipids

Studies have shown an association between lipid levels and PCa risk (100, 101). MR analyses by Anna I et al. revealed that the genetically predicted lipoprotein A concentration is correlated with the risk of PCa (102). Bull CJ et al. reported that higher low-density lipoprotein (LDL) and triglyceride levels increase aggressive PCa risk, although the evidence is weak (103). Shiqiang F evaluated the relationship between genetically proxied inhibition of LDL-cholesterol-lowering drug targets and PCa risk using MR methods. Genetically proxied proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibition may involve biological mechanisms that reduce the risk of overall and early-onset PCa through the regulation of Lp (a) (104, 105). Shusheng et al. found an association between the effect of triglycerides on PCa risk by applying IVW, suggesting that the odds of PCa increase with elevated triglyceride levels (106). MR analysis by Nabila K et al. revealed that monounsaturated fat levels were positively associated with overall PCa risk (99).

#### 3.3.2.4 Amino acids

Cancer cells often exhibit abnormal growth and proliferation in which enhanced metabolism of amino acid substances is needed. Using TSMR, Yindan et al. demonstrated that serum aspartate levels may promote the development of PCa and breast cancer. An in-depth study of the underlying biochemical mechanisms would be valuable for the early assessment and diagnosis of these two cancers and for the development of clinical intervention strategies (107). MR analysis by Shaoxue Y et al. revealed that circulating alanine concentrations were positively associated with PCa risk and that genetically predicted alanine aminotransferase levels were inversely related to the risk of PCa (108).

#### 3.3.2.5 Red blood cells and hemoglobin

An MR study by Pin et al. provided evidence that elevated mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration are potentially associated with reduced risks of developing PCa (109).

#### 3.3.2.6 Circulating cytokines

Emma et al. performed analyses using methods such as TSMR and IVW and evaluated MR hypotheses in sensitivity and colocalization analyses, providing evidence of a positive correlation between the concentration of genetic proxies for macrophage inflammatory protein 1a (MIP1a) and overall PCa risk and a negative correlation between the concentration of genetic proxies for vascular endothelial growth factor and the risk of late-stage PCa (110). An MR study by Binghui L et al. suggested that long-term IL-6 levels may increase the risk of PCa, whereas long-term IL-1ra levels may reduce this risk (111).

#### 3.3.2.7 Circulating free testosterone

Two MR analyses showed that circulating free testosterone levels were related to elevated PCa risk, whereas circulating total testosterone levels showed no association with PCa risk (112, 113).

### 3.3.3 Related diseases

Numerous studies have analyzed the potential association between other diseases and PCa risk using MR methods. For example, genetically predicted hyperthyroidism is related to a decreased risk of PCa occurrence (114). Patients with systemic lupus erythematosus have a lower risk of developing PCa (115, 116). Obstructive sleep apnea was significantly negatively associated with PCa susceptibility (117). There was a reverse causal relationship between PCa and pernicious anemia (118). The MR showed a significant association of PCa on erysipelas (119). Schizophrenia, depression and T2DM are not thought to be associated with PCa risk (120–123).

### 3.3.4 Modifiable risk factors

#### 3.3.4.1 Obesity

The increasing prevalence of obesity globally poses a major threat to public health (124). However, current research suggests that the impact of obesity on PCa is complex. The precise pathophysiological mechanisms underlying the association between obesity and PCa incidence remain incompletely elucidated, with current scientific consensus yet to be definitively established (125, 126). A meta-analysis by Discacciati et al. demonstrated that obesity potentially reduces localized PCa risk and increases the risk of advanced PCa (127). Similarly, an MR analysis by Georgios et al. suggested that obesity increases the risk of advanced PCa (128). A meta-analysis of MR studies by Susanna et al. suggested that a genetically predicted higher adult BMI is related to a reduced risk of cancers such as PCa and breast cancer (129). Moreover, Nabila K et al. showed a negative correlation between BMI and overall PCa through TSMR (99, 130). There was no strong evidence that genetically determined metabolically unfavorable adiposity, favorable adiposity or BMI were correlated with overall PCa in the study by Aurora P-C et al. (131).

As for the conflicting results of the above studies, some believe that obesity may have different effects on PCa risk at different stages throughout the lifespan (132). The conclusion that a larger BMI and waist circumference are positively correlated with the risk of PCa mainly applies to the mid-to-late life, rather than early adulthood (133). Therefore, relevant MR studies should further clarify the effects of obesity at different time points on different developmental stages of PCa. In addition, current discrepancies in obesity-PCa associations across studies may stem from methodological limitations in adiposity assessment. The sole reliance on BMI as a clinical indicator of obesity may yield incomplete characterization of this relationship, as this metric fails to account for critical parameters such as metabolic health status and body composition metrics. Incorporating regional adiposity patterns (e.g., visceral vs. subcutaneous fat distribution) and functional adiposity biomarkers (e.g., leptin/adiponectin ratio) could better elucidate the heterogeneous biological pathways through which obesity may exert differential impacts on prostate carcinogenesis and disease progression (126). Moreover, the differences in the stages and classifications of PCa selected in different studies have led to varying results. Existing research has shown that obesity is associated with advanced or fatal PCa and reduces the risk of low-grade PCa (134), making the relationship with PCa incidence more complex.

In summary, the discrepancies among the research findings may stem from inappropriate assessment methods for obesity, variations in the stages and types of PCa selected across different studies, as well as the influence of obesity on PCa incidence being associated with distinct life stages.

#### 3.3.4.2 Smoking

Cigarette smoking can have deleterious effects on humans and increase the risk of a number of diseases. However, a definitive causal relationship between smoking and PCa has not yet been established. The meta-analysis of MR studies by Susanna et al. concluded that smoking preference was negatively related to the risk of PCa (135, 136). A European pooled study showed that smokers had a lower risk of PCa, and this finding may be attributable to detection bias. In addition, smokers have a greater risk of dying from PCa, possibly due to the direct impact of smoking, which may lead to poor treatment outcomes (137). Using MVMR analysis, Yongle et al. proposed a possible explanation for these implausible findings and showed that each additional increase in the lifetime smoking index increases the risk of PCa by 95%, suggesting a definite causal relationship between smoking and PCa risk (138).

### 3.3.5 Drug targets

A MR study has shown that Sodium-glucose cotransporter 2 inhibitors inhibition is associated with an increased risk of PCa (139). Ding WJ et al. 's drug target MR study found that 3-hydroxy-3-methylglutaryl-assisted enzyme A reductase inhibitors (HMGCR) were associated with an elevated risk of PCa (140).Sun X et al., using a drug-targeted MR approach, found that genetically proxied metformin effects were associated with an increased risk of PCa (141).Sun L et al. 's MR study found that genetically proxied inhibition of PCSK9 was associated with reduced risk of PCa (105).The study by Yun Z. et al. provides strong evidence that the use of drugs that act on the renin-angiotensin system can reduce PCa risk (142). Ren F et al. proposed through MR analysis that genetically predicted KDEL containing 2, isoform CRA_a (KDELC2) is negatively associated with PCa. In addition, Kunitz-type protease inhibitor 2, Glutathione S-transferase P, and Cathepsin S may serve as potential therapeutic targets for PCa (143).

### 3.3.6 Leukocyte telomere length

Telomeres play a significant role in the development and progression of cancer. Cells with longer telomere lengths have greater proliferative potential and a greater cumulative probability of mutation (144). In addition, it has been proposed that telomere shortening can cause end-to-end chromosome fusions and attenuate the DNA damage response, thereby increasing genomic instability and causing carcinogenesis (145). In conclusion, telomeres play a dual role in cancer development, and the direction of action may depend on the type of cancer and other influencing factors. Based on the GRS and MR data, Yixin et al. concluded that a shorter LTL is inversely associated with the risk of cancers such as PCa (146). Junfeng et al. conducted a study to evaluate the relative LTL in PCa patients and its correlation with aggressive disease characteristics at diagnosis and biochemical recurrence (BCR) following aggressive treatment (radical prostatectomy and radiotherapy). Employing the MR method, they found a notable association between shorter LTL and higher Gleason scores in PCa patients. Furthermore, in localized patients undergoing prostatectomy or radiotherapy, shorter LTL and genetically predicted shorter LTL have significant positive correlations with BCR risk, i.e., patients with shorter LTL have a worse prognosis (147). A recent MR study demonstrated that a genetically determined longer LTL was associated with greater PCa risk (148).

### 3.3.7 Other factors

MR analysis revealed that many other factors, such as height (130, 149), circulating vitamin E levels (150), circulating vitamin C levels (151), circulating VD levels (152, 153), homocysteine levels (154), tryptophan (155), blood pressure (156), serum urea concentration (157), allergic diseases (158), Circulating Bilirubin Levels (159), processed meat, red meat (160), plasma phospholipid arachidonic acid concentrations (161), and circulating levels of C-reactive protein (162), are not associated with PCa risk or are weakly associated with PCa risk. Chen G et al. 's two-step MR analysis revealed that proinsulin functions as a suppressive factor in PCa, showing significant independence from insulin-like growth factor 1 (163).

The application of MR in PCa is shown in Table 3.

## 3.4 Prostatitis

According to the National Institutes of Health (NIH) classification system, prostatitis is categorized into four types: Type I (acute bacterial prostatitis), Type II (chronic bacterial prostatitis), Type III (chronic prostatitis/chronic pelvic pain syndrome, CP/CPPS), and Type IV (asymptomatic inflammatory prostatitis). Given that Type III (chronic non-bacterial prostatitis) accounts for approximately 90% of clinical cases (164), this study focuses on Type III prostatitis. Chronic prostatitis (chronic pelvic pain syndrome) is defined as pelvic pain accompanied by variable urinary symptoms and sexual dysfunction persisting for at least three months (165). Accumulated evidence confirms significant correlations between prostate inflammation development and multiple biomarkers, encompassing immune-inflammatory indicators, hormonal profiles, tumor-associated proteins, and nutritional parameters (166).The MR studies included in this article investigate the causal relationships between prostatitis and risk factors such as gut microbiota, complement C4, immune cells and thyroid function.

### 3.4.1 Gut microbiota

The physiological functions of the host organism can be modulated by gut microbiota through their regulatory effects on multiple biological pathways, encompassing immune regulation, oxidative stress response, inflammatory modulation, and the

maintenance of anabolic-catabolic equilibrium (167, 168). While direct evidence linking gut microbiota to prostate pathophysiology remains elusive, emerging research suggests that prostate health may be compromised through indirect pathological pathways, with chronic inflammatory processes likely serving as the principal mediating mechanism (169–171). In 2016, Shoskes et al. pioneered the application of MiSeq sequencing technology to delineate significant gut microbial dysbiosis in chronic nonbacterial prostatitis (CNP) patients (172). More recently, MR analyses have further advanced mechanistic insights into the gut microbiota-PCa causal axis through rigorous causal inference frameworks. According to these MR studies, the risk of prostatitis may be decreased by the presence of Methanebacteria, Methanobacteriales, Methanobacteraceae, Erysipelatoclostridium, the Eubacterium eligens group, phylum Verrucomicrobia and Parasutterella. Faecalibacterium, LachnospiraceaeUCG004, Sutterellagenus Sutterella, NB1n, Gastranaerophilales, Odoribactergenus, Odoribacter, Ruminococcaceae UCG010, Melainabacteria, genus Holdemania and Cyanobacteria play causal roles in promoting the development of prostatitis (173–176).

### 3.4.2 Other factors

Few MR analyses have been conducted on prostatitis. However, certain risk factors, such as complement C4 (177), certain T cell subsets (178), and thyroid function (179) have been identified as having causal relationships with prostatitis. Complement C4, a pivotal component of the complement system, serves as a critical mediator in innate immunity by enabling rapid recognition and clearance of pathogenic microorganisms (180), while simultaneously reflecting systemic inflammatory activity (181). Importantly, a TSMR study recently validated a positive causal link between elevated complement C4 concentrations and chronic prostatitis pathogenesis (177).

While extensive research has established potential connections between immune cell activity and prostatitis (182, 183), the causal dynamics of specific immune populations in this inflammatory process remain undetermined. A recent investigation leveraging bidirectional MR systematically explored causal relationships between immunophenotypic characteristics and prostatitis pathogenesis. The analyses identified that particular T-cell subsets-notably CD3 + CD4 + T lymphocytes and CD3 + CD8 + T cells-demonstrated significant causal associations with elevated prostatitis risk (178).

Chronic prostatic inflammation may be modulated by endocrine hormone dysregulation or metabolic abnormalities (184). Although no studies have established direct associations between thyroid hormones and prostatitis risk, a large-scale observational investigation revealed prostate volume positively correlated with free thyroxine (FT4) levels (185). Given the potential overlap in pathogenic mechanisms underlying prostatic hypertrophy and prostatitis, Huang et al. employed MR to assess causal relationships between genetically predicted thyroid function alterations and benign prostatic disorders. Their findings demonstrated that elevated thyrotropin (TSH) concentrations and hypothyroidism development were inversely associated with risks of prostatic hypertrophy and inflammatory prostatic conditions (179).

The application of MR in prostatitis is shown in Table 4.

# 4 Discussion

## 4.1 Existing problems and solutions

While these MR studies advance our understanding of male reproductive disorders, several methodological limitations persist in the field. We examine these ongoing challenges specific to andrology research and propose solutions to enhance future studies.

### 4.1.1 Multi-methodology validation

Although MR studies can suggest causal associations between risk factors and male-specific diseases, they do not reveal the underlying mechanisms of their effects. Thus, the estimated magnitude of the effect of exposure on outcomes obtained from MR analysis is not equivalent to the actual causal effect (186). It is also necessary to compare MR analyses with findings from large cohort studies or RCTs to evaluate the consistency and robustness of the evidence. Example illustrations are provided for reference:

1. Validating consistency between MR results and large-scale cohort studies.

For instance, in the manuscript section exploring the causal relationship between PCa and obesity, MR studies have reported inconsistent findings. We identified relevant prospective studies indicating that obesity during mid-to-late adulthood (but not early adulthood) showed inverse associations with localized PCa. These studies also revealed dual associations between BMI and fatal PCa - reduced risk in men with obesity during early adulthood versus increased risk in those with obesity during mid-to-late adulthood (187).

Although the effects of obesity on PCa remain complex, such cohort studies can provide longitudinal associations between obesity and PCa to verify consistency with corresponding MR findings. Therefore, MR results aligning with cohort discoveries in the manuscript may be considered relatively conclusive regarding causal relationships (though higher-level evidence remains necessary). For MR results inconsistent with cohort findings or showing methodological limitations, rigorous evaluation should be conducted regarding analytical process integrity, methodological completeness, and disease staging comprehensiveness.

2. Assessing robustness of MR causal inference using RCT evidence.

As our MR analysis suggests close associations between genetically proxied LDL-cholesterol-lowering drug targets and reduced risks of overall PCa/early-onset PCa, we referenced statins-related RCT outcomes to validate MR robustness (188). The MR approach inherently avoids confounding factors, while combining both methodologies compensates for individual limitations. This multi-level evidence integration substantially enhances result credibility.

In conclusion, researchers should not limit themselves to MR methodology alone. Concurrent collection of regional patient data for broader cross-sectional/observational studies is crucial to validate findings. Result verification constitutes an authorial responsibility rather than readers' obligation.

Additionally, MR authors must avoid selective result presentation. Objective, rigorous, and comprehensive selection of instrumental variables and datasets should be ensured. Disease-related datasets should be comprehensively incorporated, with multivariable analyses employed to guarantee result robustness.

### 4.1.2 European-dominant databases in prospective MR studies

Moreover, many prospective studies only use databases that include European populations, and there is a lack of relevant MR studies for Asian populations, which may lead to a lack of comprehensiveness and impact in the application of research results. Researchers can analyze large samples of data from different ethnic groups, taking into account population stratification, to achieve broader application of the research results. We humbly suggest some directions that might help resolve this difficulty:

1. Integration of population data.

We propose collaborating with Asian research institutions to conduct multicenter cohort studies, while advocating for government-supported transnational health data infrastructure development. This initiative should integrate educational, economic, and health datasets through standardized core variable definitions and establish a unified data collaboration platform with harmonized protocols (189).

To advance open science and data transparency, we recommend publicly sharing data preprocessing codes and statistical model parameters in research publications to enable reproducibility (190). This approach particularly encourages researchers to replicate and supplement findings with Asian population data. Furthermore, actively incorporating Asian-based studies (e.g., reports from China's National Cancer Center (191)) would help counterbalance the current European-centric literature bias, thereby enhancing the reliability and generalizability of research conclusions.

2. Population stratification design.

Prospective studies should incorporate pre-stratification by race, region, and cultural background, with Asian populations further categorized into East, Southeast, and South Asian subgroups for distinct exposure-outcome analyses (192). Furthermore, sociocultural variables should be incorporated into analyses, particularly Asia-specific factors (e.g., family structure, healthcare accessibility) that may influence disease risk profiles.

These strategies will enhance the global representativeness of research findings, providing more generalizable evidence for precision medicine and public health policies.

### 4.1.3 Survivorship bias

In addition, in male-specific disease research, when the disease of interest is associated with a risk of death, there may be survivorship bias. For example, when a long-term study of a disease is conducted, the participants in the final analysis are not a random sample because some of the study participants died earlier, which may have had some impact on the results. Researchers can identify and adjust for this bias in a variety of ways, such as using data collected in the early stages of the disease or applying weighting methods to adjust for survivorship. Nonetheless, completely eliminating survivor bias is challenging, so this issue should be carefully considered when interpreting the results of MR studies.

This paper discusses possible solutions to address this problem.

1. Integration of early cohort data: Guided by Elston's intention-to-treat principle (193), researchers should prioritize the incorporation of longitudinal cohorts with early disease phenotypes to capture participants prior to mortality-driven attrition, thereby minimizing attrition bias. When including early cohorts (such as UK Biobank baseline data (194)), focus should be placed on incident PCa cases to avoid reliance on prevalent cases that may overrepresent indolent cancers.
2. Consider implementing genetic risk stratification: Categorize subgroups through Genetic Risk Score (GRS) stratification to identify PCa cases with accelerated progression, as their shorter disease latency periods reduce survival-related attrition, thereby potentially mitigating survival bias (195, 196).
3. Methodological adjustments: To address survivor bias in mortality-related exposure effects, researchers could implement strategies under semi-parametric additive hazard models as proposed by Vansteelandt et al. (197). This approach enables dynamic adjustment for survival selection through lifetime modeling of genetic exposure effects, rather than relying solely on cross-sectional data snapshots.

### 4.1.4 Linkages between MR studies

The connections among several studies addressing similar research questions are weak, and some articles present contradictory views. For example, in studies exploring the correlation between LTL and PCa risk, one study showed that a shorter LTL was related to a reduced risk of PCa (146). However, other studies have suggested that a shorter LTL is detrimental to patient prognosis and that PCa patients with higher Gleason scores have shorter LTLs (147). The authors could review the relevant literature to discuss the plausibility of a potential causal connection between exposure and outcome, to interpret the results, or to suggest possible biological mechanisms (198). A lack of harmonization is observed among MR studies addressing similar research questions.

### 4.1.5 Standardization of reporting

Recent methodological advancements emphasize the critical need for standardized reporting frameworks in MR studies. The

STROBE-MR checklist provides a 32-item guideline to enhance methodological transparency (199, 200). These guidelines provide actionable resources for researchers to refine methodological rigor and enhance the translational value of causal inference studies.

### 4.2 Summary and outlook

There has been significant progress in applying MR to male-specific diseases, offering novel insights into etiological mechanisms and paving the way for innovative preventive and therapeutic strategies. The clinical translation of MR findings can directly inform actionable approaches, including: (1) lifestyle interventions (BMI, smoking, and dietary optimization) for personalized prevention; (2) microbiome-targeted therapies (probiotics/nutritional modulation) in high-risk groups; (3) drug repurposing and development guided by genetic evidence; (4) early screening protocols based on genetic risk networks (For example, CVD and T2DM can serve as early screening recommendations for high-risk populations of ED); and (5) precision therapies (e.g., anti-inflammatory agents) leveraging causal biomarker profiles.

With the increasing abundance of genomics data, the number of IVs that can be used in MR studies is increasing, and the accuracy and resolution of the studies will continue to improve. In addition, with the increase in computational power and the continuous improvement of statistical methods, more complex genetic modeling problems are expected to be solved, and the potential of MR in the study of male-specific diseases is promising.

Current MR studies on male-specific diseases should further improve the quality of study design, pay attention to the standardization of reporting and linkage with previous large MR studies, rigorously validate the results, and make appropriate adjustments for possible bias. In addition, interdisciplinary cooperation should combine expertise in genetics, epidemiology and clinical medicine and make reasonable assumptions and comprehensive interpretations of potential causality by combining the biological mechanisms of diseases and evidence from observational cohort studies in an effort to provide a valuable basis for research on the etiology of male-specific diseases as well as for the formulation of preventive policies in public health.

## Author contributions

QP: Conceptualization, Data curation, Methodology, Writing – original draft, Writing – review & editing. ZC: Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. HL: Investigation, Methodology, Writing – review & editing. JC: Funding acquisition, Resources, Supervision, Writing – review & editing. SM: Methodology, Project administration, Supervision, Writing – review & editing. CZ: Funding acquisition, Resources, Supervision, Writing – review & editing. ZS: Funding acquisition, Resources, Supervision, Writing – review & editing.

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# The relationship between dietary inflammation potential, dietary oxidative balance score, and female reproductive function: a mediation analysis of obesity indicators

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**Background:** Dietary inflammation and oxidative stress have been linked to reproductive health, and weight gain has also been found to play an important role in female reproductive function specifically. In this study we explore the effects of dietary inflammatory index (DII) and dietary oxidative balance score (DOBS) on infertility and sex hormones, and clarify the mediating role of obesity on these effects.

**Methods:** A total of 1,774 subjects from the 2013-2016 National Health and Nutrition Examination Survey (NHANES) were selected. The DII and DOBS were determined by aggregating data on 26 and 17 dietary components obtained by dietary recall, respectively. Infertility (yes or no, self-reported in questionnaire) and serum gonadal hormones including total testosterone (TT), estradiol ($E_2$), and sex hormone-binding globulin (SHBG) were considered as main outcome variables. Multivariate logistic regression and restricted cubic splines (RCS) were applied for further analysis, and mediation models were constructed to figure out the mediating role of obesity indicators.

**Results:** The prevalence of infertility among American women of childbearing age was 12.66%. There were significant linear relationships between the DII and DOBS, and infertility and serum SHBG ($p$ for overall < 0.05). Consuming foods with higher DII was significantly associated with higher risk of infertility (OR: 1.86; 95% CI: 1.20-2.89) and lower levels of SHBG (β: -9.98; 95% CI: -19.45–0.51). Compared to the lowest DOBS category, the adjusted beta estimates for SHBG and $E_2$ were 12.03 (95% CI: 2.01-22.04) and 134.58 (95% CI: 3.46-266.24) in the highest DOBS group. However, anti-inflammatory and anti-oxidative diets reduced the risk of infertility by 51% and increased SHBG levels by 14.54 nmol/L. Interestingly, obesity indicators mediated the associations of dietary inflammation and oxidative stress potential with infertility and SHBG.

**Conclusions:** Dietary inflammation and oxidative stress are strongly associated with the risk of infertility and serum SHBG levels, indicating that anti-inflammatory and anti-oxidative diets may mitigate fertility disorders that result from obesity.

KEYWORDS

**dietary inflammation, infertility, oxidative stress, national health and nutrition examination survey (NHANES), mediation analysis**

# 1 Introduction

Infertility is a disease of the male or female reproductive system defined by the failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse and is a major health challenge globally (1). According to a World Health Organization (WHO) report published in 2023, the lifetime prevalence of adult infertility worldwide is approximately 17.5% (1). The prevalence of infertility among married women aged 15-44 in the U.S. rose from 6.7% in 2011-2015 to 8.7% in 2015-2019, according to the National Center for Health Statistics (2). Polycystic ovary syndrome (PCOS) is the most common cause of anovulatory infertility, and sex steroid hormones and related binding proteins have been linked to its prevention and diagnosis (3–5). Inflammation and oxidative stress play important roles in reproduction generally speaking as well. Several studies have demonstrated that pro-inflammatory cytokines and reactive oxygen species alter estrous cyclicity, steroidogenesis, and ovulation, increasing the risk of infertility (6, 7), and diet also has the potential to regulate inflammation and oxidative stress (8).

Dietary inflammatory index (DII) and dietary oxidative balance score (DOBS), calculated based on the inflammatory and oxidative effects of dietary nutrients, are ideal indicators for assessing the effects of diet on inflammation and oxidative stress (9, 10) and have been shown to predict pertinent biomarkers for biological aging (11). In female adolescents in particular, strong associations between DII with SHBG and TT have been reported (12). Moreover, a longitudinal study revealed that pro-inflammatory diets can put women at risk for infertility (13). Nevertheless, the majority of prior studies focused on the individual effects of dietary anti-inflammatory potential. The combined effects of dietary inflammation and oxidative stress on reproductive health therefore warrant further investigation.

Obesity has likewise been shown to be an important factor in women's reproductive health. Previous studies have found that insulin resistance and abnormal lipid metabolism caused by being overweight and obese can affect the secretion of female reproductive hormones, lead to abnormal ovulation, and increase the incidence of female infertility (14, 15). Whether a weight-loss diet can regulate sex hormone levels and improve female infertility is also attention question worth answering. Based on the above relationship between diet, obesity, and reproductive function, one may naturally assume that the relationships between dietary inflammatory and oxidative potential and female reproductive function may be mediated by obesity.

In order to elucidate the combined effects of dietary anti-inflammatory and anti-oxidative potential on female reproductive function, we analyzed the relationships between DII, DOBS, DII&DOBS and reproductive function indicators including infertility, SHBG, $E_2$, and T. Further, obesity indicators were included into the models as mediating variables. The data were sourced from the NHANES cycles of 2013-2016.

# 2 Materials and methods

## 2.1 Study population

The NHANES is a complex, stratified, and multistage study conducted by the National Center for Health Statistics (NCHS) in an effort to understand the nutritional and health status of Americans. Detailed information about NHANES is publicly available at https://www.cdc.gov/nchs/nhanes/index.htm. Prior to the survey, all protocols received approval from the Institutional Review Board of the NCHS, and informed consent was obtained from all participants.

This cross-sectional study was based on the 2013-2016 cycle of the NHANES and included women aged 20-45 years. Individuals fulfilling any of the following criteria were excluded: (a) males (n=9,895); (b) age< 20 or age> 45 (n=7,548); (c) missing data for DII/DOBS components (n=342); or (d) missing outcomes or mediating variables (n=584). After applying these filters a total of 1,774 participants were included in the final analysis (Figure 1).

## 2.2 Assessment of DII

In the NHANES, dietary nutrient intake was assessed by a 24-hour dietary review. Participants were asked about the types and amounts of food they had consumed by professional technicians. The intake of each food component was then estimated using the University of Texas Food Intake Analysis System and the USDA Survey Nutrient Database (16).

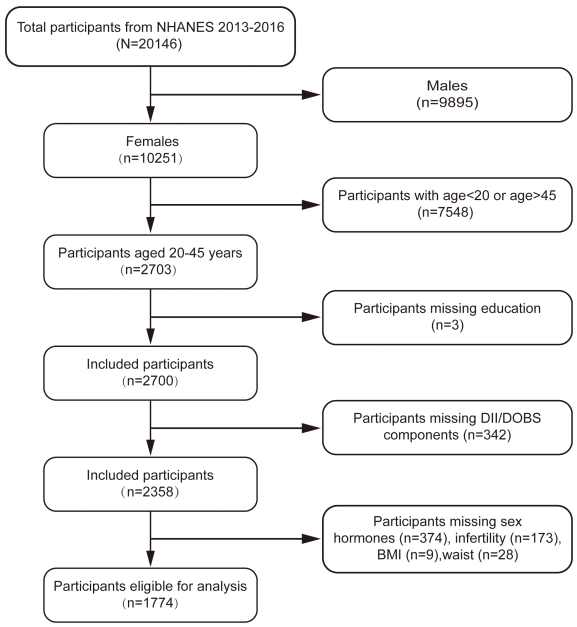


FIGURE 1
Flowchart depicting participant selection.

The DII has been described in detail in previous studies (10) and is based on literature review and population data. The score considered 45 dietary components associated with dietary inflammatory potential and their representative dietary intake ranges. In this study, 26 of the above 45 dietary components were selected for the DII calculation. Previous studies have shown that the predictive power of DII remains stable when it is calculated using fewer than 30 dietary components (17). Details of the dietary components used to calculate the DII can be found in Supplementary Table S1. First, the Z-score of a certain dietary component was calculated as follows: (daily intake - global daily mean intake)/the standard deviation * the overall inflammatory effect score. Second, this value was converted to a percentile. To achieve a symmetrical distribution with values centered on 0 and bounded between -1 (maximally anti-inflammatory) and 1 (maximally pro-inflammatory), each percentile score was doubled and then 1 was subtracted from the result. Finally, the DIIs of the 26 individual dietary components were added together to obtain the participant's DII. Higher DIIs indicate greater dietary inflammatory potential.

## 2.3 Assessment of DOBS

The DOBS was calculated by combining predetermined scores of pro- and anti-oxidative factors from the diet, considering a total of 17 nutrients, including 3 pro-oxidants and 14 anti-oxidants. These nutrients have been well-established in previous studies for their role in determining the DOBS (11, 18). Anti-oxidants were scored from 1 to 3, and pro-oxidants were scored inversely after categorizing the dietary variable into tertiles. Alcohol intake was categorized a nondrinker, nonheavy drinker, and heavy drinker, with scores of 3, 2, and 1, respectively. Additionally, vitamin C, beta-carotene, and vitamin B12 were log-transformed for better distribution approximation. Detailed information on the scoring system for DOBS components can be found in Supplementary Table S2. The participant's DOBSs were calculated as the sum of scores for each dietary ingredient selected.

We also set DII&DOBS as a variable to evaluate the combined effect of the two indicators in order to study the potential effects of diets with pro-inflammatory and pro-oxidative properties versus diets with anti-inflammatory and anti-oxidative proper-ties on female reproductive function (11, 18). Participants were divided into three groups based on their DII and DOBS values: the pro-inflammatory and pro-oxidative diet group (those in the third tertile of DII and the first tertile of DOBS), the anti-inflammatory and anti-oxidative diet group (those in the first tertile of DII and the third tertile of DOBS), and the composite diet group (those who did not fall in either of the above two groups).

## 2.4 Assessment of serum sex hormones, SHBG and infertility

The outcome variable of infertility was derived from each woman's self-report on the Reproductive Health Questionnaire (RHQ074). When asked "Have you ever attempted to become pregnant over at least a year without becoming pregnant?" Women who responded "yes" were labeled as "ever-infertile" and those who responded "no" were labeled as "fertile" (19).

Blood samples were drawn from all study participants' antecubital veins by a trained phlebotomist, and serum specimens were stored under -30°C before being shipped to the National Center for Environmental Health for testing. Serum TT and $E_2$ were measured using isotope dilution liquid chromatography tandem mass spectrometry (ID-LC-MS/MS), and SHBG was quantified based on the reaction of SHBG with immuno-antibodies and chemo-luminescence measurements of the reaction products via a photomultiplier tube. Details of the analytical methodology can be found in the laboratory procedure manual on the NHANES website (20, 21).

## 2.5 Assessment of BMI, WC, and covariates

The mediating variables considered in this study were BMI and waist circumference (WC). WC was measured at the iliac crest by a tape measure to the nearest millimeter (22), and BMI was calculated as weight in kilograms divided by the square of height in meters. According to previous research, individuals with a BMI≥30.0 kg/$m^2$ were classified as "obese" and the rest as "nonobese", and women with a WC≥ 88 cm were labeled as "high-WC" (23).

The confounders adjusted in this study included age (years), race (nonHispanic White/nonHispanic Black/Mexican American/other race/ethnicity), education level (below high school/high school degree/some college or AA degree/college graduate or above), marital status (married or living with partner/live alone),

family income to poverty ratio (PIR) (< 1/≥ 1/miss), smoking status (never smoker/former smoker/current smoker), physical activity (≥ 600MET/< 600MET), total energy intake (kcal), total cholesterol (mmol/L), triglyceride (mmol/L), LDL-cholesterol (mmol/L), HDL-cholesterol (mmol/L) and insulin resistance index (HOMA-IR). HOMA-IR = fasting insulin (mU/L) * fasting glucose (mmol/L)/ 22.5 (24).

## 2.6 Statistical analysis

Individual sample weights were established based on the NHANES recommended sample weight on dietary day-one (WTDRD1) records. During a baseline characteristic analysis, continuous variables were expressed as weighted means (standard errors), and categorical variables were expressed as sample numbers (weighted percentages). To examine differences in the characteristics of variables between the DII, DOBS, and different combinations of DII and DOBS values, univariate analysis was performed using ANOVA for differences in weighted means of continuous variables and the chi-squared test was carried out for differences in weighted percentages of categorical variables in order to characterize the total population. Moreover, to study the effects of obesity, we stratified the data by obesity and high-WC, and further analyzed the characteristics of DII, DOBS, and DII&DOBS in infertile and fertile groups.

The continuous variables SHBG, TT, and $E_2$ were transformed into binary variables according to their relation to the median. Binomial logistic regression models were then used to investigate the associations between DII, DOBS, and different combinations of DII and DOBS with infertility and sex hormones. Subsequently, the best fit dose-response curves for the correlations between DII and DOBS with indicators of reproductive function were shaped by restricted cubic spline (RCS) regression. Multivariate linear regression models were also performed to assess relationships between DII, DOBS, and different combinations of DII and DOBS with continuous indicators of reproductive function (SHBG, TT, and $E_2$) and traditional obesity (BMI and WC). All of the above regression models were adjusted for age, race, education, marital status, PIR, smoking status, physical activity and total energy intake.

Mediation analysis was used to identify the mediation effects of obesity indicators on the relationships between dietary inflammation and oxidative stress potential and female reproductive function. All statistical analysis was conducted with R 4.3.3, and a two-sided $p$ value < 0.05 was assumed to indicate a statistically significant test result for all tests.

# 3 Results

## 3.1 Baseline characteristics

Baseline characteristics of individuals grouped by DII tertile, DOBS tertile, and different combinations of DII and DOBS are shown in Supplementary Table S3. The mean age of subjects was 32.53 ± 0.30 years. Of the participants, 55.71% were non-Hispanic white, and the weighted prevalence of infertility was 12.66%. With the exception of physical activity and obesity, there were significant differences in study variables between different DII/DOBS tertiles ($p$< 0.05). Participants consuming pro-inflammatory and pro-oxidative diets were more likely to be Hispanic white, to have lower education, to have lower wealth, to have less physical activity, to have higher BMI and WC, to have lower total energy intake, to live alone, and to smoke ($p$< 0.05).

In the stratified analysis, significant differences in DII and DOBS between the infertile and fertile groups were only observed in women with obesity and high-WC ($p$< 0.05), suggesting that obesity indicators may be crucial factors (Table 1).

## 3.2 Multiple logistic regression analysis

Figure 2 shows the relationship between DII, DOBS, DII&DOBS and infertility and sex hormones as determined by binomial logistic regression. In the model that adjusts for all confounding, the highest DII tertile was associated with increased risk of infertility and low-SHBG. For DOBS, participants in T3 had significantly lower risk of infertility (OR: 0.62, 95% CI 0.39-1.01) and higher SHBG levels (OR: 1.62, 95% CI 1.20-2.19) compared to those in T1. Additionally, women who consumed anti-inflammatory and anti-oxidative diets had lower risk of infertility (OR: 0.49, 95% CI 0.28-0.82) and higher SHBG levels (OR: 1.67, 95% CI 1.19-2.33). After further adjusting the current model for total cholesterol, triglyceride, LDL-cholesterol, HDL-cholesterol and HOMA-IR, the association between anti-inflammatory and anti-oxidative diets and infertility remained significant (OR: 0.43, 95% CI 0.18-0.95). For obesity indicators, when DII increased by one unit, the risks of obesity and high-WC increased by 1.15 and 1.12 times, respectively. Furthermore, an anti-inflammatory and anti-oxidative diet was a protective factor for obesity (OR: 0.62, 95% CI 0.44-0.87) and high-WC (OR: 0.63, 95% CI 0.44-0.91) (Supplementary Figure S1).

## 3.3 RCS plots of correlations between DII/ DOBS and reproductive function indicators

In RCS regression, after adjusting for different covariates, we detected significant linear relationships between continuous DII/ DOBS and infertility risk and dichotomous SHBG ($p$ for overall <0.05, $p$ for nonlinear >0.05, Figure 3).

## 3.4 Multivariate linear regression analysis

In the multivariate linear regression models that adjusted for all confounding, there was a strong negative correlation of DII with SHBG (β=-2.85, 95% CI: -5.15–0.55) and strong positive correlations of DII with BMI (β=0.52, 95% CI: 0.27-0.77) and

TABLE 1 Characteristics of DII, DOBS, and DII&DOBS in fertile and infertile groups stratified by obesity indicators[a].

| Dietary type | Non-obese (n=1046) | | | Obese[c] (n=728) | | |
|---|---|---|---|---|---|---|
| | Fertile | Infertile | $p^d$ | Fertile | Infertile | *p* |
| **DII**[b] | 1.04 (0.10) | 1.13 (0.21) | 0.68 | 1.35 (0.12) | 1.81 (0.14) | 0.018 |
| **DOBS**[b] | 34.61 (0.35) | 35.09 (0.67) | 0.50 | 34.08 (0.45) | 32.42 (0.53) | 0.018 |
| **DII&DOBS (%)** | | | 0.24 | | | 0.11 |
| Pro-inflammatory and pro-oxidative diet | 229 (22.74) | 22 (20.13) | | 201 (29.96) | 42 (37.89) | |
| Anti-inflammatory and anti-oxidative diet | 246 (26.62) | 21 (20.99) | | 142 (23.52) | 21 (15.51) | |
| Composite diet | 479 (50.64) | 49 (58.88) | | 264 (46.52) | 58 (46.60) | |
| **Dietary type** | **Normal-WC[b] (n=671)** | | | **High-WC[c] (n=1103)** | | |
| | Fertile | Infertile | *p* | Fertile | Infertile | *p* |
| **DII** | 0.94 (0.11) | 0.94 (0.11) | 0.63 | 1.31 (0.10) | 1.64 (0.14) | 0.038 |
| **DOBS** | 34.84 (0.38) | 34.78 (1.02) | 0.96 | 34.10 (0.34) | 33.19 (0.50) | 0.11 |
| **DII&DOBS (%)** | | | 0.45 | | | 0.16 |
| Pro-inflammatory and pro-oxidative diet | 132 (20.78) | 14 (23.49) | | 298 (28.69) | 50 (32.30) | |
| Anti-inflammatory and anti-oxidative diet | 169 (28.20) | 12 (20.71) | | 219 (23.53) | 30 (16.98) | |
| Composite diet | 314 (51.02) | 30 (55.80) | | 429 (47.78) | 77 (50.72) | |

[a]Values are presented as weighted mean (SE) for continuous variables, and n (%) for categorical variables.
[b]DII, dietary inflammatory index; DOBS, dietary oxidative balance score; WC, waist circumference.
[c]Individuals with a BMI ≥30.0 kg/m$^2$ were classified as "obese", and women with a WC ≥ 88 cm were labeled as "high-WC" (23).
[d]Two independent sample *t*-test was used for differences in weighted means of continuous variables, and the chi-squared test was used for differences in weighted percentages of categorical variables.

WC (β=1.18, 95% CI: 0.61-1.75). DOBS was positively associated with SHBG (β=0.93, 95% CI: 0.28-1.59) and TT (β= 0.23, 95% CI: 0.02-0.44) and was inversely correlated with BMI (β=-0.08, 95% CI: -0.15–0.01), WC (β=-0.16, 95% CI: -0.33–0.01). An anti-inflammatory and anti-oxidative diet was positively correlated with serum SHBG and negatively correlated with BMI and WC, and the beta estimates (95% CIs) were 14.54 (3.35 to 25.74), -1.67 (-2.90 to -0.44), and -4.15 (-6.92 to -1.38). The results of the multivariate linear regression analysis are shown in Figure 4 and Supplementary Figure S2.

## 3.5 Associations between obesity indicators and reproductive function

Multivariate linear regression and logistic regression models were developed to evaluate the associations between obesity indicators and infertility risk and sex hormone levels. BMI (OR=1.03, 95% CI: 1.01-1.05) and WC (OR=1.02, 95% CI: 1.01-1.03) were strongly related to a higher risk of infertility after adjusting for confounders (Figure 5). More specifically, BMI was negatively associated with SHBG (β=-1.53, 95% CI: -1.95–1.11) and positively associated with TT (β=0.14,

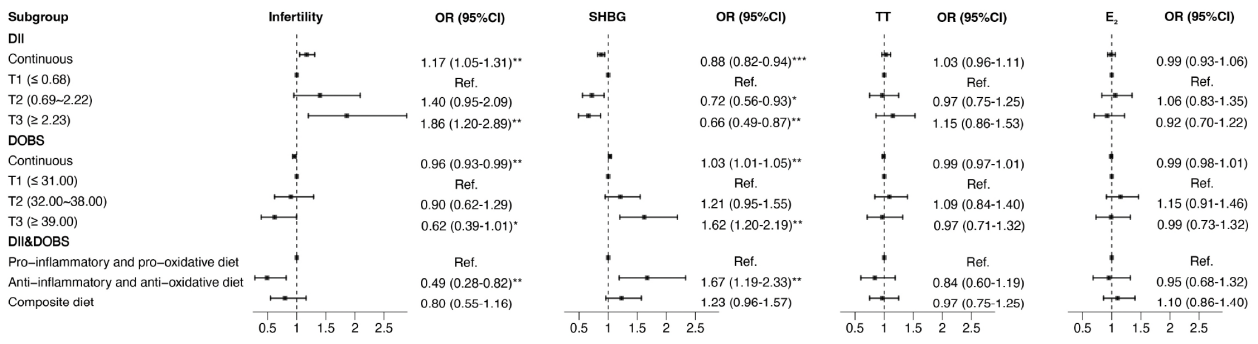


FIGURE 2
Forest plot of the associations of DII, DOBS, and DII&DOBS with indicators of reproductive function in binomial logistic regression models. SHBG, TT, and $E^2$ were transformed into binary variables by comparing them to their respective medians. Models were adjusted for age, race, education, marital status, PIR, smoking status, physical activity, and total energy intake. *$p$<0.05, **$p$<0.01, ***$p$<0.001. T, tertile; SHBG, sex hormone-binding globulin; TT, total testosterone; $E_2$, estradiol; PIR, family income-to-poverty ratio.

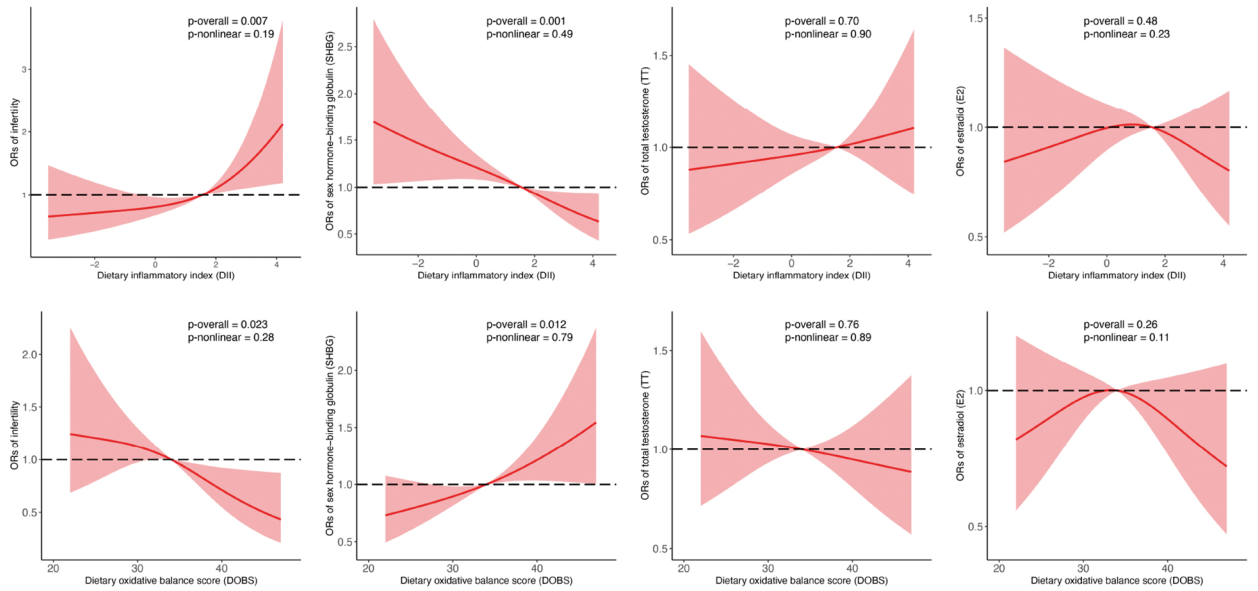


FIGURE 3
Associations between DII, DOBS, and reproductive function indicators analyzed by binomial logistic regression and RCS. SHBG, TT, and $E_2$ were transformed into binary variables by comparing them to their respective medians. Models were adjusted for age, race, education, marital status, PIR, smoking status, physical activity, and total energy intake. SHBG, sex hormone-binding globulin; TT, total testosterone; $E_2$, estradiol; PIR, family income-to-poverty ratio. Central estimates are represented by solid red lines, and shaded areas indicate 95% CIs.

95% CI: 0.01-0.27). The beta estimates (95% CI) between WC and SHBG, TT, and $E_2$ were -0.59 (-0.78 to -0.40), 0.06 (0.01 to 0.12) and 2.58 (0.10 to 5.05), respectively, after adjusting for covariates (Figure 5, Supplementary Figure S3).

## 3.6 Mediating effect analysis

Mediation statistical models were performed to ascertain whether BMI and WC had mediation effects on any of the above associations. The proportions of the indirect effects on DII, DOBS, anti-inflammatory and anti-oxidative diet, and infertility mediated by BMI were measured at 9.92, 6.49, and 7.36, respectively. Similarly, the percentages of the indirect influences on DII, anti-inflammatory and anti-oxidative diet, and infertility mediated by WC were determined to be 11.77 and 9.48, respectively (Figures 6, 7). Except for the effect of DOBS on SHGB, BMI and WC also significantly mediated the associations of dietary inflammation and oxidative stress scores with SHBG (Supplementary Figures S4, S5).

# 4 Discussion

This study demonstrates that the dietary potential for inflammation and oxidative stress, as indicated by scores derived from pro-inflammatory and anti-oxidative dietary components in an individual's diet, are linked to an increased risk of infertility and lower levels of SHBG. Additionally, the significant mediating effect

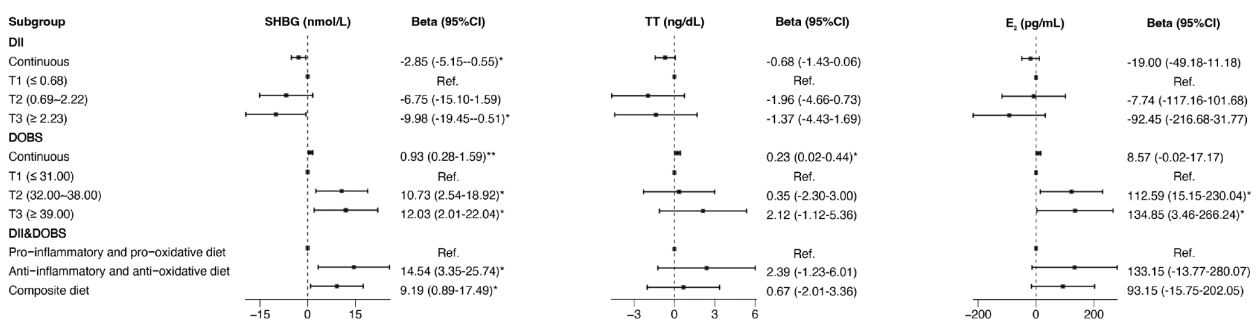


FIGURE 4
Forest plot of the associations of DII, DOBS, and DII&DOBS with SHBG and sex hormones in multivariate linear regression models. Models were adjusted for age, race, education, marital status, PIR, smoking status, physical activity, and total energy intake. *$p$<0.05, **$p$<0.01. T, tertile; SHBG, sex hormone-binding globulin; TT, total testosterone; $E_2$, estradiol; PIR, family income-to-poverty ratio.

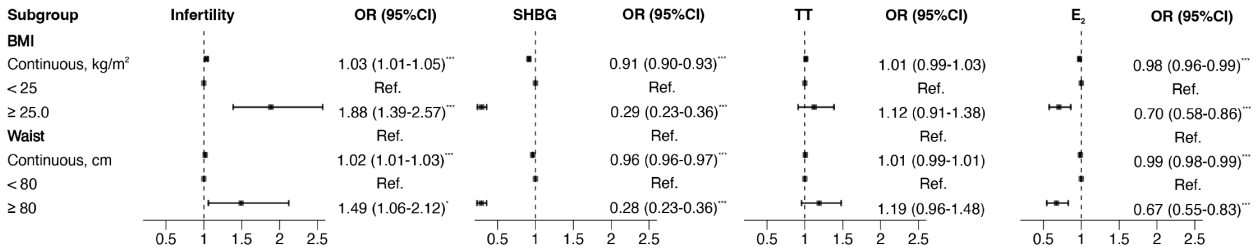


FIGURE 5
Forest plot of the associations of BMI and WC with indicators of reproductive function in binomial logistic regression models. SHBG, TT, and $E_2$ were transformed into binary variables by comparing them to their respective medians. Models were adjusted for age, race, education, marital status, PIR, smoking status, physical activity, and total energy intake. *$p$<0.05, ***$p$<0.001. SHBG, sex hormone-binding globulin; TT, total testosterone; $E_2$, estradiol; BMI, body mass index; WC, waist circumference; PIR, family income-to-poverty ratio.

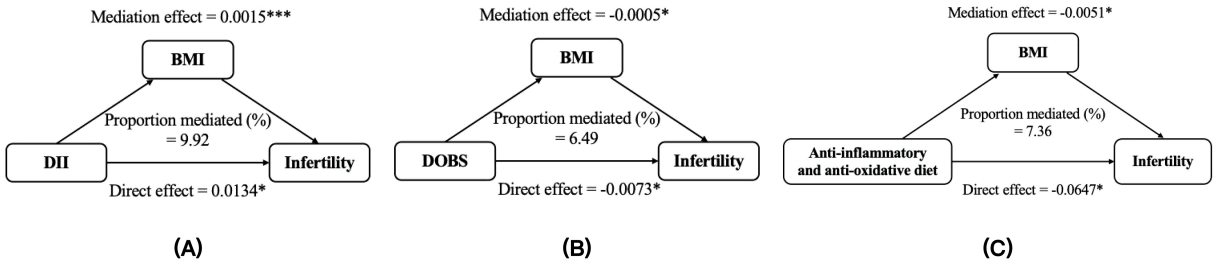


FIGURE 6
BMI mediated the relationships between DII **(A)**, DOBS **(B)**, anti-inflammatory and anti-oxidative diet **(C)** and infertility. Models were adjusted for age, race, education, marital status, PIR, smoking status, physical activity, total energy intake. * $p$ < 0.05, *** $p$ < 0.001. DII, dietary inflammatory index; DOBS, dietary oxidative balance score; BMI, body mass index.

of BMI and WC suggests that anti-inflammatory and/or anti-oxidant diets for weight loss may be an effective way to prevent infertility and low SHBG levels.

Previous studies have shown that consumption of high DII diets is associated with increased risk of chronic inflammation activation, obesity, PCOS, and infertility (25–27). DOBS is associated with rheumatoid arthritis, biological aging, and all-cause and disease-specific mortality (11, 28, 29). Teng and colleagues built a nomogram model based on anti-oxidative intake (vitamin C, vitamin E, selenium) and showed significant predictive capacity for stroke risk with an area under the curve (AUC) of 77.4% (76.3%-78.5%) (30). However, there may be a synergistic effect between dietary inflammation and oxidative stress components, and there are currently few articles that consider their joint effects. In this paper, we found that an anti-inflammatory and anti-oxidative diet may be a protective factor for female reproductive function. Compared to a pro-inflammatory pro-oxidative diet, an anti-inflammatory anti-oxidative diet reduced the risk of infertility by 51% and increased levels of SHBG by 14.54 nmol/L. Decreased SHBG levels increase the bioavailability of androgens, which in turn leads to progression of ovarian pathology, anovulation, and the phenotypic characteristics of PCOS. SHBG has been found to be an early biomarker and therapeutic target for PCOS

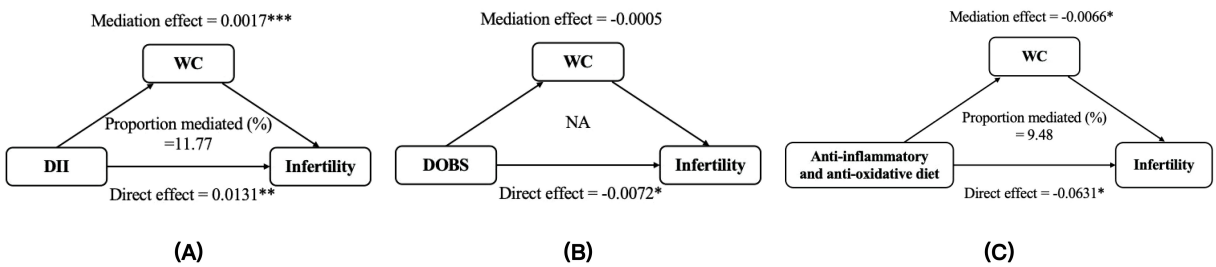


FIGURE 7
The mediation analysis of WC on the relationships between DII **(A)**, DOBS **(B)**, anti-inflammatory and anti-oxidative diet **(C)** and infertility. Models were adjusted for age, race, education, marital status, PIR, smoking status, physical activity, total energy intake. * $p$ < 0.05, ** $p$ < 0.01, *** $p$ < 0.001. DII, dietary inflammatory index; DOBS, dietary oxidative balance score; WC, waist circumference

(4). From a public health perspective, our findings suggest that an anti-inflammatory and anti-oxidative diet is a potential independent factor affecting reproductive health. Promoting such a diet may reduce the risk of infertility in the population.

Inflammation and oxidative stress in the body are influenced by dietary and lifestyle factors (31–33). One previous study has suggested that anti-inflammatory and anti-oxidative components in the diet may affect the balance of inflammation and oxidative stress (11), and positive associations have observed between DII/DOBS values and levels of CRP, IL6, TNF-α in the blood (34). Previous studies have shown that chronic inflammatory activation and oxidative stress may contribute to infertility as well (33). Oxidative stress can inhibit the expression and secretion of SHBG by down-regulating HNF-4α, which may be an important factor promoting the occurrence of hyperandrogenemia in PCOS (35).

Our study reveals the associations between dietary inflammation and oxidative stress potential and obesity. In mediating effect analysis, the significant mediation effect indicating that obesity indicators, specifically BMI and WC, serve as important pathways through which anti-inflammatory and anti-oxidative diets influence infertility. This finding underscores the importance of dietary interventions aimed at obese women, emphasizing the need to prioritize anti-inflammatory and antioxidant components to enhance reproductive health. In addition, the significant direct effect suggesting that there are other potential mechanisms besides obesity. For instance, pro-inflammatory and pro-oxidative diets such as unhealthy hypercaloric diets, excessive consumption of saturated and trans-fatty acids, and processed foods can lead to insulin resistance and diabetes, which are major causes of increased risk of infertility and hormonal disorders in women (36, 37). It has also been confirmed in mice that were fed a high-fat diet that the mRNA expression levels of oxidative stress markers (such as ROS) in oocytes were significantly increased, and the levels of anti-oxidative markers (such as GSH-Px, SOD, and CAT) were decreased. Oxidative stress was activated, which damaged mitochondrial function, led to oocyte apoptosis, and ultimately reduced oocyte quality (38).

In fact, many studies have demonstrated a causal relationship between obesity and impaired reproductive function. For example, a Mendelian randomization study showed that BMI, waist-to-hip ratio (WHR), and WHR adjusted for BMI may cause PCOS (39). Compared to age-matched controls, obese women had a higher risk of ovulatory sub-fertility and anovulatory infertility (40–42). Although anovulation can be overcome with ovarian stimulation, obese women have decreased responsiveness to gonadotropins, decreased oocyte retrieval, decreased oocyte quality, reduced rates of pre-implantation embryo development, and increased risk for miscarriage compared to their lean counterparts (15, 40, 43). Hunter et al. conducted a meta-analysis of randomized controlled trials (RCTs) on the effectiveness of weight loss interventions in improving obesity and infertility outcomes and found that women randomized to a combination of diet and exercise intervention were more likely to become pregnant, risk ratio (RR) = 1.87 (95% CI 1.20, 2.93) and achieve a live birth RR = 2.20 (95% CI 1.23, 3.94) (44). Furthermore, several studies using rodent models have shown that obesity has a negative impact on ovarian function. For example, rats fed a high-fat diet gained weight and had irregular estrus cycles, manifested by longer estrous intervals and shorter estrus periods (45, 46). Additionally, in obese mice and rats, increased ovarian granulosa cell apoptosis induces follicular atresia, which reduces egg number, egg quality, and ovaria-related hormone synthesis (47). All of these research results suggest that the impact of obesity on female reproductive function cannot be ignored.

In the univariate analysis stratified by obesity indicators, significant differences in DII and DOBS were only found in women with obesity or high-WC. Additionally, obesity indicators mediated the association between anti-inflammatory and anti-oxidative diets and infertility (BMI: 7.36% and WC: 9.48%). Our results specifically indicate that significant anti-inflammatory and anti-oxidative diets that reduce BMI and WC may be an effective way to prevent and treat infertility. Beyond that, compared with only considering a single indicator, stronger association were observed between anti-inflammatory and anti-oxidative diets and infertility, indicating a possible combined effect of DII and DOBS. In clinical interventions, particularly for obese women, we recommend the consumption of foods rich in antioxidants and anti-inflammatory properties, such as fresh fruits and vegetables, fruits, vegetables, legumes and dietary unsaturated fats (48, 49). Additionally, it is advisable to reduce the intake of high-fat, sugar, ultra-processed foods, red meat (50, 51).

The present study has several notable strengths. First, the NHANES was based on a large sample population and a complex sampling design, and the infertility rate was nationally representative. Second, this study explored the linear and nonlinear associations between dietary inflammation index and oxidative balance score with infertility and reproductive hormones, and further examined the potential mediating role of obesity, thereby providing a treatment possibility for obesity-related reproductive dysfunction.

However, the study also has some limitations. First, due to the cross-sectional study design, a causal association cannot be established and needs to be explored in future, longitudinal studies. Second, the 24-hour dietary recall is dependent on respondents' memories, making it potentially subject to recall bias. Thirdly, the generalizability of our findings needs to be further validated in populations outside of NHANES. Fourth, in addition to WC and BMI, indicators such as percentage of body fat and visceral fat area can be included in future studies to reflect obesity more comprehensively.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The studies involving humans were approved by ethics review board of the National Center for Health Statistics. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

## Author contributions

ML: Data curation, Formal analysis, Writing – original draft. XW: Validation, Visualization, Writing – review & editing. YX: Validation, Writing – review & editing. YW: Visualization, Writing – review & editing. BL: Data curation, Writing – review & editing. HX: Methodology, Software, Supervision, Writing – review & editing. ZL: Conceptualization, Methodology, Project administration, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2025.1517318/full#supplementary-material

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# Potential effects of environmental toxicants on sperm quality and potential risk for fertility in humans

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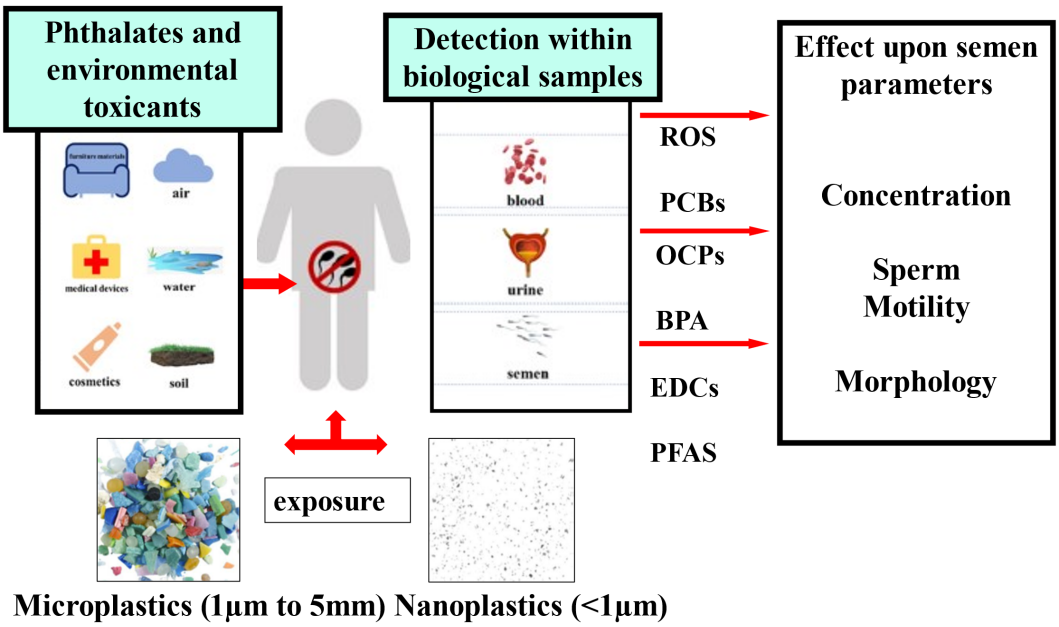
Semen quality is a key factor in male fertility, but defining normal reference values for semen parameters remain challenging. Over the past four decades, several authors have reported a noticeable decline in sperm parameters, raising concerns about male reproductive health. While the exact causes remain unclear, potential contributors include environmental pollution, endocrine disruptor chemicals (EDCs) and oxidative stress, with the latter becoming a growing concern. Environmental changes and increased exposure to EDCs, such as pesticides, herbicides, bisphenol A (BPA), phthalates, polychlorinated biphenyls (PCBs), and heavy metals, are believed to contribute significantly to the decline in sperm quality. These chemicals impact individuals from prenatal life through adulthood, potentially leading to long-term reproductive consequences. Overall, this review explores the relationship between environmental toxicants, including volatile organic compounds, EDCs, as well as oxidative stress and reduced male fertility. While a substantial body of research has found associations between these exposures and adverse fertility outcomes, some studies have reported no significant associations. The primary objective of this review is to provide a deeper understanding of the potential mechanisms between these environmental chemicals on testicular function and spermatogenesis. It also examines the broader evidence on the decline in sperm quality and explores its potential implications for overall fertility outcomes in humans. By doing so, the review will shed light on the broader public health implications of environmental pollutants and their impact on male reproductive health, emphasizing the need for further research in this critical area.

KEYWORDS

**male infertility, sperm parameters, assisted reproductive technology, environmental pollution, oxidative stress**




GRAPHICAL ABSTRACT
The figure illustrates the effect of environmental toxicants and plastics (macro and micro) on human reproduction and sperm quality. EDCs, Endocrine disrupting chemicals; PFAS, poly- and per-fluoroalkyl substances; BPA, bisphenol A; OCPs, organochlorinated pesticides; PCBs, polychlorinated biphenyls; ROS, reactive oxygen species.

## Introduction: the global human sperm decline

Several published studies have recently highlighted a relevant decline in sperm count, nearly halved over the past few decades (1–6). Though the evidence for a decline in sperm parameters may be considered equivocal, male reproduction nevertheless seems to be at high risk. Various factors seem to contribute to this, including nutrition, environmental pollution, as well as an increase in cryptorchidism and testicular cancer, potentially linked to exposure to environmental estrogen-like endocrine disruptors (7, 8). Exposure to endocrine disruptors or high estrogen levels might cause a temporary reduction in testosterone, which has been reported by several authors, and may thereby impair male fertility (1, 3, 5). Notably, men generally do not normally seek medical attention for reproductive health unless they experience issues or difficulties in becoming parents. Interestingly, data have proposed a link between male subfertility and overall health status (9–15). A Danish study of 4,712 men found that semen analysis could serve as a biological marker for long-term morbidity and mortality, particularly related to cardiovascular alterations and diabetes mellitus (16). Additionally, men with low sperm number and motility were more likely to be hospitalized for several different pathologies and illnesses compared to those men with normal semen parameters. Among these hospitalized individuals, those with a sperm concentration of 195-200 million/ml were, on average, firstly admitted to hospital seven years later than counterparts with a sperm number less than 1 million/ml. The authors concluded that those results were independent of socioeconomic status and lifestyle factors, suggesting that normal sperm assessment could be associated with general health status in adult men (17). These findings were further supported by Capogrosso and co-workers (1).

## Environmental factors and sperm quality

Increased rates of infertility appear to coincide with rising global pollution. About 8–12% of couples worldwide experience infertility, with male factors being the predominant cause in up to 50% of cases (18). Male infertility affects about 7% of men worldwide (19). It is caused by a multitude of factors, including hormonal, genetic, behavioral, iatrogenic, environmental, and lifestyle variables, as well as congenital defects (20). Given that environmental toxins are widespread in today's world, pollution has become a major factor contributing to the rising trend of male infertility (21, 22). The primary indicator of male fertility is semen quality (23). It has been observed that spermatogenesis, steroidogenesis, and sperm function are adversely affected by environmental pollution, which lowers male fertility and harms semen quality (24, 25). There is limited information regarding the direct effects of environmental chemicals on human spermatogenesis, even though chemicals found in industrial waste, pesticides, insecticides, herbicides, food additives, and

other substances seem to adversely affect spermatogenesis in adult men. The available studies are mainly conducted in workplace settings, where individuals are exposed to these chemicals at high concentrations, rather than in the general population (26, 27).

## Air pollution

Air pollution has recently become a global concern, contributing to respiratory (28), cardiovascular (29), skin-related (30), and reproductive diseases (31, 32). Recent studies indicate that air pollution has a major effect on human fertility and sperm quality (21, 22, 33, 34). In India, ranked third for air pollution and with the second largest population (35), pollutants such as particulate matter, volatile organic compounds, ozone, nitrogen oxides, sulfur dioxide ($SO_2$), carbon monoxide (CO), and radiation such as X-ray exposure, are major health threats (35, 36). Particulate matter, particularly PM10 (particles ≤ 10μm in diameter) is extremely dangerous, and enters the lungs and bloodstream after inhalation, leading to serious health issues (37). Finer particles, like PM2.5 (particles ≤ 2.5μm) present an even greater risk to health (34). Air pollution has been linked to increased sperm DNA fragmentation, sperm morphological alterations, and decreased sperm motility (38). A meta-analysis revealed a substantial negative correlation between air pollution levels and semen volume, sperm concentration, total sperm motility, morphology, and the DNA fragmentation index (22). A recent study on gaseous pollutants shows that exposure to $SO_2$ considerably reduces sperm parameters across all exposure windows (39). Both $SO_2$ and nitrogen dioxide ($NO_2$) significantly affect sperm concentration and motility, especially during the early stages of spermatogenesis. A study by De Rosa and collaborators found that tollgate workers exposed to car exhaust had lower total sperm motility than nearby residents (40). Lead and nitrogen oxides from vehicle exhaust significantly impaired sperm quality. Calogero and co-authors reported that tollgate workers had high levels of sperm DNA fragmentation and damaged sperm chromatin compared to healthy, unexposed men (41). Ozone, a major air pollutant, is linked to defective sperm morphology, with increasing numbers of men reporting infertility due to abnormal sperm morphology (42). PM2.5, a primary cause of haze, has also been implicated in male infertility (22, 33, 34, 43). Studies show that sperm exposed to PM2.5 exhibit a higher frequency of morphological defects and cytoplasmic droplets (44). Additionally, sperm motility, concentration, total sperm count, sperm head shape, and overall semen quality are negatively correlated with PM2.5 exposure (45). Although the precise mechanisms by which air pollution causes male infertility are still unclear, several factors may help explain this link:

a. Heavy Metals and PAHs: Car exhaust contains heavy metals like lead, zinc, and copper, as well as polycyclic aromatic hydrocarbons (PAHs), which have estrogenic, antiestrogenic, and antiandrogenic properties. These chemicals might impair gametogenesis and gonadal steroidogenesis, leading to infertility (46). PM2.5 accumulation in reproductive organs via placental and blood-testis barriers can also disrupt hormone levels and contribute to infertility (47).
b. Oxidative Stress: Increased oxidative stress induced generation of reactive oxygen species (ROS), which results in lipid peroxidation, fragmentation of sperm DNA, and infertility (46).
c. DNA Damage and Epigenetic Changes: Changes in gene expression and DNA methylation result in male infertility because of sperm DNA alteration brought on by the creation of DNA adducts, particularly with exposure to PAHs (46, 48).

# Endocrine-disruptor chemicals

Many chemical compounds commonly used in daily life have the potential to impact the vertebrate neuroendocrine system, which plays a crucial role in maintaining homeostasis and regulating essential processes such as development, growth, metabolism, and reproduction (49, 50). Over recent years, the release of various chemical pollutants, including pesticides, flame retardants, alkylphenols, polychlorinated biphenyls (PCBs), phthalates, and metals has significantly increased. Chemicals that mimic or interfere with the actions of naturally occurring hormones are classified as endocrine disrupting chemicals (EDCs) (51). These EDCs are defined as exogenous agents that disrupt the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body. EDCs consist of a wide range of both natural and synthetic substances, most of which are released into natural waters due to anthropogenic activities. They enter living organisms through various routes, including air, soil, water and food, with the aquatic environment serving as the primary route of transmission. Once in the water, these substances can bioaccumulate through the food chain, which increases human exposure, particularly through the consumption of fish and seafood (52). Most of the time, environmental contaminants are typically transferred to humans unintentionally during daily activities, mainly absorbed through the skin, inhalation or ingestion (53, 54). Over 90% of the overall amount of chemical exposure occurs through dietary intake, which is the primary pathway for EDCs and other compounds to enter the human body (55). The adverse effects of these chemicals on the reproductive function of aquatic species is well documented, an example being the significant decline of fish populations in freshwater systems (56–59). Also, there is evidence suggesting that EDCs may be responsible for a skewed sex ratio at birth, with a higher incidence of male births being observed in some populations (60–64). Among this group of chemicals, steroidal estrogens (e.g. estrone, 17β-estradiol and 17α-ethinylestradiol) and phenolic xenoestrogens (e.g. alkylphenols and bisphenol A) are of particular concern (65). The growing concern over environmental

chemicals is largely due to their association with various human health disorders, including testicular cancer, falling sperm counts, endometriosis, precocious puberty, and breast cancer (66). It is well-established that organisms have evolved sensitivity to both endogenous and exogenous chemical signals, allowing them to adapt to physical, chemical or biological stimuli while maintaining internal homeostasis. However, this sensitivity to environmental cues also makes organisms vulnerable to inadvertent and potentially harmful chemical signals from the surrounding environment (67). The long-term exposure to EDCs raises critical concerns about the risks to human health. As these chemicals accumulate in the environment, the risk to both wildlife and human populations becomes more evident, especially related to reproductive function. As such, the growing body of evidence underscores the need for increased regulation of these chemicals, particularly those that are known to exhibit endocrine-disrupting properties (51, 65, 68–71). Additionally, certain EDCs, also known as "obesogens," have been implicated in the promotion of obesity, insulin resistance, and increased risk of type II diabetes (72–74). These metabolic disorders, in turn, seem to be significant risk factors for cardiovascular disease (75, 76). The effects of EDCs also extend to bone metabolism. Some persistent organic pollutants (POPs) have been shown to alter the processes involved in bone development and turnover, likely through their estrogenic and anti-estrogenic actions (77, 78). Additionally, numerous EDCs have been shown to either depress the immune system or cause hyper-immunity, leading to altered immune responses to infections and an increased risk of cancer (72, 79). Although it is still up for debate whether the effects of EDCs on the immune system qualify as "endocrine" effects, there is no denying that they pose a serious risk to human health. The widespread presence of these chemicals in the environment and their ability to interfere with critical hormonal functions pose a significant threat to public health. Understanding the mechanisms by which EDCs impair reproductive function is essential for developing effective strategies to mitigate their impact on both wildlife and human populations. As research continues to reveal the full extent of these chemicals' effects, public awareness and policy action will be key in reducing exposure and minimizing health risks associated with EDCs (Green51, 70, 71).

## Effect of endocrine disruptors on sperm quality

Several studies have provided substantial evidence that EDCs can mimic or block steroid hormones by acting as their agonists or antagonists, disturbing normal hormone-regulated processes, particularly those related to sexual development and reproduction (80–82). EDCs, various compounds capable of disturbing the endocrine system in both wildlife and humans, have raised significant concern among the public and toxicologists (68, 69, 83, 84). Environmental pollutants, such as organochlorinated pesticides (OCPs) and PCBs have been linked to "endocrine disruptor" effects (85, 86). These POPs harm human health in several ways, causing birth defects and posing neurotoxic, hepatotoxic, nephrotoxic, immunotoxic, and carcinogenic consequences (72, 87). When the term "endocrine disruption" was first introduced in 1991, research mainly focused on the estrogenic effects of these chemicals, leading to their initial classification as xenoestrogens (88, 89). Various *in-vivo* and *in-vitro* studies have reported the presence of many substances with estrogenic, anti-estrogenic, androgenic, and anti-androgenic properties (90, 91). The adverse reproductive effects of EDCs are well-documented, as they interfere with endocrine function by blocking receptor activity. These regulatory processes are crucial and closely linked to sperm production (14, 15, 51, 70, 71, 92–94). Moreover, sperm production and quality are regulated at multiple levels. The hypothalamus releases gonadotropin-releasing hormone (GnRH), which in turn stimulates the anterior pituitary gland to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Disruption at any stage of this process can lead to damage in sperm quality. Figure 1 illustrates the main points where EDCs exert their influence. Testicular damage may involve increased spermatocyte apoptosis due to Sertoli cell dysfunction or the overexpression of apoptotic proteins (95, 96). Sertoli cells nourish developing spermatocytes, removing excess cytoplasm and promoting testosterone-driven spermatogenesis. When Leydig cells fail to produce testosterone, androgen receptor-mediated gene transcription necessary for spermatogenesis might be impaired. Some research indicates that EDCs such as BPA may inhibit ATP production (97), potentially by disrupting mitochondrial function, which could reduce sperm motility. Additionally, an altered hormonal environment caused by EDCs might contribute to aneuploidy in sperm and potential transgenerational effects. However, many of these proposed mechanisms require further validation through clinical studies to better understand how EDCs affect male infertility (97, 98). As a result, EDCs pose significant risks to both human and environmental health, particularly concerning reproductive function. Their ability to interfere with natural hormone function emphasizes the need for further research and stronger regulations. As evidence of their harmful effects increases, it is critical to prioritize public health policies aimed at reducing exposure and mitigating the long-term consequences of endocrine disruption on both wildlife and human populations.

## Pollution by plastics and plasticizers

Using machine learning and probabilistic material flow analysis, it has been estimated that the world produces 52 million metric tons of macroplastics per year, with significant levels of plastic pollution accumulating within our environment (99). The production of macroplastics continues unabated, despite the dire warning of their threat to reproductive health (100). Plasticizers having an approximate half-life of six hours, such as BPA, do not bioaccumulate in the body (101, 102) and are excreted through urine. Plasticizers are commonly found in populations; in the US,

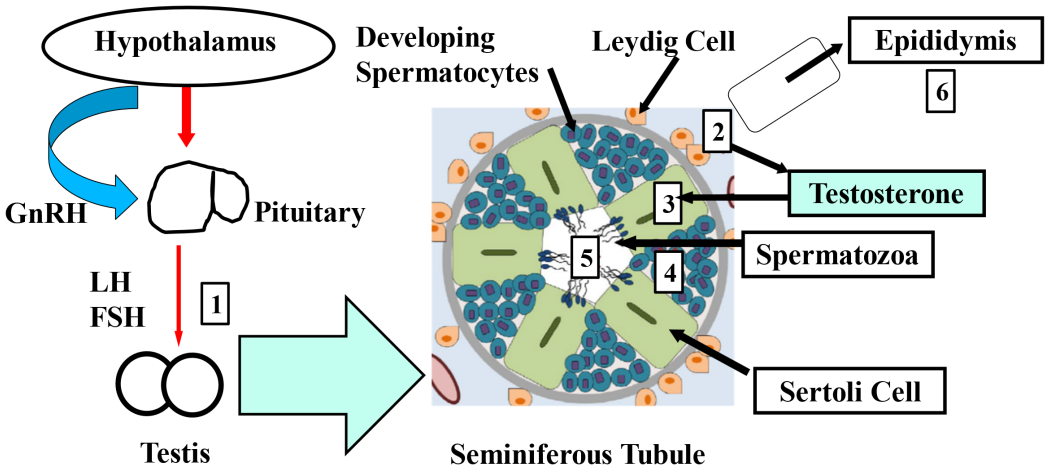



FIGURE 1
The primary mechanisms through which EDCs affect sperm quality are outlined as follows 1-6. GnRH: gonadotropin-releasing hormone, LH: luteinizing hormone, FSH: follicle-stimulating hormone. (1) Interference with testicular gonadotropin receptors, (2) disruption of Leydig cell steroidogenesis, (3) damage to Sertoli cells, (4) inhibition of spermatocyte development, (5) disruption of mature sperm, (6) alteration of epididymal sperm modification.

92.6% of individuals aged 6 and older have BPA present in their body (103). Even though plasticizers are not retained in adipose tissue, everyday exposure to these compounds raises questions about their potential to affect hormones (102). Humans may also inhale certain environmental pollutants that have volatilized and become contaminated (24, 104). Inhalation can be a significant mode of exposure, particularly for volatile and semi-volatile substances (105). Apex predators, such as polar bears, are frequently found to contain environmental contaminants. It is believed that the presence of EDCs in the tissues of animals living in remote locations indicates that these chemicals are distributed over great distances via both air and ocean currents (24, 106). An essential habitat for possible exposure to airborne particles and chemicals is the indoor living or working space. Another important way that workers in hazardous environments might become contaminated is through occupational exposure to EDCs (105). One of the primary EDCs, extensively used in the production of epoxy resins, hard polycarbonate plastics, and many other materials, is BPA. By binding competitively to many kinds of estrogen receptors, BPA imitates the effects of estrogen (107). It has also been found that BPA decreased serum levels of thyroid hormone, hormone production, and the release of hypothalamic steroid hormones (108), lowered levels of male gonadotropin hormone (109), and caused abnormal embryonic development and impaired implantation (110). In a similar vein, chronic BPA exposure has been shown to affect ovarian reserve in non-pregnant mice (111) and disrupt the estrous cycle (112). Additionally, a different investigation found that low concentrations of BPA induced oxidative stress in the testis *in-vitro* (96). Due to public health concerns about the toxic effects of BPA, its application is restricted especially in the US and is replaced by "BPA-Free" products that contain substitutes such as bisphenol-F, bisphenol-B and bisphenol-S (BPS) (113).

## Microplastics, nanoplastics and sperm quality

Microplastics (1μm to 5mm in diameter) and nanoplastics (<1μm in diameter) can either be manufactured as such (primary) or form as a result of the breakdown of larger plastics (secondary), defined by both their size and chemical composition. When exposed to natural environmental forces, such as mechanical friction, extreme heat, and ultraviolet radiation, plastics undergo physical and chemical aging, breaking down into smaller particles, typically within the nanometer to micrometer range in diameter. These smaller particles can then be widely distributed across the environment, appearing in the atmosphere, soil, oceans, and even in the food and water we consume (6). Additionally, microplastics can adsorb and release POPs and toxic heavy metals, facilitating their transport and potential bioaccumulation in the environment. Polyethylene (PE), polypropylene (PP) and polystyrene (PS) are the most common microplastic polymers found in the marine environment. Studies have confirmed the presence of microplastics in human feces (114) and urine (115), demonstrating that they can be ingested, are small enough to cross cell membranes, and can be excreted. In a study involving Italian volunteers Raman microspectroscopy identified several types of microplastics in urine, including polyethylene vinyl acetate (PVA), polyvinyl chloride (PVC), PP, and PE (115). Given our increasing exposure to microplastics in daily life, there is a growing concern regarding their potential negative impacts on reproductive health and male fertility (116). With respect to neuroendocrine control of male reproduction, a significant inverse correlation between the dosage and duration of exposure to PS microplastics and serum levels of FSH, LH and testosterone, has been observed in male rats and mice (117–119). After just 24 hours exposure to

environmental levels (100μg/L and 1mg/L) of PS microplastics within the drinking water, PS microparticles accumulated within the testis, with chronic exposure leading to testicular inflammation, disruption of the blood-testis barrier (BTB), and a decline in testosterone serum levels (117). Furthermore, sperm morphology, DNA integrity and viability were also impaired. *In-vitro* studies using primary cultures of mouse Leydig cells showed that PS microplastics adhered to and were internalized by these cells, causing downregulation of the LH receptor, steroidogenic acute regulatory protein (StAR), and steroidogenic enzymes, resulting in a decrease in testosterone production (119). The BTB is essential for maintaining male reproductive function and is generally considered impermeable to most toxicants (120). However, nanoplastics in particular, have been shown to accumulate within Sertoli cells (121). Interestingly, several studies have demonstrated that PS microplastics reduce the expression of various proteins critical to BTB integrity, including basal ectoplasmic specialization protein, β-catenin, claudin-11, connexin-43, N-cadherin, occludin, and zona occludens-1 (122, 123). Moreover, PS microplastics induce oxidative stress, damage seminiferous tubules, and cause apoptosis in spermatogenic cells, which results in reduced sperm concentration and motility, as well as increased abnormal sperm morphology (122). One potential mechanism by which microplastics compromise BTB integrity is through the suppression of the mammalian target of rapamycin (mTOR)/protein kinase B (also known as Akt) pathway via their generation of ROS. In this respect, mTOR and focal adhesion kinase (FAK) regulate F-actin organization within the cytoskeleton of the BTB (124), and PS microplastics have been shown to disrupt this regulation via generation of ROS (123). PS microplastics have also been detected in the epididymis of all bulls tested, with a mean concentration of 0.37μg/mL (125). Furthermore, *in-vitro* exposure to comparable concentrations of PS reduced bovine sperm motility and impaired blastocyst development, with evidence of increased formation of ROS and apoptosis (125). Using the mouse model, several studies have demonstrated that both PS micro- and nanoplastics can disrupt perinatal testicular development, reduce fertility, and even cause infertility in the male (126–128). In a more recent study, it was demonstrated that daily oral ingestion of PS microplastics (1mg/dL or 3mg/dL) for 28 or 56 days resulted in their detection within the testis (129). After 56 days of exposure to either concentration of PS, there was a significant decrease in sperm count and motility, along with a marked increase in sperm morphological abnormalities. Clinical studies on the impact of microplastics on male factor infertility are scarce. However, a multi-site study conducted in China examined the association between mixed exposure to microplastics and dysfunction of spermatogenesis (130). Semen and urine samples were collected from 113 participants across three regions. Using Raman microscopy, microplastics were detected in all semen and urine samples, with the highest detection rates for PS, PE and PP. Interestingly, polytetrafluoroethylene (PTFE) exposure was significantly associated with decreased semen quality, including decreased sperm count and concentration. Additionally, multi-linear regression analysis showed that exposure to each additional polymer type correlated with a significant decrease in total sperm count, concentration and progressive motility (130). Another recent study used advanced sensitive pyrolysis-gas chromatography/mass spectrometry to quantify 12 types of microplastics within the testis of the human and the dog (131). Microplastics were found to be present within all testis samples, with significant inter-individual variability. The mean concentration of microplastics was 122.63 μg/g in the dog testis and 328.44μg/g in the human testis. Interestingly, a negative correlation was observed between the presence of specific polymers such as PVC and polyethylene terephthalate (PET) and the normalized weight of the testis (131). Of even greater concern, nanoplastics are likely to be more pervasive than microplastics due to their smaller size and larger surface area-to-volume ratio, which enhances their ability to adsorb and release EDCs and toxic heavy metals. Consequently, further research is urgently required to determine whether different particle sizes and different polymers have differential impacts on male and female fertility.

## Perfluoroalkyl and polyfluoroakyl substances and sperm assessment

One diverse group of POPs, known as poly- and per-fluoroalkyl substances (PFAS), is represented by thousands of synthetic perfluorinated organic chemicals (PFCs) typically used in the manufacture of non-stick cookware and food packaging. Though some more persistent longer carbon chain PFAS, such as perfluorooctanoate (PFOA) and perfluorooctane sulfonate (PFOS), known as “forever chemicals”, have been phased out in manufacturing since the turn of the century, their past usage on a grand scale has resulted in their bioaccumulation and ubiquitous persistence within the environment. Alarmingly, epidemiological evidence has long associated exposure to PFAS with testicular dysgenesis, including testicular cancer and impaired semen quality (132). Though a later systematic review of their impact on human fertility proved equivocal, in the male at least (133), a more recent meta-analysis has revealed that concentrations of PFOA and perfluorononanoic acid (PFNA) are inversely associated with sperm progressive motility (134). Furthermore, exposure to PFOA *in utero*, measured in maternal blood samples from week 30 of pregnancy, has been associated with higher levels of gonadotrophins (FSH and LH) in the systemic circulation, and reduced sperm count and concentration in 169 adult male offspring (135). A similar investigation of 864 young men from the Fetal Programming of Semen Quality (FEPOS) cohort was conducted recently (136). First trimester plasma samples from their mothers were retrieved from the Danish National Biobank and were analyzed for the presence of up to 15 PFAS. Using weighted quantile sum regression and negative binomial regression, combined maternal exposure to PFAS was associated with lower sperm concentration, count, and higher non-progressive sperm motility and immotility in their offspring (136). Therefore, coincident with the global decline in male fertility, the enduring presence of PFAS should be of great concern to reproductive health specialists (137). Epidemiological studies are, by nature, plagued by

multiple confounding factors, making it difficult to assign causality but, nevertheless, provide large data sets for examining possible associations between PFAS and sperm quality. One such study, investigated the possible association between the serum levels of 10 different perfluoroalkyl acids (PFAAs) and testicular function in 105 men from the general population (138). Using liquid chromatography-tandem mass spectrometry with electrospray ionization, it was found that men with high combined levels of PFOA and PFOS had a significantly lower median total normal sperm count of 6.2 million in their ejaculate versus 15.5 million in men with a low combined level of PFOA and PFOS (138). A multi-geographical study investigated PFAS and their possible association with reproductive hormones and sperm quality in 604 partners of pregnant women (139). There was a slight increase in sex hormone-binding globulin (SHBG) and *in situ* terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL), suggestive of reduced bioavailability of testicular steroids and increased sperm DNA fragmentation. However, no consistent evidence was found for a significant correlation between exposure to PFAS and sperm DNA fragmentation, apoptosis, or reproductive hormones (139). However, a later study did find a significant negative association between exposure to PFCs and sperm quality (140). Contamination with PFCs was observed within the whole blood of 58% of subjects and this was associated with a significant increase in alteration of semen parameters compared to those in whom PFCs were not detected. Furthermore, using fluorescent *in situ* hybridization (FISH) for chromosomes 18, X and Y, and TUNEL coupled to flow cytometry for sperm DNA fragmentation, sperm disomy and diploidy rates, and the DNA fragmentation index were significantly increased in PFC-positive versus PFC-negative individuals (140). Another study included the male partners of 501 couples planning pregnancy (141). Men had blood collected and provided a baseline semen sample plus another approximately one month later. Using tandem mass spectrometry, seven PFCs (perfluorosulfonates, perfluorocarboxylates, and perfluorosulfonamides) were quantified within the serum. After adjusting for confounders and modelling repeated semen samples, linear regression analysis showed that perfluorooctane sulfonamide (PFOSA) was associated with smaller sperm heads, lower DNA stainability and higher bicephalic and immature spermatozoa (141). A study specifically focused on the Pearl River delta, a region in China labelled one of the "world factories," investigated PFAAs within the blood and semen of 103 participants (60). These men were found to have higher levels of PFAAs than men in other regions within China. Also, there was a significant inverse correlation between the levels of perfluoro-n-pentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluorobutanoic acid (PFBA), perfluorobutanesulfonic acid (PFBS), perfluorohexanesulfonic acid (PFHS), PFOA, and PFOS with sperm motility (60). In a broader study, matched semen and serum samples were collected from 664 men from a cross-sectional population of couples undergoing their first assessment of fertility (142). Using mass spectrometry, 16 target PFAS were analyzed and their association with semen quality parameters was evaluated by multivariable linear regression analysis. Seminal PFOA, PFOS and emerging chlorinated polyfluorinated ether sulfonate (6:2 Cl-PFESA) were significantly associated with a lower percentage of progressively motile spermatozoa and a higher percentage of sperm DNA fragmentation (142). The mechanism by which PFAS impair sperm quality is largely unknown. However, a recent *in-vitro* study using exposure of spermatozoa to environmentally relevant concentrations of a cocktail of PFAS has attempted to address this in the mouse model (143). Interestingly, a three-hour exposure to PFAS *in-vitro* did not affect the sperm functional profile, in terms of capacitation or fertilization rates, but did significantly delay the developmental progression of *in-vitro* fertilized day 4 preimplantation embryos, which suggested an alternative stress-mediated impact at fertilization. Clearly, further research is warranted to identify the mechanisms and threats that PFAS present to male fertility and human health.

## Oxidative stress and sperm quality

Recent studies have highlighted that one of the major causes of testicular damage is oxidative stress, which leads to an increase in the production of ROS (Figure 2). This imbalance can result in significant changes in protein production and DNA damage in testicular cells (144, 145). The production of ROS is a condition in which the natural equilibrium between oxidants and antioxidants is disrupted, causing the generation of free radicals. These free radicals contain an uneven number of electrons, making them highly reactive with other chemical compounds or molecules. This reactivity triggers a cascade of chemical reactions than can be toxic to cells and tissues, including gametes and embryos (146–149). Increased ROS at the level of the testis have also been associated with dysfunction in site-specific hypermethylation. This occurs either through the upregulation of DNA methyltransferases (DNMTs) or the formation of new complexes involving these enzymes (148, 150, 151). It is important to emphasize that sperm epigenetic changes due to oxidative stress may be secondary to additional factors, including sperm manipulation during ART treatment, or individual patient characteristics, such as age, health conditions, or lifestyle (150, 152, 153). Nevertheless, research indicates that oligozoospermic men tend to experience more pronounced epigenetic changes compared to men having normal sperm parameters (154–156). Moreover, an increase in seminal ROS production and a corresponding decrease in antioxidant enzyme activity have been linked to a variety of sperm alterations, including chromosomal abnormalities, micronuclei formation, changes in sperm membrane potential, as well as an increased rate of apoptosis and DNA fragmentation (148, 149, 151, 157–159). Sperm DNA damage caused by ROS has serious implications for embryogenesis, potentially leading to increased risks of implantation failure or miscarriage. The extent of sperm DNA damage, whether through single or double-strand breaks, can influence the ability of the oocyte to repair the damage at fertilization or post fertilization, impacting the overall chances of a successful pregnancy (160). The primary targets of ROS-induced damage in the testis include Leydig

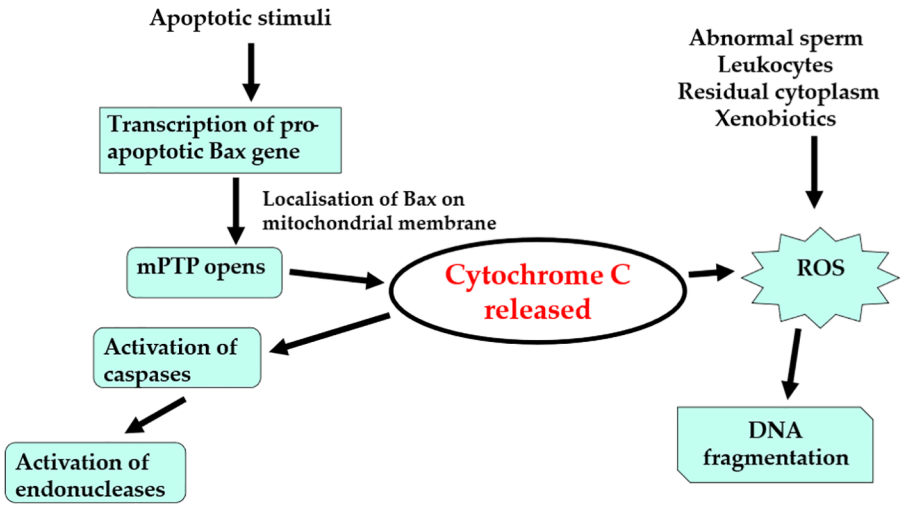



FIGURE 2
Concurrent pathways involved in ROS, and sperm DNA damage/fragmentation. Adapted from (144). ROS: reactive oxygen species; DNA: deoxyribonucleic acid; mPTP: mitochondrial permeability transition pore.

cells, seminiferous tubules, and spermatozoa. ROS disrupt the normal function of Leydig cells, leading to decreased testicular steroidogenesis and reduced testosterone, which in turn affects spermatogenesis and can lead to infertility (149, 157, 158, 160). In a study by Desai and collaborators (161), it was shown that increased ROS levels can induce sperm DNA damage and even sperm death. Another study found a strong association between oxidative stress, increased lipid peroxidation, and dysfunction in the body's antioxidant defense functions. The sperm plasmalemma is particularly vulnerable to lipid peroxidation due to its high content of polyunsaturated fatty acid. The byproducts of lipid peroxidation can damage the sperm plasmalemma, disrupt the function of mitochondrial proteins involved in the electron transport chain, and ultimately impair sperm motility and fertilization potential (162). It is well established that sperm motility, acrosome reaction, and binding to the oocyte are highly sensitive to oxidative stress. ROS can induce a loss of membrane fluidity and compromise the integrity of the sperm membrane, both of which are crucial for successful fertilization (163). This loss of membrane integrity can significantly impair sperm function, reducing motility, compromising the acrosome reaction, and hindering the sperm's ability to bind effectively with the oocyte during fertilization. As a result, oxidative stress plays a critical role in both male fertility and reproductive outcomes, influencing sperm quality and the ability to conceive. Interestingly, recent research has focused on the potential for antioxidants to mitigate the effects of ROS on sperm and overall testicular health. Antioxidant supplementation, such as with vitamins C and E, has been shown to reduce oxidative stress in sperm, potentially improving sperm motility and DNA integrity. However, the effectiveness of such interventions remains debated, and more research is needed to understand the optimal dosages and specific antioxidants that may benefit male fertility (164, 165). Moreover, the balance between oxidative stress and antioxidant defense mechanisms is highly dynamic, and what might be beneficial in one context may not be effective in another. Ultimately, the growing body of evidence underscores the importance of oxidative stress as a major factor influencing male fertility. Both environmental and lifestyle factors can increase ROS production, leading to sperm DNA damage and compromised fertility. The complex interplay between oxidative stress, epigenetic modifications, and sperm function highlights the need for further research into the mechanisms underlying male infertility. By understanding these processes more thoroughly, strategies can be developed to mitigate oxidative stress and its impact on sperm quality, providing hope for improved fertility treatments and outcomes for men struggling with infertility.

## Oxidative stress and mitochondrial function

Mitochondrial function is vital for reproductive health, as mitochondria provide the energy needed for sperm motility, essential for navigating the female reproductive tract and fertilizing the oocyte. Disruptions in mitochondrial metabolism, particularly within the electron transport chain, can be caused by an excessive production of ROS (166, 167). Increased mitochondrial ROS production is a key factor in sperm DNA fragmentation, reducing sperm viability and fertilization potential (168). DNA damage is a significant cause of male infertility, as it affects embryo development and increases the risk of miscarriage. The impact of mitochondrial dysfunction extends beyond DNA damage and motility issues. ROS can also disrupt important sperm functions by overwhelming antioxidant defense systems. Seminal enzymes like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) protect sperm by neutralizing ROS. These antioxidants help maintain a balance between ROS production and clearance. However, when mitochondrial ROS production exceeds the capacity of these

protective enzymes, sperm cells become vulnerable to oxidative damage (163, 169). Damaged sperm membranes further impair motility, survival, and the acrosome reaction, a crucial process for fertilization. If the sperm cannot undergo the acrosome reaction, it cannot penetrate the egg's outer layers, making fertilization impossible. In ART, where sperm quality is critical, oxidative stress poses a significant challenge, and sperm impairment might lead to a decrease in fertilization rate and poor embryo development. Managing ROS levels is crucial in ART: and usage of antioxidants can protect sperm from oxidative damage by neutralizing excess ROS and supporting mitochondrial function. Supplementation with antioxidants such as vitamins C and E, coenzyme Q10, and N-acetylcysteine has been explored as a way to reduce oxidative stress and improve sperm quality in men undergoing fertility treatments (164, 170–172). By restoring the balance between ROS production and antioxidant defense, these strategies may enhance sperm quality and increase the chances of successful fertilization. However, the clinical use of antioxidants is still under investigation. Optimal dosages, specific antioxidants, and timing of supplementation require further research to understand how best to support mitochondrial function and sperm quality in assisted reproduction. Finally, mitochondrial function is critical for sperm health: excessive ROS production damages sperm DNA, impairs motility, and compromises membrane integrity, negatively impacting fertility. Understanding and managing the balance between ROS and antioxidants is essential for improving outcomes in ART. By addressing oxidative stress with targeted antioxidant therapies, it may be possible to mitigate ROS effects on sperm function, ultimately enhancing fertility treatments for couples struggling with infertility.

## Conclusive remarks

This manuscript addresses the growing concern that environmental pollution negatively affects sperm quality, contributing to the decline in male reproductive health. Environmental toxins, such as heavy metals, pesticides, industrial chemicals, EDCs, microplastics, and oxidative stress, may impair hormonal balance, sperm production, and fertility. Over time, these toxins accumulate in the body, leading to reduced sperm count, motility, and DNA fragmentation, which significantly affect fertility (164). These toxins are commonly found in contaminated air, water, and food, posing a persistent risk to male reproductive health. Air pollution is another major factor influencing male infertility. Prolonged exposure to pollutants like particulate matter, $NO_2$, and CO can harm sperm quality. These pollutants generate ROS, which might damage sperm DNA, membranes, and mitochondria. Oxidative stress reduces sperm motility, viability, and the likelihood of successful fertilization (163, 173). With air pollution widespread in urban areas, its impact on male fertility must be further investigated. EDCs, found in pesticides, plastics, and personal care products, disrupt hormonal systems involved in reproduction. These chemicals mimic or block hormones like estrogen and testosterone, which are critical for sperm production and function. Phthalates, BPA, and other plasticizers have been linked to reduced sperm count, motility, and testosterone levels (174, 175). Given the ubiquity of EDCs in everyday products, avoiding exposure is challenging, and their long-term effects on male fertility are still under investigation. Microplastics, present in nearly every ecosystem, pose a growing threat to male reproductive health. Their small size allows them to be ingested by humans and animals through food and water. Recent studies show that microplastics can accumulate in human tissues, including the testis, potentially contributing to oxidative stress and disrupting sperm function. Oxidative stress is a central mechanism through which many environmental factors, such as toxins, air pollution, EDCs, and microplastics, contribute to male infertility. An imbalance between ROS production and antioxidant defenses damages sperm DNA, impairs motility, and reduces fertilization potential. Increased exposure to environmental pollutants exacerbates oxidative stress, further compromising sperm quality. In response to these concerns, scientists and public health experts urge governments to prioritize male reproductive health by increasing research funding and implementing policies to reduce harmful environmental exposures. Large-scale studies are necessary to establish definitive links between environmental factors and male infertility. Governments must also regulate substances like industrial chemicals, air pollution, and plastics to protect male reproductive health. Urgent action is needed to mitigate these environmental hazards and safeguard reproductive health for future generations.

## Author contributions

RS: Conceptualization, Writing – original draft, Writing – review & editing. PG: Conceptualization, Data curation, Investigation, Visualization, Writing – original draft. EG: Conceptualization, Data curation, Investigation, Writing – original draft. LT: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft. FE: Conceptualization, Data curation, Investigation, Resources, Visualization, Writing – original draft. SF: Conceptualization, Methodology, Writing – review & editing.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# The association between season and meteorological factors on clinical outcomes after fresh embryo transfer: a cohort study from Central Plains, China

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**Objective:** To investigate whether seasonal variations and meteorological factors influence pregnancy outcomes in women undergoing *in vitro* fertilization/Intracytoplasmic sperm injection and embryo transfer(IVF/ICSI-ET) treatment.

**Materials and methods:** We conducted a retrospective cohort study of women who underwent IVF/ICIS-ET for the first time at the Reproductive Medicine Center of the First Affiliated Hospital of Zhengzhou University from January 1, 2011, to December 1, 2021. A total of 24420 cycles were collected. They were divided into four groups according to the oocyte retrieval date. The main outcome measures were clinical pregnancy rate and live birth rate. Binary logistic regression was used to explore the factors affecting clinical pregnancy rate and live birth rate.

**Results:** In this study, the live birth rate of cycles with oocyte retrieval performed in summer was significantly higher than those in spring, autumn, and winter (61.24% [4859/7934] vs. 59.09% [3074/5202] vs. 58.89% [3676/6242] vs. 57.70% [2909/5042]; $P<0.001$), with the summer group also exhibiting the lowest abortion rate among the four seasons. Notably, despite these differences, no statistically significant variations were observed in biochemical pregnancy rates or clinical pregnancy rates across the groups ($P>0.05$). Taking spring as the reference, the live birth rate in summer was higher (aOR=1.079,95% CI: 1.004-1.160), and the abortion rate was lower (aOR = 0.841,95% CI: 0.746-0.948). The live birth rate and abortion rate in autumn and winter were not significantly different from spring. Multivariate logistic regression analysis showed higher daily average temperature, and humidity at the time of oocyte retrieval increased the live birth rate (OR = 1.005, 95% CI = 1.002–1.007; OR = 1.004, 95% CI = 1.001–1.006).

**Conclusions:** In women who undergo IVF/ICSI treatment, the season ambient temperature, and humidity on the date of the oocyte retrieval may impact embryo development and live birth.

KEYWORDS

**art, season, temperature, live birth rate, clinical pregnancy rate**

# Introduction

Assisted reproductive technology (ART) is a medical technique used to treat infertility, including *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), and embryo transfer (ET) The success rate of ART is affected by many factors, including the patient's age, health status, reproductive system condition and treatment plan. The observed seasonality of natural human pregnancy and birth rates is widely recognized by epidemiologists (1). This phenomenon raises the question of whether ART also exhibits seasonal patterns—a subject that has garnered interest within the research community. Fluctuations in annual ART pregnancy rates have been noted by many experts, presenting variations that are challenging to ascribe merely to technological advancements or laboratory culture conditions. Consequently, the focus has shifted to extrinsic factors, including seasonal shifts and light exposure, which may influence these rates. Some studies deny any correlation between the seasons and reproductive outcomes (2–4), In contrast, other research reports a definitive seasonal influence (5–7), with some attributing it to temperature changes (6), and others pointing to the variation in daylight hours (7). Such disparate findings could stem from differences in research design, geographical settings, ethnic backgrounds, climate conditions, and protocols for ovulation induction. To provide clarity on this matter, our study conducts a retrospective analysis of the relationship between clinical pregnancy outcomes and meteorological factors among patients undergoing ART at our center. Spanning 11 years, this investigation seeks to discern if there is indeed a connection between the meteorological environment of Central China and the success rates of assisted reproduction in this locale.

# Materials and methods

## Data source

The clinical records of patients who received assisted reproductive treatment at the Reproductive Center of the First Affiliated Hospital of Zhengzhou University from January 2011 to December 2021 were collated for this retrospective study. Inclusion criteria (1): Couples who underwent *in vitro* fertilization-embryo transfer (IVF-ET) or intracytoplasmic sperm injection-embryo transfer (ICSI-ET) within the first cycle (2); Couples who successfully underwent oocyte retrieval, embryo formation, and fresh transfer after controlled ovarian stimulation (COS) (3); Men and women with comprehensive body mass index (BMI) data and documented follow-ups. Exclusion criteria include (1): Sperm donation, oocyte donation, or thawing cycles (2); Pre-implantation genetic diagnosis/screening (PGD/PGS) cycles (3); Intrauterine insemination cycles (4); Cycles with incomplete follow-up data (5); Women and/or men with chromosomal abnormalities and/or genetic diseases.

## Climate data

The meteorological information for Zhengzhou was obtained from the Resources and Environment section of the China Statistical Yearbook, with a focus on the monthly average temperature, humidity, and total sunlight hours. Grouping by Meteorological Seasons: For a more accurate reflection of the climate's influence, patients were grouped based on the date of oocyte retrieval corresponding to meteorological seasons: spring (March to May), summer (June to August), autumn (September to November), and winter (December to February of the subsequent year).Climatic Factors Analysis: To assess the impact of climate on reproductive outcomes, the monthly temperature, humidity, and cumulative sunlight exposure were categorized into specific groups. Temperature was divided into low (<10°C), medium (10-20°C), and high (>20°C) temperature groups. Humidity was categorized using tertiles into low (<53%), medium (53-61%), and high (>61%) humidity groups. Cumulative sunlight was segmented into low (≤133.2h), medium (134.8-172h), and high (≥172.3h) sunlight groups.

## IVF protocol

Our research employed controlled ovarian stimulation cycles using diverse strategies, such as long luteal phase, long follicular phase, and antagonist protocols. The selection of these protocols was primarily based on individual factors like women's serum hormone levels, age, and ovarian functionality. This tailored approach guided the administration of ovulation induction treatments, ensuring that drug dosages were adjusted in accordance with each patient's specific circumstances throughout the treatment period. In the downregulation stage, at the onset of the menstrual cycle (day 2–3), we administer 3.75 mg of a long-acting gonadotropin-releasing hormone (GnRH) agonist (Diphereline, Beaufour Ipsen, France) via subcutaneous injection to achieve pituitary downregulation. Following a period of 30–42 days, we conduct a vaginal ultrasound and measure serum levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estrogen (E2), and progesterone (P). This assessment ensures that patients meet the downregulation criteria, characterized by the absence of functional cysts larger than 10 mm in diameter, FSH levels below 5 IU/L, and LH levels below 3 IU/L. Once these standards are met, controlled ovarian hyperstimulation is initiated. For ovulation induction and oocyte retrieval, the gonadotropin (Gn) dosage (GONAL-f, Merck Serono, Germany) is tailored to each patient's age, anti-Müllerian hormone (AMH) level, antral follicle count (AFC), BMI, and baseline serum FSH levels. Adjustments to the Gn dosage and the inclusion of human menopausal gonadotropin (LeBold, Zhuhai Livzon Pharmaceutical, China) depend on follicle size and hormone levels. Ovulation is triggered when one dominant follicle reaches at least 20 mm and a minimum of three dominant follicles are 17 mm or more in diameter. For this purpose, we use 250 mg of Azer (Merck Serono, Italy) combined with 2000 IU of human chorionic gonadotropin (hCG)

(Zhuhai Livzon Pharmaceutical, China). Oocyte retrieval is then performed under vaginal ultrasound guidance, 36–37 hours after administering the trigger injection.

## Embryo culture and transfer

The follicles were retrieved under ultrasound guidance 36–37 hours after triggering. On the same day, semen specimens were collected from male partners via masturbation following standardized protocols. All study participants adhered to a prescribed sexual abstinence period of 2 to 7 days prior to semen collection, following established protocols. IVF and ICSI inseminations were both done at 38–40 h after triggering. Embryos were then cultured in a continuous medium in an incubator maintained at 37°C with a CO2 concentration of 6%. And insemination was assessed on the first day (the day after insemination), and the quality of embryos at the Cleavage stage was assessed on the third day. Fertilization was assessed on the first day following oocyte retrieval, with the presence of a double pronucleus (2PN) indicating normal zygotes. These zygotes were then transferred to cleavage fluid for further culture. On the third day, we evaluated cleavage and development, assessing embryo quality based on blastomere number, diameter, morphology, and developmental rate. Fresh embryo transfer was conducted based on embryo quality, endometrial condition, and patient factors.

## Outcome measures

The primary outcomes were clinical pregnancy and live birth, while secondary outcomes included biochemical pregnancy and early miscarriage. Clinical pregnancy was confirmed by ultrasonographic visualization of one or more gestational sacs or definitive clinical signs. Live birth was defined as the birth of a fetus that showed any signs of life post-expulsion or extraction, regardless of gestational age. Biochemical pregnancy refers to a pregnancy identified solely through hCG detection in serum or urine without clinical development. Early miscarriage was characterized as the spontaneous loss of a clinical pregnancy before 12 completed gestational weeks. Evaluation measures included: 1) ovarian response indicators: such as total and duration of Gn usage, and number of oocytes retrieved; 2) embryo quality indicators: including the number and rate of 2PN fertilizations; and 3) pregnancy outcomes: rates of biochemical pregnancy, clinical pregnancy, early miscarriage, and live birth.

## Statistical analysis

Statistical analysis was performed using SPSS 26.0 software. Continuous variables were expressed as mean ± standard deviation and categorical variables as frequency and percentage. We compared baseline characteristics across four groups. For continuous variables, one-way ANOVA or Kruskal-Wallis tests were used for homogeneous variance groups, while the LSD-t test was utilized for within-group pairwise comparisons. In cases of unequal variances, the Games-Howell test was applied. Multivariable logistic regression analysis helped assess the BMI-outcome relationship for dichotomous results, with odds ratios (ORs) and 95% confidence intervals (CI). A p-value of <0.05 was considered statistically significant (two-sided).

# Result

## Clinical characteristics of four groups of patients according to the season of oocyte retrieval date

Table 1 describes the clinical character of patients in 24,420 IVF/ICSI treatment cycles corresponding to different seasons, all of

TABLE 1 Clinical characteristics of four groups of patients according to the season of oocyte retrieval date.

| Parameter | Spring Group | Summer Group | Autumn Group | Winter Group | *P* |
|---|---|---|---|---|---|
| Cycle | 5202 | 7934 | 6242 | 5042 | |
| Female Age (year) | 30.44 ± 4.56 | 30.40 ± 4.50 | 30.44 ± 4.39 | 30.57 ± 4.56 | 0.214 |
| Male Age (year) | 31.64 ± 5.30 | 31.45 ± 5.27$^{c}$ | 31.51 ± 5.04$^{c}$ | 31.75 ± 5.43 | 0.01 |
| Female BMI (kg/m$^2$) | 22.86 ± 3.14$^{bc}$ | 22.80 ± 3.24$^{b}$ | 22.59 ± 3.17$^{c}$ | 22.71 ± 3.06 | 0.001 |
| Male BMI (kg/m$^2$) | 24.96 ± 3.47 | 25.04 ± 3.58 | 24.97 ± 3.64 | 25.08 ± 3.56 | 0.224 |
| Basal FSH (IU/L) | 6.98 ± 2.32$^{b}$ | 6.97 ± 2.37$^{b}$ | 7.08 ± 2.38 | 7.00 ± 2.25 | 0.017 |
| AFC | 12.97 ± 6.26$^{abc}$ | 13.53 ± 6.47$^{c}$ | 13.41 ± 6.49 | 13.24 ± 6.42 | 0.001 |
| Total sperm count*10$^6$ | 182.89 ± 2.71 | 173.91 ± 2.07 | 160.98 ± 2.39 | 172.65 ± 2.84 | 0.001 |
| PRM | 27.85 ± 26.97$^{abc}$ | 25.60 ± 27.49$^{bc}$ | 22.60 ± 26.59$^{c}$ | 23.30 ± 26.34 | 0.001 |
| NRP | 14.41 ± 10.01$^{b}$ | 14.44 ± 10.49$^{b}$ | 13.58 ± 10.27$^{c}$ | 14.17 ± 10.35 | 0.001 |
| I'M | 37.76 ± 25.74$^{b}$ | 37.02 ± 25.91$^{bc}$ | 39.71 ± 26.80 | 38.87 ± 26.08 | 0.001 |

Data are presented as mean ± S.D. a indicates P<0.05, compared with the summer group; b indicates P<0.05, compared with the autumn group; c indicates P<0.05, compared with the winter group. PRM, progressive motility; NPR, non-progressive motility; IM, immotility.

which met the study's inclusion criteria. The number of cycles varies across different seasons, with the highest count in summer (7934) and the lowest in winter (5042). There is no significant difference in female age among different seasonal groups, while the male age in the winter group is slightly higher than that in other seasonal groups (P=0.010). The female BMI in the spring group is the highest and significantly higher than that in the summer and autumn groups (P < 0.010), but there is no statistical difference in male BMI. However, summer stands out as the season with the lowest baseline FSH levels and the highest Antral Follicle Counts (AFC) among patients, a notable distinction supported by statistical significance (P<0.05). Furthermore, our study identified a clear seasonal pattern in sperm quality: both total sperm count and progressive motility displayed the lowest values in the autumn group and peaked in the spring group, with autumn also recording the highest number of immotile sperm (P<0.05). This seasonality may be attributed to the approximately 74-day cycle of spermatogenesis, with adverse effects of high summer temperatures on sperm quality becoming evident in the autumn, whereas the optimal conditions of winter contribute to better sperm quality observed in the spring.\

## Assisted pregnancy outcomes of four groups according to the season of oocyte retrieval date.

Figure 1 and Table 2 illustrates the laboratory and clinical data of patients grouped by season. Spring emerged as the season with the shortest gonadotropin (GN) administration duration for oocyte retrieval and the lowest follicle-stimulating hormone (FSH) usage (p<0.05). However, this group also had the thinnest endometrial lining post-ovulation induction, though not significantly thinner (p>0.05). In regards to oocyte retrieval, despite patients in summer recording the highest antral follicle count (AFC), the actual yield was the lowest. In contrast, the autumn group demonstrated the highest oocyte retrieval count compared to other seasons (*P*<0.05). The data did not reveal significant variances in IVF fertilization rates, IVF-2PN (pronuclei) rates, or ICSI-2PN rates among the seasonal groups (*P*>0.05). This indicates that the primary factor affecting the quantity of IVF and ICSI fertilized oocytes and 2PN rates was the number of oocytes retrieved, rather than seasonal differences. The comparison of biochemical and clinical pregnancy rates across all groups showed no statistically significant discrepancies (all *P*>0.05). However, miscarriage and live birth rates manifested notable seasonal differences (*P*<0.01 for miscarriage rates; *P*<0.001 for live birth rates). Detailed analysis pointed out higher miscarriage rates in the spring [15.42% (572/3708)] and winter [15.16% (543/3581)] when compared to the summer [13.19% (754/5718), *P*<0.01]. No conspicuous differences were noted in miscarriage rates between other groups. Live birth rates in the spring [59.09% (3074/5202)], autumn [58.89% (3676/6242)], and winter [57.70% (2909/5042)] proved to be inferior to those in the summer [61.24% (4859/7934), *P*<0.001], while comparisons among the remaining groups again showed no significant differences.

## The multivariate logistic regression analysis of season and pregnancy outcomes of the four groups

Table 3 elaborates on the impact of the oocyte retrieval season on pregnancy outcomes, employing binary logistic regression to control for confounders. The study considered variables such as biochemical pregnancy, clinical pregnancy, miscarriage, and live birth, adjusted for the age of both female and male partners, baseline AFC, motile sperm count, and the season of oocyte retrieval (categorized as spring, summer, autumn, and winter, with spring serving as the reference group). Our findings indicate that the summer group exhibited a significantly higher rate of miscarriages compared to the spring group (aOR=0.841, 95% CI: 0.746-0.948). On the other hand, the miscarriage rates for both the autumn group (aOR=0.905, 95% CI: 0.798-1.025) and winter group (aOR=0.974, 95% CI: 0.856-1.110) were not statistically different from those in the spring group, pointing to a degree of seasonality in reproductive outcomes. Analysis of live birth rates revealed that the summer group had a statistically significant higher rate compared to the spring group (aOR=1.079, 95% CI: 1.004-1.160, *P*<0.05),

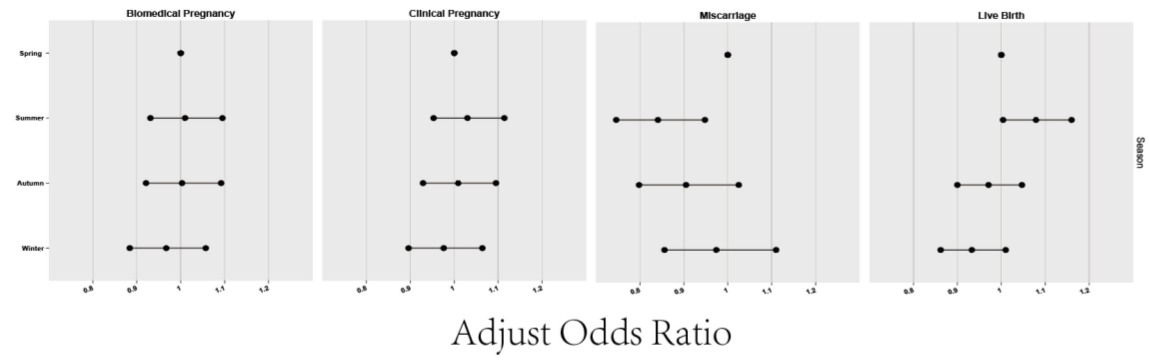


FIGURE 1
Relation between the season of oocyte retrieval date and pregnancy outcomes.

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TABLE 2 Assisted pregnancy outcomes of four groups according to the season of oocyte retrieval date.

| Parameter | Spring Group | Summer Group | Autumn Group | Winter Group | *P* |
|---|---|---|---|---|---|
| Gn day | 12.00 ± 2.19[abc] | 12.51 ± 2.25 | 12.52 ± 2.24 | 12.53 ± 2.27 | 0.001 |
| The total dose of FSH | 1968.91 ± 724.61 [abc] | 1997.77 ± 736.32[b] | 2043.23 ± 784.05[c] | 2012.50 ± 732.79 | 0.001 |
| Endometrial thickness(mm) | 12.25 ± 3.62 | 12.26 ± 3.23 | 12.25 ± 3.16 | 12.32 ± 4.27 | 0.752 |
| No. of retrieved oocytes | 12.51 ± 5.82 [b] | 12.46 ± 5.70 [b] | 12.73 ± 5.96 | 12.59 ± 5.89 | 0.048 |
| Number of fertilized oocytes | 12.07 ± 5.77 [b] | 11.96 ± 5.79 [bc] | 12.34 ± 5.95 | 12.25 ± 5.94 | 0.005 |
| IVF Fertilization rate(%) | 94.63 ± 18.63 | 93.45 ± 20.62 | 92.51 ± 22.13 | 93.61 ± 20.08 | 0.638 |
| IVF- No. of 2PN | 7.98 ± 4.31 | 7.88 ± 4.30 [b] | 8.09 ± 4.40 | 8.01 ± 4.40 | 0.086 |
| IVF-2PN rate(%) | 71.10 ± 19.96 | 71.69 ± 21.33 | 72.90 ± 19.47 | 78.28 ± 90.13 | 0.23 |
| ICSI- Number of fertilized oocytes | 11.94 ± 5.50 | 12.29 ± 5.37 | 12.35 ± 5.72 | 12.01 ± 5.58 | 0.142 |
| ICSI- Fertilization rate(%) | 84.17 ± 16.37 | 82.04 ± 16.70 | 78.84 ± 19.43 | 83.40 ± 17.23 | 0.18 |
| ICSI- No. of 2PN | 8.09 ± 4.13 | 8.12 ± 4.07 | 8.21 ± 4.24 | 7.98 ± 4.14 | 0.572 |
| ICSI-2PN rate(%) | 72.86 ± 16.83 | 80.87 ± 16.02 | 69.14 ± 18.39 | 70.89 ± 18.17 | 0.063 |
| Biomedical pregnancy, rate(%) | 74.47 ± 0.44 | 74.96 ± 0.43 | 74.99 ± 0.43 | 74.49 ± 0.44 | 0.659 |
| Clinical pregnancy rate (%) | 71.16 ± 0.45 | 72.03 ± 0.45 | 71.74 ± 0.45 | 70.90 ± 0.45 | 0.489 |
| Miscarriage rate (%) | 15.45 ± 0.36[a] | 13.20 ± 0.34[c] | 14.00 ± 0.35 | 14.64 ± 0.36 | 0.007 |
| Live birth rate(%) | 58.80 ± 0.49 [a] | 61.38 ± 0.49[bc] | 58.57 ± 0.49 | 58.15 ± 0.49 | 0.001 |

Data are presented as mean ± S.D. a indicates *P*<0.05, compared with the summer group; b indicates *P*<0.05, compared with the autumn group; c indicates *P*<0.05, compared with the winter group.

suggesting a noteworthy seasonal influence. Conversely, the autumn (aOR=0.971, 95% CI: 0.900-1.047) and winter (aOR=0.933, 95% CI: 0.862-1.010) groups did not demonstrate a statistically significant difference in live birth rates when compared to the spring, indicating a more complex interaction between seasonality and reproductive outcomes. The seasonality of oocyte retrieval appears to notably affect both miscarriage and live birth rates.

TABLE 3 The multivariate logistic regression analysis of season and pregnancy outcomes of the four groups.

| Parameter | Spring | Summer | Autumn | Winter |
|---|---|---|---|---|
| Biomedical pregnancy rate | Reference | 1.010 (0.931-1.095) | 1.003 (0.921-1.092) | 0.967 (0.884-1.057) |
| Clinical pregnancy rate | | 1.030 (0.953-1.114) | 1.009 (0.929-1.095) | 0.976 (0.896-1.064) |
| miscarriage rate | | 0.841 (0.746-0.948) | 0.905 (0.798-1.025) | 0.974 (0.856-1.110) |
| Live birth rate | | 1.079 (1.004-1.160) | 0.971 (0.900-1.047) | 0.933 (0.862-1.010) |

Data were expressed as OR (95%CI). All models are adjusted according to female age, male age, AFC, and PNR.

## The multivariate logistic regression analysis of individual meteorological factors and pregnancy outcomes

Beyond the raw delineation of seasons, we delved into various environmental factors such as temperature, humidity, and daylight duration, which inherently differ across seasons, to discern their potential effects on pregnancy outcomes. For this purpose, climate factors were categorized into three groups to elucidate their individual and combined impact. Figure 2 and Table 4 presents a detailed analysis of the relationship between individual meteorological factors and pregnancy outcomes, following adjustments for potential confounders using binary logistic regression. The study specifically considered observation indicators such as biochemical pregnancy, clinical pregnancy, miscarriage, and live birth, while controlling for factors including the female and male age, baseline AFC, motile sperm count, along with meteorological factors associated with oocyte retrieval (temperature, humidity, daylight), all compared to a reference lower data group Regression analysis showed a positive correlation between higher temperatures and a greater live birth rate when compared to cooler conditions (OR=1.099, 95% CI:

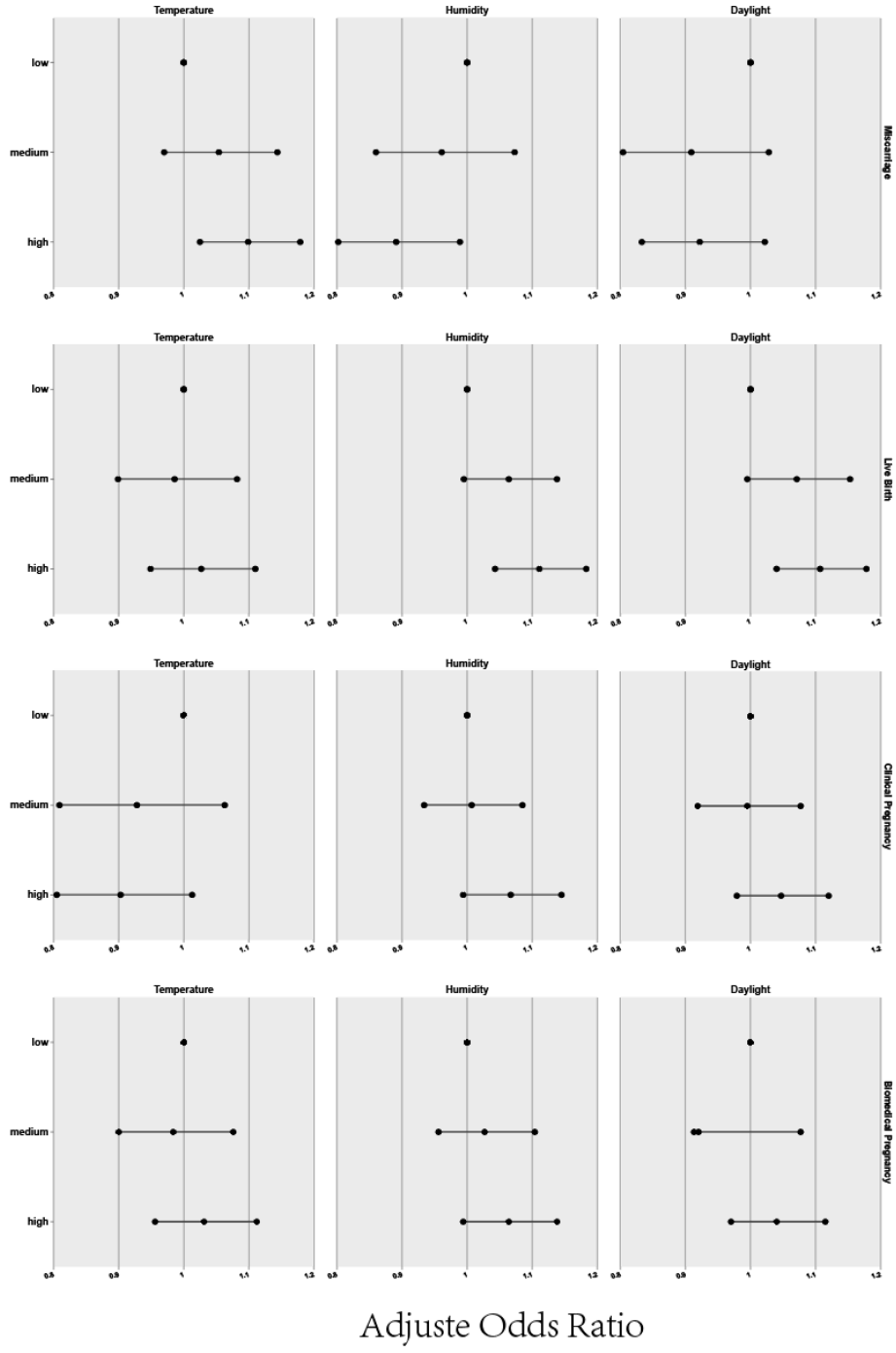


FIGURE 2
Relation between individual meteorological factors and pregnancy outcomes.

1.025-1.179), while increased humidity was associated with both reduced miscarriage rates and higher live birth rates (miscarriage rate OR=1.099, 95% CI: 1.025-1.179; live birth rate OR=1.111, 95% CI: 1.043-1.183) as opposed to lower humidity levels. Furthermore, longer daylight hours also corresponded with improved live birth rates vis-a-vis shorter daylight durations (OR=1.107, 95% CI: 1.040-1.178).

Table 5 shows that when assessing temperature, humidity, and daylight duration as continuous variables, our analysis suggests that for each 1°C temperature increase, the live birth rate could improve by 0.5%, and each 1% increase in humidity potentially increases the biochemical pregnancy rate by 0.3% and the live birth rate by 0.4%. However, variations in daylight duration did not yield a statistically significant effect on pregnancy outcomes.

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TABLE 4 The multivariate logistic regression analysis of individual meteorological factors and pregnancy outcomes.

| Parameter | temperature | | | humidity | | | daylight | | |
|---|---|---|---|---|---|---|---|---|---|
| | low | medium | high | low | medium | high | low | medium | high |
| Biomedical pregnancy rate | Reference | 0.986(0.899-1.082) | 1.027(0.949-1.110) | Reference | 1.007(0.934-1.085) | 1.067(0.994-1.145) | Reference | 0.92(0.913-1.077) | 1.040(0.970-1.115) |
| Clinical pregnancy rate | | 0.984(0.900-1.076) | 1.031(0.956-1.112) | | 1.027(0.956-1.104) | 1.064(0.994-1.138) | | 0.995(0.919-1.077) | 1.047(0.979-1.120) |
| Miscarriage rate | | 0.928(0.809-1.063) | 0.903(0.805-1.013) | | 0.961(0.860-1.073) | 0.891(0.802-0.989) | | 0.909(0.804-1.028) | 0.922(0.833-1.022) |
| Live birth rate | | 1.054(0.970-1.144) | 1.099(1.025-1.179) | | 1.064(0.995-1.138) | 1.111(1.043-1.183) | | 1.071(0.995-1.153) | 1.107(1.040-1.178) |

Data were expressed as OR (95%CI). All models are adjusted according to female age, male age, AFC, and PNR.

## Discussion

Extensive research has explored the myriad influences on pregnancy outcomes, yet the underlying mechanisms frequently remain elusive. Factors traditionally held in high regard include the woman's age, embryo quality, and endometrial receptivity—especially during the critical window of embryo implantation. In this study, we compared live birth rates as the primary pregnancy outcome, live birth is the most immediate outcome that patients care about, and it is also the ultimate indicator for evaluating the effectiveness of assisted reproductive technologies such as *in vitro* fertilization (IVF). In ART, the literature presents a dichotomy, with some domestic and international studies suggesting possible seasonal effects, while others dispute the existence of such trends, leading to mixed conclusions. Ambiguities in existing research—stemming from diverse ovarian stimulation protocols, varying criteria for patient inclusion based on season, and different climatic backgrounds—make it challenging to directly compare findings across studies. Moreover, prior investigations often overlook the specific elements of seasonality, such as temperature, humidity, and daylight duration.

Our investigation probed the association between pregnancy outcomes after fresh embryo transfer and multiple parameters, including the season of oocyte retrieval, average monthly temperature, humidity, and sunlight exposure on the day of oocyte retrieval. The study concludes that temperature, humidity, and sunlight rank as significant meteorological factors influencing pregnancy outcomes. Notably, seasons with higher average temperatures, humidity, and sunlight exposure—typically summer—show an enhanced rate of pregnancy compared to seasons with generally lower levels of these variables, such as winter. Meteorological elements such as temperature, humidity, and sunlight were dissected to reveal an association with reproductive outcomes. Higher monthly averages of these components correlated with increased live birth rates. Further analysis, treating these factors as continuous variables, pinpointed that upticks in temperatures and humidity are linked with a rise in live birth rates. This gradient relationship underscores the independent effects of summer's intensified warmth, humidity, and sunlight on live births. Consequently, the synergy of these three factors in summer could explain the season's particularly high live birth rates. Parallel findings emerged from Wood and colleagues' study on IVF and ICSI outcomes in the UK, which indicated that summer surpasses winter in fostering the optimal conditions for pregnancy (8).

The fertility implications of seasonal changes are two-fold: weather conditions and temperature play a crucial role in male sperm quality (9) while sunlight exposure is a determinant of female ovulation patterns. Of note is the germinal epithelium in the testes, which is acutely temperature-sensitive, making it vulnerable to even slight increments in heat that can inhibit sperm production. For optimal spermatogenesis, the testes must maintain a temperature of 2-6°C below the body's core temperature. Deviations from this thermal niche can be detrimental to spermatogenesis, thereby influencing conception rates (10, 11)., High temperatures have been shown to negatively impact male fertility by inhibiting

TABLE 5 The relationship between individual meteorological factors(continuous variable) and pregnancy outcomes.

| parameter | temperature | | humidity | | daylight | |
|---|---|---|---|---|---|---|
| | OR 95%CI | P | OR 95%CI | P | OR 95%CI | P |
| Biomedical pregnancy rate | 1.002 (0.999-1.005) | 0.239 | 1.003 (1.000-1.005) | 0.037 | 1.000 (1.000-1.001) | 0.304 |
| Clinical pregnancy rate | 1.002 (0.999-1.005) | 0.201 | 1.002 (1.000-1.005) | 0.059 | 1.000 (1.000-1.001) | 0.39 |
| Miscarriage rate | 0.996 (0.992-1.000) | 0.073 | 0.997 (0.993-1.001) | 0.148 | 1.000 (0.999-1.001) | 0.645 |
| Live birth rate | 1.005 (1.002-1.007) | 0.001 | 1.004 (1.001-1.006) | 0.002 | 1.001 (1.000-1.001) | 0.057 |

Data were expressed as OR (95%CI). All models are adjusted according to female age, male age, AFC, and PNR.

sperm formation and hormone synthesis, resulting in decreased sperm count and compromised quality in ejaculation. This association is supported by existing research indicating that sperm concentration and rapid motility typically peak in spring, with a notable decline in autumn—an observation that is consistent with our own findings (12). From a practical standpoint, the seasonal variation in semen quality may seem to pose a challenge for IVF/ICSI outcomes. However, the IVF/ICSI process involves rigorous semen preparation to isolate the most viable sperm for fertilization, rendering the seasonal influence less significant. Even in cases where viable sperm quantities are adversely affected by the season, ICSI can still provide a successful fertilization rate by focusing on selecting individual sperm. It is also apparent that the ambient temperature at the time of oocyte retrieval is an important factor affecting IVF/ICSI success rates during different seasons. Despite the observed relationship, the influence of ambient temperatures on ovarian function and its implications for reproductive technology remains an area for further investigation. A study assessing temperature's impact on ovarian function revealed a negative correlation between high temperatures and AFCs among infertile patients, suggesting a decrease in ovarian reserve (13). Contrary to prior evidence, our data demonstrate that AFCs during summer oocyte retrieval cycles were significantly elevated compared to spring, autumn, and winter cycles, a finding that conflicts with established literature on seasonal influences in ovarian response. Although AFC is a marker of ovarian reserve, it may not directly reflect oocyte quality. Interestingly, there could be a positive relationship between higher ambient temperatures and both increased AFCs and potentially improved oocyte quality. Moreover, our study's timelines differ from previous investigations. Gaskins. Et (13) measured body temperature 90 days prior to testing the ovarian reserve, coinciding with follicle recruitment phases. In contrast, our temperature measurements were concurrent with oocyte extraction, providing a current snapshot of conditions during the crucial 36-hour window of oocyte maturation. Thus, our data suggest that higher ambient temperatures might influence oocyte maturation and quality more directly.

Melatonin (14), a hormone with antioxidant properties has been shown to interact with the reproductive system. Secreted by the pineal gland, melatonin's production is higher during periods of darkness, regulating seasonal reproductive changes by controlling gonadotropin-releasing hormone (GnRH) secretion. Seasonal variations in melatonin could lead to changes in neurotransmitter release and influence the plasticity of GnRH neurons. In humans, melatonin concentrations in follicular fluid before ovulation are higher than in serum, and these levels align with seasonal light-dark variations. This indicates melatonin's potential role in sex hormone production and its impact on oocyte maturation and quality. Granulosa cells within mature follicles are known to secrete melatonin. For individuals with Polycystic Ovary Syndrome (PCOS), who may require oocyte *in vitro* maturation (IVM), supplementing the IVM culture medium with melatonin has been found to enhance cytoplasmic maturation and clinical outcomes (15) A study involving low-quality oocytes demonstrated that the addition of melatonin, inositol, and folic acid to the treatment protocol significantly improved oocyte quality and subsequent pregnancy success rates (16). The majority of participants in these studies underwent pituitary down-regulation with GnRH analogs and received exogenous gonadotropins for ovarian stimulation. This treatment approach suggests that any direct influence of light exposure on the participants may be mitigated due to the induced suppression of GnRH neuron activity. Moreover, melatonin receptors are widely distributed in peripheral tissues, suggesting a potentially more immediate regulatory role of melatonin outside the central nervous system. Studies indicate that the length of daylight (photoperiod) affects melatonin levels, subsequently influencing steroid hormone secretion. This may have a consequential effect on the endometrium's receptivity, further impacting reproductive outcomes.

Human 7-dehydrocholesterol can be transformed into vitamin D through exposure to ultraviolet rays, with summer typically yielding higher vitamin D levels in the serum due to increased sunlight. The relationship between vitamin D levels and *in vitro* fertilization (IVF) success rates, however, remains a subject of debate. While some studies associate elevated vitamin D with a greater likelihood of IVF success (17–20) others do not support this correlation (21–23). A systematic review and meta-analysis incorporating findings from 11 cohort studies reported a positive relationship between higher vitamin D concentrations and improved chances of clinical pregnancy and live birth in women utilizing assisted reproductive technologies (24).

Despite these findings, the inconsistency among studies means that the individual contributions of vitamin D and melatonin to folliculogenesis are yet to be definitively determined. As a result, the current body of evidence does not allow for definitive claims regarding a causal link between these substances and the quality of oocytes in response to ovarian stimulation. To date, no studies have examined the effects of ambient temperature and humidity on

serum melatonin and vitamin D levels. Moreover, sunlight's role in IVF treatments may involve other elements such as vitamin D3, serotonin, dopamine, and opioids (17, 25–27). These insights propose that seasonal changes could indirectly affect pregnancy outcomes, not only by potentially impacting the quality of sperm and oocytes but also through other pathways. Our research posits that the summer season, characterized by prolonged sunlight exposure and likely increased vitamin D levels, is associated with better pregnancy outcomes post-IVF/ICSI treatment. While the detailed mechanisms by which seasons affect the outcomes of assisted reproductive pregnancy cycles remain elusive, the concept of photoperiodism in humans is evident. The duration of sunlight seems to forge a connection between the seasons and enhanced clinical success in fresh embryo transfers. Indeed, seasonal variables, including climate and environmental factors, likely play a role in IVF treatment results.

Air quality has shown a significant correlation with reproductive outcomes, with adverse effects being observed in both natural and assisted conceptions. Particulate matter, especially Particulate Matter(PM)10 (28) and PM2.5 (29), is associated with decreased fertility, an increased risk of infertility, lower live birth rates, and higher miscarriage rates among IVF patients. Elevated nitrogen dioxide ($NO_2$) exposure correlates with increased miscarriage rates in the general population and reduced live birth rates in those undergoing IVF. Additionally, sulfur dioxide ($SO_2$) exposure is linked to *in vitro* DNA damage and a rise in miscarriage occurrences. Research from a regional institution underscores that atmospheric levels of PM10 and $SO_2$ have tangible effects on pregnancy outcomes (30).

Our study's extensive dataset, analyzing over 24,000 ART cycles and encompassing a broad range of ovulation induction protocols and infertility causes, ensured a robust sample size across most BMI categories, enhancing the relevance and generalizability of our findings. All cycle data were sourced from a single large institution, which helped maintain consistency in physician practices, treatment plans, and patient profiles. We focused on the first fresh ART cycle for each patient to reduce confounding factors from repeated or frozen cycles, thereby providing a more precise estimation of outcomes. However, our study is not without limitations. Firstly, the major limitation of this study is its retrospective nature. Secondly, we collected retrospective data from a single center, the First Affiliated Hospital of Zhengzhou University. Therefore, the research findings may be difficult to apply to other regions with different climates. In addition, indoor heating is quite common in some areas of Henan Province, which may have influenced the research results. Similar to previous studies, we selected the seasons and meteorological factors related to specific dates during the *in vitro* fertilization (IVF) process as representatives of the overall environment during the treatment period, which may introduce biases into the research findings. Thirdly, factors such as smoking, alcohol consumption, metabolic health, and the presence of diabetes were not considered, potentially affecting the study's broad applicability. Additionally, a gap remains in our understanding of the underlying mechanisms, which warrants further investigation.

## Conclusion

A summation of data suggests that the autumn season yields a greater number of retrieved oocytes compared to other seasons. The cohort from summer exhibits the lowest rate of miscarriage and the highest live birth rate, contributing to the most favorable pregnancy outcomes. It appears that summer conditions—characterized by high temperature, high humidity, and abundant sunlight—serve as independent positive factors for live birth rates. Therefore, with respect to ART treatments, strategically timing ovulation induction and oocyte retrieval may prove advantageous. Specifically, scheduling oocyte retrieval during the summer months could potentially improve pregnancy outcomes.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The studies involving humans were approved by Life Science Ethics Review Committee of Zhengzhou University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

## Author contributions

HL: Conceptualization, Formal Analysis, Methodology, Writing – original draft. XL: Investigation, Writing – review & editing. JL: Resources, Writing – review & editing. YM: Software, Validation, Writing – review & editing. GL: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# The impact of inoculation with the inactivated COVID-19 vaccine on the outcomes of *in vitro* fertilization and embryo transfer: a cohort study of 1,258 women from Sichuan, China

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**Objective:** This study aimed to assess the impact of inoculation with the inactivated coronavirus disease 2019 (COVID-19) vaccine on the outcomes of *in vitro* fertilization and embryo transfer (IVF-ET).

**Methods:** From January 2021 to December 2022, patients undergoing their first cycle of IVF-ET at the Reproductive Medicine Center of Sichuan Provincial Women's and Children's Hospital were prospectively enrolled. Based on inoculation with inactivated COVID-19 vaccines before ovarian stimulation (OS) by a gonadotrophin-releasing hormone (GnRH) antagonist or agonist protocol, the patients were divided into the vaccinated group ($n$ = 713) and the unvaccinated group ($n$ = 545). The vaccinated group were sub-grouped based on the dose of inoculation (single dose, $n$ = 74; double dose, $n$ = 275; and triple dose, $n$ = 126) and the interval between the first inoculation and OS (<3 months, $n$ = 65; 3–6 months, $n$ = 123; and >6 months, $n$ = 287).

**Results:** The rates of mature oocytes, normal fertilization, cleavage embryo, high-quality cleavage embryo, blastocysts, and high-quality blastocysts were not significantly different between the vaccinated and unvaccinated groups ($p$ > 0.05). For fresh embryo transfer, the implantation rate (IR), the clinical pregnancy rate (CPR), the live birth rate (LBR), the gestational age at delivery, and the birth weight of infants were not significantly different between the two groups ($p$ > 0.05). The IR, CPR, LBR, and birth weight of infants were not significantly different for both the dose and interval subgroups ($p$ > 0.05).

**Conclusion:** Inactivated COVID-19 vaccines may not affect the outcomes of IVF-ET.

KEYWORDS

SARS-CoV-2, inactivated vaccine, *in vitro* fertilization, embryo transfer, live birth

# 1 Introduction

Since the end of 2019, the coronavirus disease 2019 (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus had become a global issue, which severely burdened the world's public health and economy. Consequently, various vaccine types have been designed and manufactured (1, 2). The inactivated COVID-19 vaccine is produced from the virus killed by chemical or physical methods to eliminate the risk of viral reversion (3). The attenuated virus vaccine is produced from viruses with decreased pathogenesis and may induce a strong immune response; however, a major concern from it has been the toxicity after vaccination (4). For viral vector vaccines, adenovirus is used to insert the COVID-19 viral gene into the human body. This vaccine is safe and effective, but the construction of the adenovirus vector is challenging (5). For the messenger ribonucleic acid (mRNA) vaccine, the risk of viral infection is low, and it has the virtue for being economical and effective (6).

Compared with non-pregnant women, pregnant women infected with SARS-CoV-2 have shown great risks of mechanical ventilation, intensive care unit admission, and death (7). Worsening maternal infection may in turn result in adverse neonatal outcomes associated with preterm birth (8, 9). Fortunately, vaccination of the mother could provide passive immunization for the fetus through the placenta (10, 11). Therefore, since the end of 2020, the World Health Organization and other international health institutions have granted approval for the inoculation of the COVID-19 vaccine in women who are pregnant, breastfeeding, or planning to conceive naturally or by assisted reproductive technology (ART) (12). Studies found that COVID-19 mRNA vaccination did not affect the outcomes of ART (13–21). The ovarian reserve, the response to ovarian stimulation (OS), and the outcomes of early pregnancy were not affected by the COVID-19 mRNA vaccine during *in vitro* fertilization and embryo transfer (IVF-ET) (13–16). Neither SARS-CoV-2 infection or the mRNA vaccine nor the immune response to these has detrimentally affected the function of follicles, manifested as a measurable change of the heparan sulfate proteoglycans (the major estrogen-binding protein) in the follicular fluid (17–19). The serum level of the anti-Müllerian hormone (AMH), the endometrial receptivity, and the sustained implantation rate (IR) were not affected by the COVID-19 mRNA vaccine (16, 20). Miller et al. (21) reported that the live birth rates (LBRs), the gestational weeks, and the birth weights were similar between those with or without COVID-19 mRNA vaccination ($n$ = 38 and 10, respectively).

The inactivated COVID-19 vaccine comprised almost half of all doses vaccinated globally and has been crucial in fighting the COVID-19 pandemic. In China, it was also the most widely used and has been proven to be safe for individuals over 18 years (22). Some have reported that the ovarian response, the embryo quality, and the ongoing pregnancy rates were not affected by the inactivated COVID-19 vaccine during IVF-ET (23–26). There are also other reports that the inactivated COVID-19 vaccine did not undermine the biochemical pregnancy rates, the clinical pregnancy rates (CPRs), and the abortion rates during frozen–thawed embryo transfer (FET) cycles (27, 28). LBR is an important outcome in IVF-ET; however, only one study has reported that it was not affected by the inactivated COVID-19 vaccine in IVF-ET, but without information on infants and subgroup analysis by interval or dose of vaccination (29). From April 2021 to January 2023, there was a widespread mass vaccination campaign in China using the inactivated COVID-19 vaccine. By now, all pregnant women inoculated with the inactivated vaccine have delivered (or terminated their pregnancy). Against this backdrop, we have the opportunity to compare the LBR and the outcomes of IVF-ET treatment and to analyze the subgroups that had different doses and intervals of vaccination.

# 2 Materials and methods

## 2.1 Study populations

Patients aged 20–45 years undergoing their first IVF-ET treatment at the Reproductive Medicine Center of Sichuan Provincial Women's and Children's Hospital from January 2021 to December 2022 were prospectively enrolled. The OS protocol was the gonadotrophin-releasing hormone (GnRH) antagonist or agonist protocol. The exclusion criteria were: history of SARS-CoV-2 infection; oocyte and/or sperm donation; pre-implantation genetic testing (PGT); oocyte frozen; adenomyosis, submucosal, or intramural uterine fibroids; uterine abnormalities; and inaccurate information on vaccination. The patients were divided into the vaccinated group (CoronaVac, an inactivated COVID-19 vaccine produced by Sinovac Biotech Ltd., Beijing, China) and the unvaccinated group based on the inoculation status before OS.

The vaccinated group was sub-grouped based on the dose of inoculation (single dose, double dose, and triple dose) or the interval between the first inoculation and OS (<3 months, 3–6 months, and >6 months). In China, the full course of vaccination consists of three doses: the first dose, the second dose (given 1 month after the first dose), and the third dose (a booster dose, given 6 months after the second dose). Prior to the OS, information on the vaccination (i.e., date of vaccination, type, and manufacturer) was recorded by a nurse via Tianfutong (an application program of the Sichuan health database) installed on mobile phones. For all patients, nucleic acid is routinely detected to exclude SARS-CoV-

2 infection before OS. The flowchart is shown in Figure 1. This prospective cohort study was approved by the Medical Ethics Committee of Sichuan Provincial Women's and Children's Hospital. All procedures in this study complied with the ethical standards of the relevant national and institutional committees on human experimentation and the Helsinki Declaration 1975 (2013 revision). The trial was registered in the Chinese Clinical Trial Registry (ChiCTR2200055721; https://www.chictr.org.cn/,16 January 2022).

The body mass index (BMI), the antral follicle count (AFC), and the serum levels of the follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol ($E_2$), progesterone (P), total testosterone (TT), prolactin, and AMH were measured as described previously (30).

## 2.2 Ovarian stimulation

Patients with decreased ovarian reserve (DOR) or polycystic ovary syndrome (PCOS) were mainly treated with the GnRH antagonist protocol, while others were generally treated with the long-acting GnRH agonist protocol.

With the GnRH antagonist protocol, 125–300 IU/day of recombinant FSH (rFSH) (Gonal-F, Merck-Serono KGaA, Darmstadt, Germany; Jinsai Heng, Jinsai Pharmaceuticals, China; Puregon®, Merck Sharp & Dohme, Rahway, NJ, USA) was injected daily from day 2 to day 3 of the menstrual cycle until the trigger day. The rFSH dose was determined based on the AFC, AMH, BMI, and age of the patient and was adjusted according to follicle development and the serum level of $E_2$. When the diameter of the

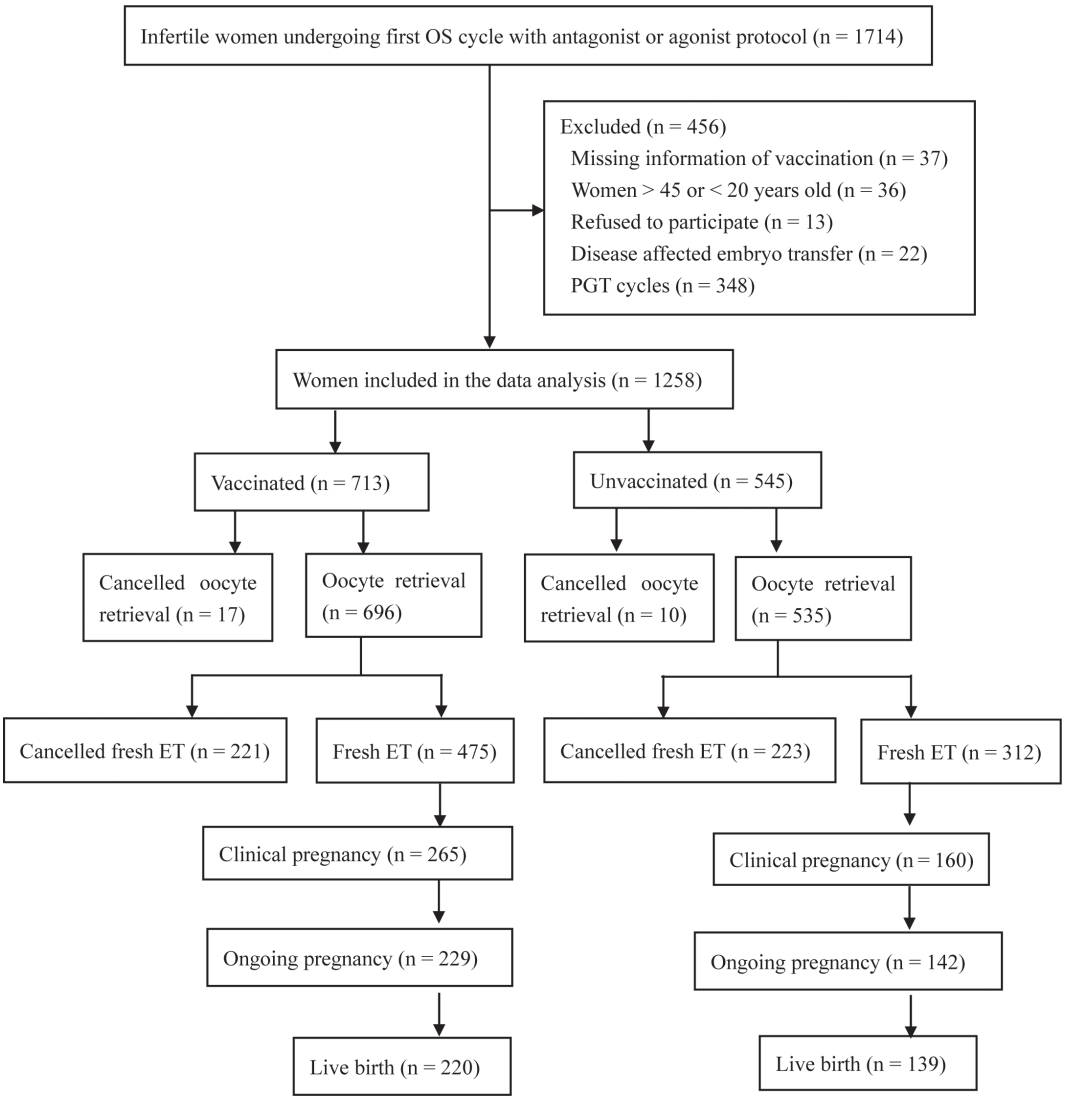


FIGURE 1
Flow chart for participants recruitment of this study.

leading follicle reached 12 mm or the serum level of LH ≥10 mIU/ml, 0.25 mg GnRH antagonist (Ganirelix, Ocalon, FL, USA) was subcutaneously injected daily until the trigger day. When the diameter of at least one or two follicles has reached 18 mm, 250 μg of recombinant human chorionic gonadotrophin (rHCG; Merck-Sheranova, Darmstadt, Germany) or 0.2 mg of triptorelin (Jinsai Pharmaceuticals, Changchun, China) was injected as the trigger, with the latter used only for all frozen embryo cycles. Transvaginal oocyte retrieval was performed 36.5 h later.

With the GnRH agonist protocol, 3.75 mg of leuprorelin acetate (Beiyi; Shanghai Livzon Pharmaceutical Co., Ltd., Shanghai, China) was injected subcutaneously from day 2 to day 3 of the menstrual cycle. After 28–38 days, 125–300 IU/day rFSH was injected daily when the diameter of most follicles was 5 mm and with serum levels of $E_2$ <50 pg/ml and LH and FSH <5 mIU/ml. The standard of the rFSH initiation dose and adjusted dose and the timing of the trigger and ovulation were consistent with the GnRH antagonist protocol.

Oocyte retrieval was cancelled for any of the following conditions: follicular growth failure (10 days after OS, diameter of the leading follicle <10 mm), premature ovulation before oocyte retrieval, and personal reasons. Ovarian hyperstimulation syndrome (OHSS) was diagnosed and graded according to Navot et al. (31).

## 2.3 *In vitro* fertilization and embryo culture

*In vitro* fertilization (IVF) was carried out 39 h after the trigger for all retrieved oocytes, while intracytoplasmic sperm injection (ICSI) was carried out 3–4 h after the retrieval of mature oocytes. A mature oocyte was defined as being at the metaphase II (MII) stage with the first polar body visible in the cytoplasm. A normal fertilized oocyte was confirmed as containing two pronuclei (2PN). The embryo was cultured to cleavage and blastocyst stage in sequential G1-plus/G2-plus medium (Vitrolife, Gothenburg, Sweden) at 37°C in a culture environment containing 6.0% $CO_2$ and 5% $O_2$. Day 3 cleavage embryo was scored based on the number of blastomeres and the degree of fragmentation, with high-quality embryo categorized as grade I or II (32). On day 5 or day 6, morphological scoring was carried out based on the Gardner and Schoolcraft's system (32). Blastocysts were considered as usable with a grade over 4CC and as high quality for those over 4BB.

## 2.4 Fresh embryo transfer

Most of the patients were transferred with two cleavage embryos with the highest morphological scores. One embryo was transferred for those with a scarred uterus or with a body height of <1.5 m, and one blastocyst was transferred when eligible (with the number of high-quality cleavage embryos ≥3); otherwise, one cleavage embryo was transferred. Luteal phase support was started on the day after oocyte retrieval by daily injection of 60 mg progesterone oil (Zhejiang Xianju Pharmaceutical Co., Ltd., Taizhou, China) or 90 mg vaginal progesterone (Crinone 8% gel; Merck, Darmstadt, Germany), and 30 mg dydrogesterone (Duphaston; Abbott Healthcare Products B.V., Weesp, the Netherlands) was taken daily.

Embryo implantation, clinical pregnancy, and early miscarriage were defined as previously described (30). Ectopic pregnancy was defined as implantation at any site out of the uterine cavity. Late miscarriage was defined as loss of pregnancy between the 12th and 28th weeks of gestation. Ongoing pregnancy was defined as the detection of fetal heartbeat at the 12th week of gestation. Live birth was defined as delivery of a live fetus after 28 weeks of gestation.

Fresh embryo transfer was cancelled for any of the following conditions: failed oocyte retrieval, no transplantable embryo formation, serum level of P >1.5 ng/ml on the trigger day, diagnosis of OHSS, prevention of OHSS (with number of oocytes retrieved >18 or serum level of $E_2$ >5,000 pg/ml on the trigger day), and personal reasons. All of the embryos were frozen, and frozen–thawed embryo was transferred at least 1 month later. Only the outcomes of fresh embryo transfer were analyzed.

## 2.5 Outcomes

The primary outcome was the LBR. The secondary outcomes were the rates of MII, 2PN, cleavage embryo, high-quality cleavage embryo, blastocyst, high-quality blastocyst, implantation, clinical pregnancy, early miscarriage, and ongoing pregnancy.

The above outcomes were calculated as follows: MII rate = number of MII oocytes/number of retrieved oocytes; 2PN rate = number of 2PN oocytes/number of oocytes for fertilization; cleavage embryo rate = number of D3 cleavage embryos/number of cleaved embryos on day 2; high-quality cleavage embryo rate = number of high-quality D3 cleavage embryos/number of cleaved embryos on day 2; blastocyst rate = number of blastocysts/number of D3 cleavage embryos cultured for blastocyst; high-quality blastocyst rate = number of high-quality blastocysts/number of D3 cleavage embryos cultured for blastocyst; IR = number of gestational sacs/number of transferred embryos; CPR = number of clinical pregnancy cycles/number of fresh embryo transfer cycles; ongoing pregnancy rate = number of ongoing pregnancy cycles/number of fresh embryo transfer cycles; early miscarriage rate = number of early miscarriage cycles/number of clinical pregnancy cycles; and LBR = number of live birth cycles/number of fresh embryo transfer cycles.

## 2.6 Statistical analysis

SPSS v26.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. Continuous variables are expressed as the mean ± standard deviation and were analyzed using one-way ANOVA. Categorical variables are expressed as frequency and were compared using chi-square or Fisher's exact test. Two-tailed $p < 0.05$ was considered as statistically significant, as we had no prior evidence indicating the effect of vaccination on the outcomes of IVF-ET.

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# 3 Results

## 3.1 Baseline characteristics of the study population

A flowchart of the recruitment is shown in Figure 1. Infertile women undergoing their first OS cycle with an antagonist or agonist protocol ($n$ = 1714) were included as participants. Women with missing information on vaccination ($n$ = 37), women >45 or <20 years old ($n$ = 36), those who refused to participate ($n$ = 13), and those with disease-affected embryo transfer ($n$ = 22) and PGT cycles ($n$ = 348) were excluded. Finally, 1,258 patients were enrolled. They were divided into the vaccinated group ($n$ = 713) and the unvaccinated group ($n$ = 545) according to their vaccination status before OS.

TABLE 1 Baseline clinical characteristics of the vaccinated and unvaccinated groups.

| Clinical characteristics | Vaccinated group (n = 713) | Unvaccinated group (n = 545) | *P* value |
|---|---|---|---|
| Age (year) | 31.85 ± 4.64 | 31.51 ± 4.43 | 0.184 |
| Duration of infertility (year) | 3.55 ± 2.99 | 3.31 ± 2.98 | 0.148 |
| Type of infertility (%) | | | 0.293 |
| Primary | 49.23% (351/713) | 46.24% (252/545) | |
| Secondary | 50.77% (362/713) | 53.76% (293/545) | |
| Etiology of infertility (%) | | | 0.368 |
| Tubal factor | 22.02% (157/713) | 16.88% (92/545) | |
| Ovulatory disorder | 4.07% (29/713) | 4.04% (22/545) | |
| Endometriosis | 0.84% (6/713) | 1.28% (7/545) | |
| DOR | 3.37% (24/713) | 3.67% (20/545) | |
| Male factor | 11.36% (81/713) | 11.74% (64/545) | |
| Mixed factor | 51.89% (370/713) | 54.13% (295/545) | |
| Unexplained infertility | 6.45% (46/713) | 8.26% (45/545) | |
| BMI ($kg/m^2$) | 22.37 ± 2.87 | 22.21 ± 2.76 | 0.304 |
| AFC | 15.01 ± 8.47 | 15.01 ± 8.75 | 0.989 |
| AMH (ng/mL) | 3.31 ± 2.99 | 3.54 ± 3.04 | 0.175 |
| Basal FSH (mIU/mL) | 6.91 ± 2.64 | 6.97 ± 2.93 | 0.713 |
| Basal LH (mIU/mL) | 5.28 ± 3.27 | 5.28 ± 2.94 | 0.995 |
| Basal $E_2$ (pg/mL) | 39.29 ± 17.56 | 38.94 ± 17.36 | 0.723 |
| Basal P (ng/mL) | 0.45 ± 0.18 | 0.44 ± 0.19 | 0.234 |
| TT (ng/mL) | 0.26 ± 0.16 | 0.27 ± 0.17 | 0.641 |
| PRL (uIU/mL) | 333.77 ± 127.78 | 333.04 ± 118.95 | 0.953 |

DOR, decreased ovarian reserve; BMI, body mass index; AFC, antral follicle count; AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; $E_2$, estradiol; P, progesterone; TT, total testosterone; PRL, prolactin.

The age, duration, type and etiology of infertility, BMI, AFC, and serum levels of AMH and basal sex hormones were not significantly different between the vaccinated and unvaccinated groups ($p > 0.05$) (Table 1).

## 3.2 Outcomes of OS and embryo culture

The proportion of OS protocol; the types of trigger and fertilization; the dose and duration of rFSH; the serum levels of $E_2$ and P on the trigger day; the numbers of retrieved oocytes, MII, 2PN, cleavage embryos, and high-quality cleavage embryos; the rates of MII, 2PN, cleavage embryo, high-quality cleavage embryo, blastocyst, and high-quality blastocyst; the incidence of mild, moderate, and severe OHSS; and the rates of cancelled oocyte retrieval were not significantly different between the vaccinated and unvaccinated groups ($p > 0.05$) (Table 2).

## 3.3 Outcomes of fresh embryo transfer in the vaccinated and unvaccinated groups

Respectively 221 and 223 patients from the vaccinated and unvaccinated groups had cancelled fresh embryo transfer. As a result, 475 and 312 patients from the vaccinated and unvaccinated groups, respectively, underwent fresh embryo transfer. The thickness of the endometrium; the number and age of embryo transferred; and the IR, CPR, multiple pregnancy rate, early miscarriage rate, ongoing pregnancy rate, LBR, gestational age at delivery, and birth weight of infants were not significantly different between the vaccinated and unvaccinated groups ($p > 0.05$) (Table 3). We further calculated the power to illustrate false negative errors. For the power calculation, we set the target difference for categorical variables as 20%. The results showed that the powers for LBR, CPR, and ongoing pregnancy rate were 0.69, 0.77, and 0.71, respectively, which suggest that the false negative errors for our results were acceptable.

In the vaccinated group, nine patients had ectopic pregnancies and nine patients had late miscarriages, compared with five and three patients, respectively, from the unvaccinated group. There were 282 (137 boys and 145 girls) and 171 (87 boys and 84 girls) live-born infants in the vaccinated and unvaccinated groups, respectively. No birth defects were found in the study. The mean gestational age at delivery was 38 weeks (28–41 weeks), and the mean birth weight was 2,847 g (500–4,430 g).

## 3.4 Outcomes of fresh embryo transfer in the vaccinated subgroups

In both the dose and interval subgroups, no significant differences were found in the IR, CPR, LBR, rates of early miscarriage and ongoing pregnancy, gestational age at delivery, and birth weight of infants ($p > 0.05$) (Table 4).

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TABLE 2 Outcomes of OS and embryo culture of the vaccinated and unvaccinated groups.

| Variables | Vaccinated (n = 713) | Unvaccinated (n = 545) | *P* value |
|---|---|---|---|
| OS protocol (%) | | | 0.198 |
| GnRH antagonist | 43.62% (311/713) | 40.00% (218/545) | |
| GnRH agonist | 56.38% (402/713) | 60.00% (327/545) | |
| rFSH duration (day) | 10.51 ± 1.82 | 10.67 ± 1.87 | 0.130 |
| rFSH dose (IU) | 2367.84 ± 795.28 | 2291.90 ± 895.05 | 0.114 |
| Serum $E_2$ on the trigger day (pg/mL) | 2748.82 ± 2165.37 | 2823.79 ± 2029.01 | 0.537 |
| Serum P on the trigger day (ng/mL) | 1.11 ± 0.67 | 1.11 ± 0.79 | 0.952 |
| Type of trigger (%) | | | 0.676 |
| rHCG | 93.04% (655/704) | 92.42% (500/541) | |
| triptorelin | 6.96% (49/704) | 7.58% (41/541) | |
| Type of fertilization (%) | | | 0.116 |
| IVF | 77.28% (534/691) | 80.98% (430/531) | |
| ICSI | 22.72% (157/691) | 19.02% (101/531) | |
| Oocytes retrieved | 11.55 ± 7.24 | 11.91 ± 7.27 | 0.393 |
| MII oocytes | 9.61 ± 6.25 | 10.02 ± 6.22 | 0.254 |
| 2PN oocytes | 6.41 ± 4.49 | 6.56 ± 4.68 | 0.564 |
| Cleavage embryos | 6.39 ± 4.32 | 6.70 ± 4.74 | 0.233 |
| High-quality cleavage embryos | 1.97 ± 2.22 | 2.21 ± 2.64 | 0.087 |
| MII rate (%) | 82.56% (6638/8040) | 83.49% (5319/6371) | 0.142 |
| 2PN rate (%) | 57.44% (4430/7712) | 56.88% (3485/6127) | 0.506 |
| Cleavage embryo rate (%) | 87.01% (4413/5072) | 86.50% (3556/4111) | 0.475 |
| High-quality cleavage embryo rate (%) | 26.83% (1362/5072) | 28.53% (1173/4111) | 0.073 |
| Blastocyst rate (%) | 50.31% (1603/3186) | 51.69% (1329/2571) | 0.298 |
| High-quality blastocyst rate (%) | 15.47% (493/3186) | 14.51% (373/2571) | 0.308 |
| Incidence of OHSS (%) | | | |
| mild | 2.52% (18/713) | 3.49% (19/545) | 0.317 |
| moderate | 1.82% (13/713) | 0.73% (4/545) | 0.097 |
| severe | 0.70% (5/713) | 0.92% (5/545) | 0.669 |
| Cancelled oocyte retrieval rate (%) | 2.38% (17/713) | 1.83% (10/545) | 0.505 |

OS, ovarian stimulation; GnRH, gonadotrophin-releasing hormone; rFSH, recombinant follicle-stimulating hormone; $E_2$, estradiol; P, progesterone; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection; MII, metaphase II; 2PN, two pronuclei; OHSS, ovarian hyperstimulation syndrome.

TABLE 3 Outcomes of fresh embryo transfer of the vaccinated and unvaccinated groups.

| Variables | Vaccinated (n = 475) | Unvaccinated (n = 312) | *P* value |
|---|---|---|---|
| Thickness of endometrium (mm) | 11.06 ± 2.37 | 10.82 ± 2.34 | 0.158 |
| Number of embryo transferred | 1.82 ± 0.38 | 1.83 ± 0.38 | 0.833 |
| Age of embryo transferred (%) | | | 0.102 |
| Cleavage | 92.63% (440/475) | 95.51% (298/312) | |
| Blastocyst | 7.37% (35/475) | 4.49% (14/312) | |
| IR (%) | 38.73% (335/865) | 34.21% (195/570) | 0.083 |
| CPR (%) | 55.79% (265/475) | 51.28% (160/312) | 0.215 |
| Multiple pregnancy rate (%) | 26.42% (70/265) | 21.88% (35/160) | 0.293 |
| Early miscarriage rate (%) | 10.19% (27/265) | 8.13% (13/160) | 0.480 |
| Ongoing pregnancy rate (%) | 48.21% (229/475) | 45.51% (142/312) | 0.458 |
| LBR (%) | 46.32% (220/475) | 44.55% (139/312) | 0.627 |
| Gestational age at delivery (week) | 37.68 ± 2.01 | 37.86 ± 1.92 | 0.386 |
| Birth weight (g) | 2820.74 ± 601.58 | 2889.38 ± 625.04 | 0.247 |

IR, implantation rate; CPR, clinical pregnancy rate; LBR, live birth rate.

# 4 Discussion

This study found that the inactivated COVID-19 vaccine did not affect the ovarian response to OS, the quality of oocytes and embryos, the LBR, and the birth weight of infants, and neither did the dose and interval of vaccination. Therefore, the vaccine appears to have no effect on the outcomes of IVF-ET.

Ovarian response is an important factor affecting the success rate of IVF-ET (33). In this study, the duration and dose of rFSH, the number of oocytes, the rates of blastocyst and high-quality blastocyst, and the numbers and rates of MII, 2PN, cleavage embryos, and high-quality cleavage embryos were similar for the vaccinated and unvaccinated groups. Another study also found no significant differences in the duration and dose of gonadotrophin between women with or without inoculation with the inactivated COVID-19 vaccine (214 *vs.* 340) (23). Dong et al. found no significant differences in the number of oocytes retrieved, the rates of fertilization, and the cleavage embryo, high-quality embryo, and blastocyst among four groups (both partners vaccinated with the COVID-19 vaccine or not, and only women or men vaccinated) (23). Other studies also reported similar numbers of oocytes, MII oocytes, 2PN, and cleaved embryos and high-quality embryos, as well as blastocyst rates, between vaccinated and unvaccinated groups (24, 25). A recent study has even shown that more oocytes were retrieved following inoculation

TABLE 4 Outcomes of fresh embryo transfer in vaccinated subgroups.

| Variables | Dose of inoculation | | | | Interval between the first inoculation and OS | | | |
|---|---|---|---|---|---|---|---|---|
| | Single dose (n = 74) | Double dose (n = 275) | Triple dose (n = 126) | *P* value | < 3 months (n = 65) | 3 ~ 6 months (n = 123) | > 6 months (n = 287) | *P* value |
| IR (%) | 46.67% (63/135) | 37.15% (185/498) | 37.50% (87/232) | 0.119 | 45.45% (55/121) | 34.51% (78/226) | 39.00% (202/518) | 0.134 |
| CPR (%) | 62.16% (46/74) | 54.18% (149/275) | 55.56% (70/126) | 0.470 | 63.08% (41/65) | 51.22% (63/123) | 56.10% (161/287) | 0.293 |
| Early miscarriage rate (%) | 6.52% (3/46) | 11.41% (17/149) | 10.00% (7/70) | 0.631 | 7.32% (3/41) | 6.35% (4/63) | 12.42% (20/161) | 0.323 |
| Ongoing pregnancy rate (%) | 56.76% (42/74) | 46.18% (127/275) | 47.62% (60/126) | 0.268 | 56.92% (37/65) | 45.53% (56/123) | 47.39% (136/287) | 0.300 |
| LBR (%) | 56.76% (42/74) | 44.00% (121/275) | 45.24% (57/126) | 0.143 | 56.92% (37/65) | 43.09% (53/123) | 45.30% (130/287) | 0.167 |
| Gestational age at delivery (week) | 37.62 ± 1.58 | 37.64 ± 2.17 | 37.81 ± 1.98 | 0.852 | 37.62 ± 1.83 | 37.72 ± 2.32 | 37.68 ± 1.94 | 0.976 |
| Birth weight (g) | 2755.42 ± 537.38 | 2851.39 ± 620.85 | 2810.00 ± 613.51 | 0.576 | 2750.69 ± 551.88 | 2882.01 ± 604.02 | 2817.50 ± 616.15 | 0.500 |

IR, implantation rate; CPR, clinical pregnancy rate; LBR, live birth rate.

of the COVID-19 mRNA vaccine, although the number of MII oocytes remained similar (34). Therefore, we propose that the inactivated COVID-19 vaccine may not significantly impact the ovarian response and the quality of oocytes and embryos in IVF-ET.

LBR is an important outcome of IVF-ET. Endometrial receptivity is a key factor that affects the LBR in IVF-ET (35). Although SARS-CoV-2 has not yet been isolated from human endometrium, the endometrium may still be susceptible to SARS-CoV-2 infection, particularly during the period of implantation (36, 37). The expression of the *ACE2* and *TMPRSS4* genes (both associated with viral infection) in the human endometrium may facilitate SARS-CoV-2 infection. During embryo implantation, their increased expression from the proliferative phase to the secretory phase may confer an increased risk for SARS-CoV-2 infection (36). Therefore, vaccination may be beneficial to reducing the risk of SARS-CoV-2 infection of the endometrium. However, it remains unclear whether the inactivated COVID-19 vaccine could affect the receptivity of the human endometrium. Studies have reported that women vaccinated with the inactivated COVID-19 vaccine had comparable LBR, rates of ongoing pregnancy, and clinical pregnancy compared with those unvaccinated in FET (27, 28). For the IVF/ICSI-ET treatment cycle, no influence on LBR was found (29). We also found that LBR was not affected by the inactivated COVID-19 vaccine, which suggests no significant risk of the inactivated COVID-19 vaccine to endometrial receptivity and pregnancy. Nevertheless, these results should be interpreted with caution considering the moderate power value of LBR (0.69); moreover, a larger sample size is required for further study.

There has been no consensus over the optimal interval between the vaccination and IVF-ET. The European Society of Human Reproduction and Embryology has recommended postponing the ART treatment for up to 2 months after the vaccination (38). The American Society for Reproductive Medicine has suggested that, given the time for recovery from the common side effects of the vaccine, vaccination should be avoided at least 3 days prior and after the oocyte retrieval and embryo transfer (39). Experts from China have recommended couples with stable immune response to undergo ART treatment 1 month after vaccination (40). We found similar CPRs for the three interval groups with IVF treatment (<3, 3–6, and >6 months), which is in keeping with a previous report (23). Therefore, we propose that IVF-ET should not be postponed due to the vaccination.

A study in China found that neither a single nor a double dose of an inactivated COVID-19 vaccine impacted the LBR and the birth height and weight of newborns in FET, but did not report on the triple dose as most patients were not yet inoculated with the third vaccine (27). We also found no significant differences in the IR, CPR, early miscarriage rate, ongoing pregnancy rate, LBR, gestational age at delivery, and birth weight of infants with the single, double, and triple doses of inoculations. We propose that the administration of a booster dose of an inactivated COVID-19 vaccine may be safe before IVF-ET. However, further studies are warranted to confirm these results and to explore their long-term effects.

This study compared the effects of vaccination with different doses and intervals on the LBR, which has not been reported previously. It has provided evidence of the safety of inactivated COVID-19 vaccines and could contribute to the improvement of existing studies. The limitations of this study included the small sample size for the single-dose group and the interval groups (<3 months) and that all patients were from a single center. Therefore, our findings should be confirmed in multicenter studies with a larger sample size. Furthermore, this study was conducted only on Chinese women, and the results may not be directly extrapolated for other ethnic/racial populations and/or vaccine types. More studies should be conducted on the follow-ups and collect additional data (cumulative pregnancy outcomes) for a more comprehensive analysis.

## 5 Conclusion

In summary, this study found that inoculation with the inactivated COVID-19 vaccine before IVF-ET did not affect the ovarian response, the quality of oocytes and embryos, the LBR, and

the birth weight of infants, and neither did the dose and interval of vaccination. Therefore, the inactivated COVID-19 vaccine is safe for patients undergoing IVF-ET and should be inoculated before IVF-ET.

## Data availability statement

The data that support the findings of this study are available on request from the corresponding author upon reasonable request. Requests to access these datasets should be directed to YG, gongyan0619@163.com.

## Ethics statement

The study was approved by the Medical Ethics Committee of Sichuan Provincial Women's and Children's Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

## Author contributions

J-jW: Writing – original draft, Supervision. YQ: Data curation, Writing – review & editing. ML: Data curation, Writing – review & editing. F-rC: Formal analysis, Writing – review & editing. M-yL: Data curation, Investigation, Writing – review & editing. XD: Data curation, Investigation, Writing – review & editing. R-nM: Data curation, Investigation, Writing – review & editing. JH: Data curation, Investigation, Writing – review & editing. JL-L: Writing – review & editing. YG: Supervision, Writing – review & editing, Funding acquisition.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# The role of oxidative balance lifestyle factors in reducing female infertility risk: insights from a population-based study

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**Background:** Diet, lifestyle, and oxidative stress have been linked to female infertility, with the Oxidative Balance Score (OBS) serving as a comprehensive indicator of an individual's oxidative and antioxidant status. This study aims to investigate the correlation between OBS and female infertility.

**Methods:** The National Health and Nutrition Examination Survey (NHANES) from 2013 to 2020 were utilized. Weighted multivariate regression analyses and restricted cubic splines (RCS) were employed to analysis. Additionally, subgroup analyses and multiple imputat6ions (MI) were carried out as sensitivity analyses to ensure the strength and reliability of the findings.

**Results:** A total of 3,905 individuals were involved in the study, the prevalence of female infertility was 11.96%. Individuals with infertility exhibited significantly lower OBS compared to those with normal fertility (19.74 ± 0.37 vs. 21.42 ± 0.20). The OBS dietary and lifestyle components also had lower scores, with averages of 15.98 ± 0.33 vs. 17.12 ± 0.18 and 3.76 ± 0.11 vs. 4.29 ± 0.05, respectively. Weighted logistic regression results revealed that a one-point increase in OBS score was associated with a 3% decrease in infertility risk (Odds Ratio (OR) 0.97, 95% Confidence Interval (CI): 0.95, 0.99). Similarly, a one-point increase in OBS lifestyle score was linked to a 15% decrease in infertility risk (OR: 0.85, 95% CI: 0.75, 0.96), and a one-point increase in OBS dietary score was associated with a 2% decrease in infertility risk (OR: 0.98, 95% CI: 0.96, 0.99). Subgroup analyses revealed that individuals with no prior history of pregnancy benefited more from OBS and OBS lifestyle in terms of infertility risk reduction compared to those with a history of pregnancy.

**Conclusion:** OBS is found to have a negative correlation with infertility, particularly in cases of primary infertility. The results of this study indicate that adopting an antioxidant-rich diet and lifestyle could potentially lower the risk of infertility.

KEYWORDS

**oxidative balance score, infertility, antioxidant, lifestyle, NHANES**

# Introduction

Infertility, defined as the inability to conceive after at least 12 months of consistent, unprotected sexual activity (1) affects approximately 15% of couples worldwide during their reproductive years. This rate is on a steadily increasing year by year. Previous studies (2, 3) on the global burden of infertility from 1990 to 2017 showed a 0.37% yearly rise in the age-standardized prevalence of infertility. Beyond its direct impact on reproductive health, female infertility has been identified as a potential risk factor for the development of cancers in the reproductive system and metabolic disorders (4, 5). This escalating prevalence, coupled with its broad-ranging implications, underscores the urgent need to address infertility as a critical global health issue.

The etiology of female infertility encompasses multiple factors, including ovulatory dysfunction, fallopian tube pathology, endometriosis, uterine abnormalities, and unexplained causes (6, 7). While medical conditions contribute significantly to infertility, modifiable lifestyle and environmental factors are increasingly recognized for their role. Many of these factors exert their effects through oxidative stress pathways, which may present opportunities for targeted intervention.

Oxidative stress, characterized by an imbalance between reactive oxygen species (ROS) production and the body's antioxidant defense mechanisms, is recognized as a crucial factor in the development of various chronic diseases, including cardiovascular disease, type 2 diabetes, chronic kidney disease, and osteoarthritis (8–12). To assess this complex interplay, the Oxidative Balance Score (OBS) was developed (13, 14). The OBS is a comprehensive measure that integrates dietary and lifestyle factors to evaluate an individual's oxidative and antioxidant status. Unlike individual biomarkers, the OBS provides a holistic assessment by incorporating both pro-oxidant and antioxidant dietary components, alongside lifestyle factors such as physical activity, body mass index (BMI), alcohol consumption, and smoking status. The OBS is calculated based on a weighted combination of these factors, with specific weights assigned to each component according to its relative contribution to oxidative stress. Over time, the OBS has been refined to enhance its predictive accuracy and applicability across diverse populations, emphasizing its growing importance as an epidemiological tool.

Additionally, the OBS offers valuable insights into the potential benefits of lifestyle modifications in reducing oxidative stress. Our study aims to fill this gap by providing a comprehensive evaluation of the relationship between OBS and female infertility.

Although previous studies (15–17) have examined isolated aspects of oxidative stress in relation to fertility, the association between comprehensive oxidative balance and female infertility remains largely unexplored. We hypothesize that a higher OBS, indicative of better oxidative balance, is associated with a lower risk of infertility, potentially with differential effects based on age and pregnancy history. This hypothesis is supported by the known age-related changes in oxidative stress and the varying etiologies of infertility across different age groups. This population-based study leverages NHANES data to investigate the relationship between OBS and female infertility, with a particular focus on examining variations across different subgroups of women. Our findings may have significant clinical implications, suggesting that targeted lifestyle interventions aimed at improving oxidative balance could be an effective strategy for reducing the risk of infertility.

# Methods

## Study population

This cross-sectional study leveraged data from the NHANES, conducted between 2013 and 2020. The NHANES, managed by the National Center for Health Statistics (NCHS), is carried out every two years to assess the health and nutritional status of the non-institutionalized civilian population in the United States. Using a complex, multistage stratified sampling approach, NHANES collects data from a diverse group of participants through surveys, physical exams, and lab tests, offering a thorough and scientifically sound evaluation of public health. The initial screening encompassed 15,689 participants. After excluding females outside the age range of 20 to 45 years (n=10,380), those without reproductive health data (n=223), and participants lacking obstetric service information (n=1,181), the final study population comprised 3,905 women.

## Measurement of OBS

The OBS is derived from a comprehensive assessment that integrates 16 dietary components and 4 lifestyle elements, encompassing 15 antioxidants and 5 pro-oxidants (13, 14). Supplementary Table 1 illustrates the detailed scoring system for OBS. Dietary antioxidants are scored from 0 to 2, with the highest tertile receiving a score of 0 and the lowest tertile a score of 2 for pro-oxidants. Lifestyle factors were assessed using a standardized scoring system: individuals received 0 points for engaging in less than 400 MET-minutes per week, 1 point for 400 to 1,000 MET-minutes, and 2 points for exceeding 1,000 MET-minutes weekly in terms of physical activity. For alcohol consumption, a score of 0 was assigned for intakes above 30 g/day, 1 point for 0 to 30 g/day, and 2 points for abstainers. Body Mass Index (BMI) was categorized as 0 points for obesity, 1 point for overweight, and 2 points for normal weight individuals. Serum cotinine levels were scored as 0 points for concentrations above 0.038 ng/mL, 1 point for levels between 0.038 to 1.13 ng/mL, and 2 points for values below 1.13 ng/mL. This systematic scoring system facilitated the evaluation of lifestyle-related health risks in the study population (18, 19).

## Measurement of infertility

The primary outcome variable of this study was infertility. Infertility status was determined based on self-reported data from

women who participated in the NHANES Reproductive Health Questionnaire (RHQ). Specifically, the response to item RHQ074, "Have you ever attempted to become pregnant over a period of at least a year without becoming pregnant?" was used to classify women. Those who answered "yes" to this question were categorized as having infertility (20).

## Covariables

The collection of variables in this study includes demographic characteristics such as age, BMI, race, marital status, education level, poverty-income ratio (PIR), history of ever pregnancies. Comorbid conditions such as diabetes, hypertension, metabolic syndrome, as well as laboratory biochemical indicators like estimated glomerular filtration rate (eGFR), HDL cholesterol, HbA1c, and albumin.

Diabetes mellitus was identified based on a combination of factors: a hemoglobin A1c (HbA1c) concentration of 6.5% or greater, a fasting plasma glucose (FPG) level exceeding 126 mg/dL, documented use of hypoglycemic agents, or a self-reported medical history of diabetes. Hypertension was diagnosed when systolic and diastolic blood pressure measurements reached or surpassed 140/90 mmHg, patients were on antihypertensive therapy, or there was a self-reported history of hypertension. For the identification of metabolic syndrome in the adult population, the criteria established by the National Cholesterol Education Program's Adult Treatment Panel III (NCEP-ATP III) (21, 22) were applied. These criteria provide a standardized approach for the clinical assessment and diagnosis of these conditions.

## Statistical analysis

Data processing in our study adhered to NHANES analysis guidelines, ensuring that no variable had missing data exceeding 10%. All analyses incorporated appropriate sampling weights. Continuous variables were presented as weighted means accompanied by standard errors, while categorical variables were depicted as weighted percentages with their respective standard errors. Disparities in continuous baseline attributes were assessed using Student's t-test, and for categorical variables, the chi-square test was applied. A weighted multiple logistic regression model was utilized to investigate the association between OBS and infertility, adjusting for various demographic and health-related variables such as age, race, marital status, educational attainment, PIR, DM, hypertension, MetS, eGFR, HDL cholesterol, HbA1c, and albumin. OBS was categorized into quartiles for trend analysis and subgroup analyses were conducted to explore relationships within different demographic and health characteristic strata. Interaction tests were performed to assess consistency across subgroups. Nonlinear relationships were examined using RCS and multiple imputation techniques were employed for reliability. Statistical analyses were carried out using R software (version 4.3.0) and Free Statistics software (version 1.9.2).

# Results

## Baseline characteristics

Figure 1 shows a flowchart of the data integration procedure. Table 1 presents the initial demographic and characteristics of the participants in the study, stratified by the presence of infertility. Participants with infertility had significantly lower scores in the OBS, OBS dietary, and OBS lifestyle categories compared to their fertile counterparts. Infertile participants showed distinct characteristics, such as older age and a higher prevalence of prior pregnancies. Furthermore, this group had a higher occurrence of comorbidities like diabetes mellitus, hypertension, and metabolic syndrome. On the other hand, individuals in the infertile group had lower levels of high-density lipoprotein (HDL) cholesterol and albumin compared to fertile individuals. These results indicate an intricate relationship between fertility status and different health factors.

## Association between oxidative balance score and infertility

Table 2 presents the results of a weighted regression analysis that examines the relationship between the OBS and the prevalence of infertility. The analysis revealed that for each one-unit increase in the OBS, there was a corresponding decrease in the likelihood of experiencing infertility, with an OR of 0.97 and a 95%CI of 0.95 to 0.98. These findings suggest a small but statistically significant protective impact of higher OBS against infertility. This association was consistent across different models, with the OR remaining stable. In the fully adjusted model, an increment of one point in OBS corresponded to a 3% decrease in the likelihood of infertility (OR: 0.97, 95% CI: 0.95, 0.99).

When OBS was modeled as a quadratic variable, the unadjusted model showed that, compared to the first quartile, the adjusted ORs for the second, third, and fourth quartiles were 0.7 (95% CI: 0.54-0.91), 0.7 (95% CI: 0.51-0.96), and 0.42 (95% CI: 0.30-0.59), respectively. After adjusting for relevant covariates, the analysis found no statistically significant disparities between the second (Q2) and third (Q3) quartiles compared to the first quartile (Q1). However, the fourth quartile (Q4) displayed a significant decrease (Model 3 adjusted OR: 0.47, 95% CI: 0.31-0.72), suggesting a linear trend for OBS in relation to infertility diagnosis (trend $p < 0.05$).

Additionally, we examined the distinct relationships between OBS dietary and OBS lifestyle components with infertility. The data in Table 2 indicate that both OBS dietary and OBS lifestyle components are significantly correlated with infertility in both unadjusted and adjusted models.

## Subgroup analyses

Subgroup and interaction analyses were performed, stratified by age, diabetes, hypertension, history of previous pregnancies, and

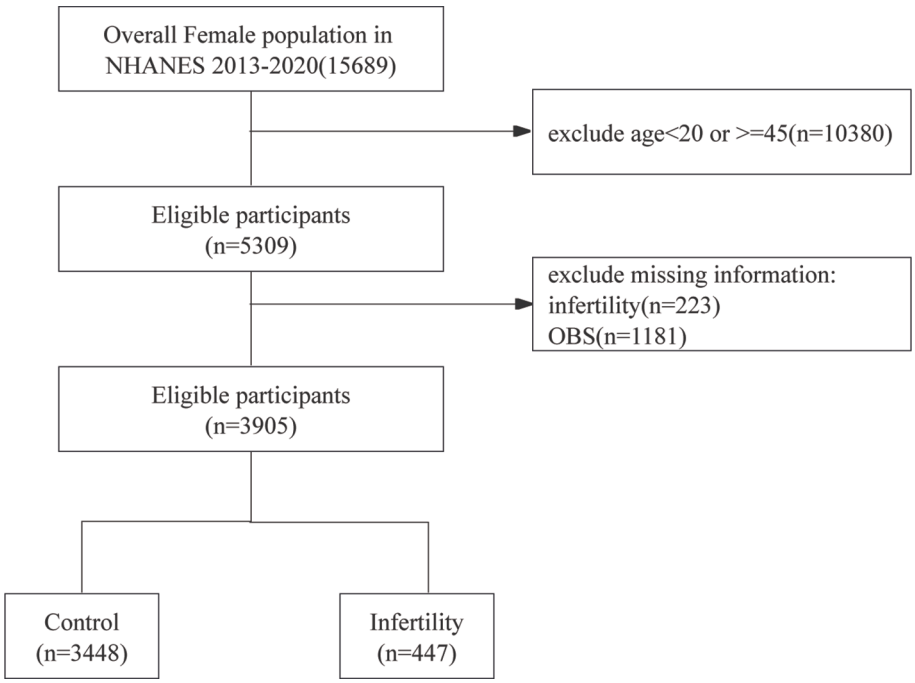


FIGURE 1
Flow chart of participants selection. NHANES, National Health and Nutrition Examination Survey.

metabolic syndrome, to evaluate the relationship between the OBS and infertility and to identify potential modifiers of this relationship. Table 3 indicates no statistically significant differences in the associations with age, diabetes, hypertension, or metabolic syndrome. However, a significant interaction was observed for the history of previous pregnancies (interaction $p < 0.05$).

Among individuals without a history of previous pregnancies, OBS showed a stronger negative correlation with infertility [OR 0.95, 95% CI (0.89, 1.01)] than among those with such a history [OR 0.98, 95% CI (0.96, 1)]. A significant interaction between age and history of previous pregnancies was noted in the OBS lifestyle component (interaction $p < 0.05$) (Table 4). For individuals under 35 years, OBS demonstrated a significantly stronger negative correlation with infertility [OR 0.75, 95% CI (0.65, 0.85)] compared to those aged 35 and above [OR 0.98, 95% CI (0.84, 1.16)]. Additionally, in individuals without a history of previous pregnancies, OBS lifestyle had a notably stronger negative correlation with infertility [OR 0.63, 95% CI (0.48, 0.83)] than among those with a history [OR 0.92, 95% CI (0.82, 1.05)]. In the case of OBS dietary (Table 5), no interaction was observed, yet individuals without a history of previous pregnancies showed a slightly stronger negative association with infertility [OR 0.97, 95% CI (0.91, 1.03)] compared to those with a history [OR 0.98, 95% CI (0.96, 1)].

Figure 2 illustrates that OBS was significantly higher in individuals without a history of previous pregnancies compared to the infertile population, a difference not observed in those with a history of previous pregnancies. Similar findings were noted for OBS dietary.

## Restricted cubic spline analysis

To investigate the potential non-linear association between the OBS and infertility, we performed RCS analysis. Employing a logistic regression model with smooth curve fitting, after controlling for additional factors, we found that the non-linear connection between OBS and infertility was not statistically significant. (Figure 3). Additionally, the non-linear associations between OBS dietary, OBS lifestyle components, and infertility were also found to be non-significant.

## Sensitivity analysis

Supplementary Table 2 presents the findings from a re-analysis of the raw data without multiple imputing missing values, which corroborates the main study findings. Regardless of how OBS was considered - as either a continuous or categorical variable - a significant negative correlation with infertility was observed.

# Discussion

Our analysis revealed a significant negative correlation between the OBS and female infertility. Specifically, we found that higher OBS levels were associated with a decreased incidence of infertility, with each one-point increase in OBS corresponding to a 3% reduction in infertility risk. This relationship remained consistent across multiple adjusted models, suggesting a robust association independent of demographic and clinical confounders.

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TABLE 1 Baseline characteristics of participants, weighted(N=3905).

| Variable | Total | Fertility | Infertility | P value |
|---|---|---|---|---|
| OBS | 21.22 ± 0.20 | 21.42 ± 0.20 | 19.74 ± 0.37 | < 0.0001 |
| OBS.dietary | 16.99 ± 0.18 | 17.12 ± 0.18 | 15.98 ± 0.33 | < 0.001 |
| OBS.lifestyle | 4.23 ± 0.05 | 4.29 ± 0.05 | 3.76 ± 0.11 | < 0.0001 |
| Age(%) | | | | < 0.0001 |
| <35 year | 59.98(0.02) | 61.68(1.22) | 47.49(3.31) | |
| >=35 year | 40.02(0.02) | 38.32(1.22) | 52.51(3.31) | |
| Race (%) | | | | 0.28 |
| Non-Hispanic White | 56.06(0.03) | 55.48(2.13) | 60.33(3.40) | |
| Non-Hispanic Black | 13.74(0.01) | 13.85(1.22) | 12.97(1.56) | |
| Mexican Ameirican | 11.73(0.01) | 11.83(1.19) | 10.95(1.82) | |
| Other Race | 18.47(0.01) | 18.84(1.19) | 15.75(2.03) | |
| Marital status(%) | | | | < 0.0001 |
| Solitude | 38.26(0.02) | 48.83(1.45) | 26.00(2.90) | |
| Cohabitation | 44.56(0.02) | 51.17(1.45) | 74.00(2.90) | |
| Education level(%) | | | | 0.17 |
| Less than or high school | 29.99(0.01) | 29.64(1.51) | 32.63(2.35) | |
| Above high school | 69.99(0.03) | 70.36(1.51) | 67.37(2.35) | |
| PIR(%) | | | | 0.23 |
| <1.3 | 25.68(0.01) | 28.03(1.20) | 24.14(2.45) | |
| 13.,3.5 | 33.67(0.02) | 35.61(1.24) | 39.86(2.99) | |
| >=3.5 | 33.85(0.02) | 36.36(1.67) | 36.00(2.98) | |
| Ever pregnant(%) | | | | < 0.0001 |
| No | 31.45(0.02) | 33.63(1.28) | 15.53(1.85) | |
| Yes | 68.49(0.02) | 66.37(1.28) | 84.47(1.85) | |
| DM(%) | | | | 0.001 |
| No | 85.11(0.03) | 90.04(0.61) | 84.84(1.70) | |
| Pre DM | 4.20(0.00) | 4.37(0.47) | 4.74(1.15) | |
| DM | 5.86(0.00) | 5.59(0.41) | 10.42(1.49) | |
| Hypertension(%) | | | | 0.004 |
| No | 86.47(0.03) | 87.21(0.77) | 81.25(2.19) | |
| Yes | 13.49(0.01) | 12.79(0.77) | 18.75(2.19) | |
| MetS(%) | | | | < 0.001 |
| No | 75.00(0.03) | 80.05(1.11) | 69.41(2.97) | |
| Yes | 20.17(0.01) | 19.95(1.11) | 30.59(2.97) | |
| eGFR | 111.06 ± 0.62 | 111.31 ± 0.64 | 109.26 ± 1.14 | 0.07 |
| HDL(mmol.L) | 1.48 ± 0.01 | 1.49 ± 0.01 | 1.39 ± 0.03 | 0.001 |
| HbA1c | 5.33 ± 0.01 | 5.31 ± 0.01 | 5.48 ± 0.03 | < 0.0001 |
| Albumin(g.L) | 41.05 ± 0.10 | 41.10 ± 0.10 | 40.64 ± 0.24 | 0.07 |

Values are mean +/- SD (continuous variables) or n% (categorical variables) are weighted.
OBS, Oxidative balance score; BMI, Body mass index; PIR, Poverty to income ratio; DM, Diabetes mellitus; MetS, Metabolic syndrome; eGFR, estimated Glomerular Filtration Rate.

TABLE 2 Association between OBS with infertility.

| Character | Crude model (95%CI) | Model 1 (95%CI) | Model 2 (95%CI) | Model 3 (95%CI) |
|---|---|---|---|---|
| OBS | 0.97 (0.95,0.98)* | 0.96 (0.94,0.98)* | 0.97 (0.95,0.99)* | 0.97 (0.95,0.99)* |
| OBS quantile | | | | |
| Q1 | ref | ref | ref | ref |
| Q2 | 0.70 (0.54,0.91)* | 0.77 (0.55,1.06) | 0.74 (0.53,1.02) | 0.76 (0.54,1.07) |
| Q3 | 0.70 (0.51,0.96)* | 0.71 (0.48,1.05) | 0.74 (0.50,1.11) | 0.80 (0.53,1.21) |
| Q4 | 0.42 (0.30,0.59)* | 0.40 (0.27,0.60)* | 0.45 (0.29,0.69)* | 0.47 (0.31,0.72)* |
| p for trend | <0.0001 | <0.0001 | <0.001 | 0.001 |
| OBS lifestyle | 0.82 (0.76,0.89)* | 0.82 (0.74,0.91)* | 0.85 (0.76,0.95)* | 0.85 (0.75,0.96)* |
| OBS lifestyle quantile | | | | |
| Q1 | ref | ref | ref | ref |
| Q2 | 0.80 (0.56,1.13) | 0.76 (0.50,1.14) | 0.79 (0.53,1.17) | 0.82 (0.53,1.25) |
| Q3 | 0.61 (0.43,0.86)* | 0.58 (0.38,0.90)* | 0.65 (0.42,1.00)* | 0.64 (0.41,1.01) |
| Q4 | 0.36 (0.21,0.60)* | 0.38 (0.21,0.71)* | 0.44 (0.23,0.85)* | 0.45 (0.23,0.88)* |
| p for trend | <0.0001 | <0.001 | 0.01 | 0.01 |
| OBS dietary | 0.97 (0.96,0.99)* | 0.97 (0.95,0.99)* | 0.97 (0.95,0.99)* | 0.98 (0.96,0.99)* |
| OBS dietary quantile | | | | |
| Q1 | ref | ref | ref | ref |
| Q2 | 0.63 (0.48,0.81)* | 0.67 (0.48,0.93)* | 0.68 (0.49,0.95)* | 0.68 (0.49,0.95)* |
| Q3 | 0.84 (0.64,1.11) | 0.86 (0.60,1.21) | 0.85 (0.59,1.22) | 0.90 (0.62,1.32) |
| Q4 | 0.50 (0.35,0.69)* | 0.46 (0.31,0.70)* | 0.51 (0.33,0.78)* | 0.52 (0.35,0.80)* |
| p for trend | <0.001 | 0.002 | 0.01 | 0.01 |

Crude model: no covariates were adjusted.
Model 1, age, marital status, race, education level, PIR and ever pregnant were adjusted.
Model 2, Model 1+DM and hypertension were adjusted.
Model 3, Model 2+MetS, eGFR, alumin, HDL and HbA1c were adjusted.
*mean p <0.05.



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TABLE 3 Subgroup analysis between OBS with infertility.

| Character | Crude model (95%CI) | Model 1 (95%CI) | Model 2 (95%CI) | Model 3 (95%CI) | p for interaction |
|---|---|---|---|---|---|
| Age | | | | | 0.48 |
| <35 | 0.95 (0.93,0.98) | 0.96 (0.93,0.99) | 0.96 (0.93,0.99) | 0.96 (0.93,0.99) | |
| >=35 | 0.98 (0.95,1.00) | 0.97 (0.94,1.00) | 0.97 (0.94,1.00) | 0.97 (0.95,1.00) | |
| DM | | | | | 0.9 |
| No | 0.97 (0.95,0.99) | 0.97 (0.94,0.99) | 0.97 (0.95,0.99) | 0.97 (0.95,0.99) | |
| PreDM | 0.92 (0.85,1.00) | 0.91 (0.80, 1.03) | 0.91 (0.83, 1.01) | 0.91 (0.82, 1.02) | |
| DM | 1.00 (0.94,1.06) | 0.98 (0.89, 1.08) | 0.98 (0.88, 1.08) | 1.00 (0.89, 1.11) | |
| Hypertension | | | | | 0.99 |
| No | 0.97 (0.95,0.98) | 0.96 (0.94,0.98) | 0.97 (0.95,0.99) | 0.97 (0.95,1.00) | |
| Yes | 0.97 (0.94,1.01) | 0.96 (0.92,1.01) | 0.96 (0.91, 1.01) | 0.96 (0.92,1.01) | |
| Ever pregnant | | | | | 0.01 |
| No | 0.92 (0.88,0.95) | 0.93 (0.89, 0.98) | 0.93 (0.88, 0.98) | 0.95 (0.89, 1.01) | |
| Yes | 0.98 (0.97,0.99) | 0.97 (0.95,0.99) | 0.98 (0.96,1.00) | 0.98 (0.96,1.00) | |
| MetS_ATP | | | | | 0.47 |
| No | 0.97 (0.95,0.99) | 0.97 (0.95,1.00) | 0.97 (0.95,1.00) | 0.97 (0.95,1.00) | |
| Yes | 0.96 (0.92,0.99) | 0.96 (0.92,1.00) | 0.96 (0.92,1.00) | 0.96 (0.92,1.00) | |

Crude model: no covariates were adjusted.
Model 1, age, marital status, race, education level, PIR and ever pregnant were adjusted.
Model 2, Model 1+DM and hypertension were adjusted.
Model 3, Model 2+MetS, eGFR, alumin, HDL and HbA1c were adjusted.
*mean p <0.05.



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TABLE 4 Subgroup analysis between OBS lifestyle with infertility.

| Character | Crude model (95%CI) | Model 1 (95%CI) | Model 2 (95%CI) | Model 3 (95%CI) | p for interaction |
|---|---|---|---|---|---|
| Age | | | | | < 0.001 |
| <35 | 0.74 (0.67,0.82) | 0.73 (0.65,0.83) | 0.74 (0.64,0.84) | 0.75 (0.65,0.85) | |
| >=35 | 0.94 (0.83,1.06) | 0.92 (0.78,1.09) | 1.00 (0.86,1.16) | 0.98 (0.84,1.16) | |
| DM | | | | | 0.15 |
| No | 0.83 (0.75,0.91) | 0.83 (0.74,0.94) | 0.84 (0.74,0.94) | 0.82 (0.73,0.93) | |
| PreDM | 0.96 (0.64,1.44) | 0.77 (0.45, 1.31) | 0.91 (0.50, 1.66) | 0.97 (0.44, 2.16) | |
| DM | 1.09 (0.88,1.36) | 1.07 (0.72, 1.60) | 1.06 (0.70, 1.62) | 1.08 (0.71, 1.63) | |
| Hypertension | | | | | 0.3 |
| No | 0.81 (0.75,0.89) | 0.81 (0.73,0.91) | 0.84 (0.75,0.94) | 0.83 (0.74,0.94) | |
| Yes | 0.94 (0.77,1.14) | 0.93 (0.73,1.19) | 0.95 (0.73, 1.24) | 0.97 (0.74,1.27) | |
| Ever pregnant | | | | | < 0.001 |
| No | 0.64 (0.54,0.75) | 0.63 (0.51, 0.78) | 0.65 (0.53, 0.80) | 0.63 (0.48, 0.83) | |
| Yes | 0.90 (0.82,1.00) | 0.88 (0.79,0.99) | 0.93 (0.83,1.04) | 0.92 (0.82,1.05) | |
| MetS_ATP | | | | | 0.08 |
| No | 0.85 (0.77,0.93) | 0.83 (0.74,0.94) | 0.83 (0.74,0.94) | 0.79 (0.69,0.90) | |
| Yes | 0.92 (0.79,1.08) | 0.97 (0.80,1.18) | 0.98 (0.81,1.19) | 1.01 (0.84,1.22) | |

Crude model: no covariates were adjusted.
Model 1, age, marital status, race, education level, PIR and ever pregnant were adjusted.
Model 2, Model 1+DM and hypertension were adjusted.
Model 3, Model 2+MetS, eGFR, alumin, HDL and HbA1c were adjusted.
*mean p <0.05.





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TABLE 5 Subgroup analysis between OBS dietary with infertility.

| Character | Crude model (95%CI) | Model 1 (95%CI) | Model 2 (95%CI) | Model 3 (95%CI) | p for interaction |
|---|---|---|---|---|---|
| Age | | | | | 0.94 |
| <35 | 0.97 (0.94,0.99) | 0.97 (0.94,1.00) | 0.98 (0.95,1.01) | 0.97 (0.94,1.01) | |
| >=35 | 0.98 (0.95,1.00) | 0.97 (0.94,1.00) | 0.97 (0.94,1.00) | 0.97 (0.94,1.00) | |
| DM | | | | | 0.9 |
| No | 0.97 (0.95,0.99) | 0.97 (0.95,1.00) | 0.97 (0.95,1.00) | 0.98 (0.96,1.00) | |
| PreDM | 0.92 (0.84,1.01) | 0.92 (0.81, 1.04) | 0.92 (0.82, 1.02) | 0.92 (0.82, 1.02) | |
| DM | 0.99 (0.93,1.06) | 0.98 (0.88, 1.08) | 0.97 (0.88, 1.08) | 0.99 (0.89, 1.11) | |
| Hypertension | | | | | 0.78 |
| No | 0.97 (0.96,0.99) | 0.97 (0.95,0.99) | 0.97 (0.95,1.00) | 0.98 (0.95,1.00) | |
| Yes | 0.98 (0.94,1.01) | 0.96 (0.92,1.01) | 0.96 (0.91, 1.01) | 0.96 (0.92, 1.01) | |
| Ever pregnant | | | | | 0.14 |
| No | 0.93 (0.89,0.98) | 0.95 (0.91, 1.01) | 0.95 (0.90, 1.01) | 0.97 (0.91, 1.03) | |
| Yes | 0.98 (0.97,1.00) | 0.97 (0.96,0.99) | 0.98 (0.96,1.00) | 0.98 (0.96,1.00) | |
| MetS_ATP | | | | | 0.26 |
| No | 0.98 (0.96,1.00) | 0.98 (0.95,1.01) | 0.98 (0.95,1.01) | 0.98 (0.96,1.01) | |
| Yes | 0.96 (0.92,1.00) | 0.95 (0.91,1.00) | 0.96 (0.92,1.00) | 0.96 (0.91,1.00) | |

Crude model: no covariates were adjusted.
Model 1, age, marital status, race, education level, PIR and ever pregnant were adjusted.
Model 2, Model 1+DM and hypertension were adjusted.
Model 3, Model 2+MetS, eGFR, alumin, HDL and HbA1c were adjusted.
*mean p <0.05.

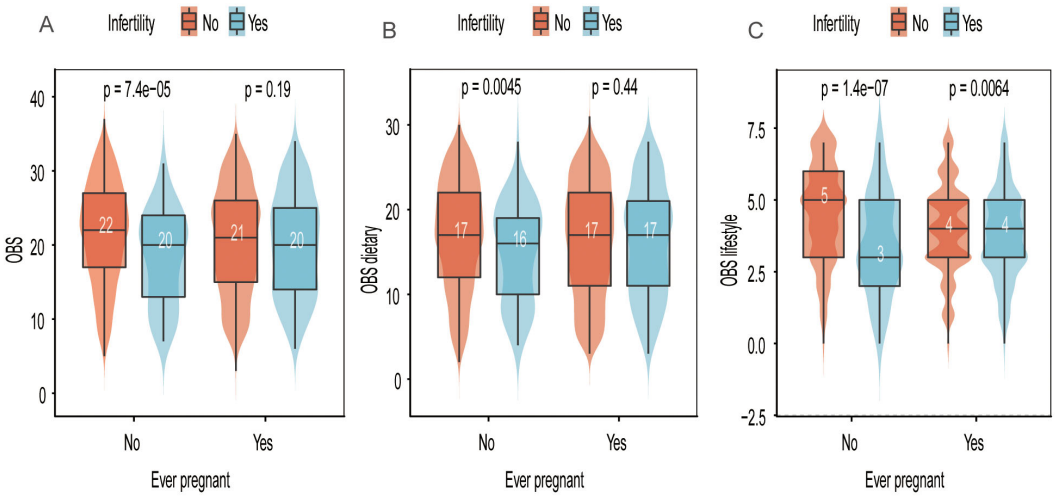


FIGURE 2
Distribution of OBS, OBS dietary, and OBS lifestyle scores in relation to "ever pregnant" and infertility status. **(A)** OBS scores stratified by infertility and pregnancy history. **(B)** OBS dietary scores stratified by infertility and pregnancy history. **(C)** OBS lifestyle scores stratified by infertility and pregnancy history.

The subgroup analyses yielded particularly insightful findings. The protective effect of OBS was significantly more pronounced among women under 35 years compared to their older counterparts. While the total OBS showed similar trends across age groups, the lifestyle component of OBS demonstrated a significant interaction with age, with younger women experiencing a substantially stronger protective effect (25% risk reduction per point) than women aged 35 and older. This age-

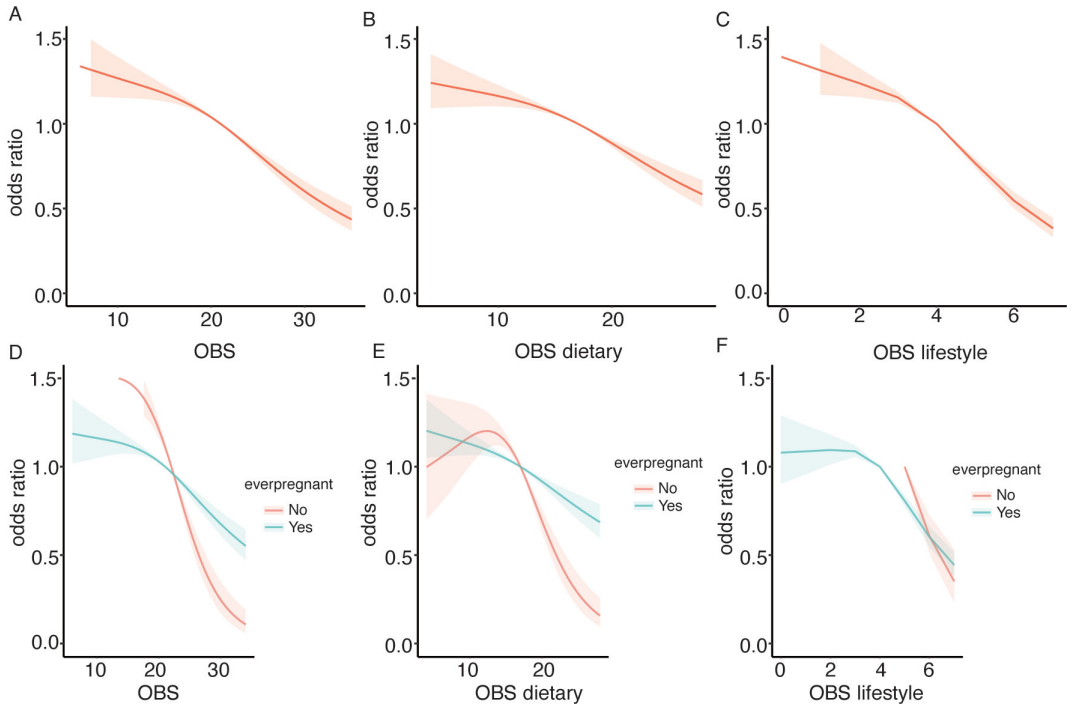


FIGURE 3
Displays the association between the OBS and infertility risk using RCS. **(A)** for the overall OBS, **(B)** for the dietary component, and **(C)** for the lifestyle component. Stratified analyses by ever pregnant status are presented in **(D–F)**, with adjustments for confounding variables.

dependent relationship reflects the differing etiologies of infertility across age groups. In younger women, conditions potentially more responsive to oxidative balance modulation—such as polycystic ovary syndrome (PCOS), endometriosis, and unexplained infertility—predominate. Oxidative stress in PCOS affects ovarian follicles, disrupting their development and maturation, which can impair fertility (23–25). Similarly, oxidative stress is implicated in endometriosis and unexplained infertility, leading to poor endometrial receptivity and negatively affecting pregnancy outcomes (26–28). Conversely, in women over 35, age-related decline in ovarian reserve becomes the primary driver of infertility, a process less amenable to improvement through oxidative balance enhancement.

Younger women benefit more from lifestyle interventions that enhance oxidative balance due to their greater biological plasticity and antioxidant capacity. Studies demonstrate that while older women can improve oxidative stress markers through resistance training, the improvements are more substantial in younger women due to their naturally higher baseline antioxidant capacity (29–31). The biological mechanisms underlying this age-dependent response involve multiple pathways. Younger women possess more robust antioxidant defense systems, greater mitochondrial efficiency, and enhanced cellular repair mechanisms (2, 32). Oxidative stress negatively affects reproductive processes by causing DNA damage and mitochondrial dysfunction, which are more pronounced in older women, while younger women are better equipped to manage oxidative stress due to more efficient cellular repair mechanisms (33–35). Antioxidants play a critical role in protecting oocytes from oxidative damage, and younger women benefit more from these protective mechanisms, which help maintain oocyte quality and fertility (36, 37).

Another key finding was the differential impact of OBS based on previous pregnancy history. The protective association between OBS and infertility was significantly stronger in women with primary infertility compared to those with secondary infertility. This suggests that women who have never achieved pregnancy represent a distinct population in which oxidative stress plays a more prominent pathophysiological role. Primary infertility may involve more significant underlying oxidative-mediated pathologies that could potentially be mitigated through antioxidant-rich diets and lifestyle modifications. Conversely, women with secondary infertility may have different etiologies less responsive to oxidative balance modulation.

Women with primary infertility exhibit significantly higher levels of oxidative stress compared to fertile women, with elevated levels of malondialdehyde (MDA), a marker of lipid peroxidation (28). This finding is consistent across various studies (38, 39), highlighting the role of oxidative stress in infertility. While data on secondary infertility are limited, the general impact of oxidative stress on reproductive health suggests it plays a role in both conditions, though potentially through different mechanisms or to varying degrees.

These findings align with our understanding of the mechanistic role of oxidative stress in female reproduction. Physiological levels of reactive oxygen species (ROS) are essential for normal follicular development, ovulation, and fertilization (40–42). However,

excessive ROS production can damage cellular components, impair mitochondrial function, and trigger apoptotic pathways in reproductive tissues. Low ROS levels in follicular fluid correlate with successful pregnancy outcomes in IVF treatments (43), suggesting a potential prognostic indicator for IVF efficacy. Our study extends these mechanistic insights by demonstrating that a comprehensive measure of oxidative balance correlates with fertility outcomes at the population level, particularly in specific subgroups of women.

The individual components of OBS provide further insights into potential intervention strategies. Dietary antioxidants, including vitamins C and E, protect reproductive cells against oxidative damage.

Amini et al (44) demonstrated that supplementation with vitamins C and E can reduce serum ROS levels compared to a control group, resulting in increased antioxidant concentrations in serum and follicular fluid. This leads to enhanced oocyte and IVF embryo quality, as well as improved pregnancy outcomes (45). Research (46) from the NHANES indicated a negative correlation between dietary fiber intake and female infertility. Li et al (47) found that Erastin, an inducer of ferroptosis, elevated ROS levels in mice under iron accumulation conditions, potentially triggering cell death mechanisms that could alleviate endometriosis and improve fertility. A Nigerian study (48) comparing serum metal levels in women with unexplained infertility to those with normal fertility revealed lower copper, zinc, and selenium levels in the infertile group. These micronutrients serve as essential cofactors for antioxidant enzymes, and their deficiency may compromise oxidative defense mechanisms in reproductive tissues.

Lifestyle factors within the OBS showed particularly strong associations with fertility outcomes. Smoking, for instance, induces oxidative stress in reproductive tissues, with women who smoke exhibiting lower success rates in assisted reproductive technologies (ART). Smoking increases ROS levels and reduces antioxidant defenses, affecting both sperm and oocyte quality (28, 49, 50). Meta-analyses show that smoking decreases live birth rates and clinical pregnancy rates per ART cycle, while increasing miscarriage rates. These effects are significant and highlight the detrimental impact of smoking on ART success (51–53).

Alcohol consumption similarly promotes oxidative stress through its metabolism, with even moderate intake potentially affecting fertility outcomes (28, 54). Obesity, another pro-oxidant factor in the OBS, is associated with both systemic inflammation and oxidative stress, contributing to ovulatory dysfunction and decreased endometrial receptivity (55–57). A cohort study by Jose Bellver et al (58) indicates that obesity may impair endometrial receptivity, contributing to infertility and negative outcomes in ART.

Our study has several methodological strengths. The use of a nationally representative NHANES population enhances generalizability, while the comprehensive adjustment for confounding variables increases confidence in the observed associations. Furthermore, our approach, which examines both total OBS and its components, provides greater insight into the relative contributions of dietary and lifestyle factors to fertility outcomes.

Nevertheless, this research has limitations that warrant consideration. The cross-sectional design precludes establishment of causality, and the reliance on self-reported infertility data may introduce recall bias. Additionally, while we adjusted for numerous potential confounders, residual confounding cannot be entirely ruled out. The NHANES database lacks comprehensive data on environmental exposures, such as air pollution, pesticides, and endocrine-disrupting chemicals, which may influence both oxidative stress and fertility.

In conclusion, our findings demonstrate that higher OBS is associated with lower infertility risk, with particularly strong effects observed in younger women and those with primary infertility. These results suggest that optimizing oxidative balance through dietary and lifestyle modifications may represent an effective strategy for reducing infertility risk in specific populations. Future longitudinal studies and clinical trials are needed to establish causality and determine whether targeted interventions to improve oxidative balance can enhance fertility outcomes in women attempting conception

## Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: https://wwwn.cdc.gov/nchs/nhanes/search/default.aspx.

## Ethics statement

The studies involving humans were approved by National Center for Health Statistics Research Ethics Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

## Author contributions

PX: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Visualization, Writing – original draft. XC: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Visualization, Writing – original draft. RL: Resources, Validation, Writing – review & editing. XS: Investigation, Supervision, Writing – review & editing. YY: Resources, Validation, Writing – review & editing. LL: Funding acquisition, Project administration, Supervision, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2025.1444832/full#supplementary-material

SUPPLEMENTARY TABLE 1
Oxidative balance score assignment scheme.

SUPPLEMENTARY TABLE 2
Sensitivity Analysis of the Association between OBS and Infertility after Multiple Imputation Crude model: no covariates were adjusted. Model 1, age, marital status, race, education level, PIR and ever pregnant were adjusted. Model 2, Model 1+DM and hypertension were adjusted. Model 3, Model 2+MetS, eGFR, alumin, HDL and HbA1c were adjusted. * mean p <0.05.

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# Using Life's Essential 8 and heavy metal exposure to determine infertility risk in American women: a machine learning prediction model based on the SHAP method

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**Background:** Fertility status is a marker of future health, and female infertility has been shown to be an important medical and social problem. Life's Essential 8 ("LE8") is a comprehensive cardiovascular health assessment proposed by the American Heart Association. The assessment indicators include 4 health behaviors (diet, physical activity, nicotine exposure, and sleep health) and 4 health factors (body mass index, blood lipids, blood glucose, and blood pressure). LE8 and heavy metal exposure have both been shown to be associated with infertility. However, the association between LE8 and heavy metal exposure and female infertility has not been investigated. The aim of this study was to develop a machine learning prediction model for LE8 and heavy metal exposure and the risk of female infertility in the United States.

**Methods:** The National Health and Nutrition Examination Survey ("NHANES") is a nationally representative program conducted by the National Center for Health Statistics to assess the health and nutritional status of the U.S. population. For this study, 873 women between the ages of 20 and 45 were selected from the 2013–2018 NHANES dataset. The association between LE8 and heavy metal exposure and risk of infertility was assessed using logistic regression analysis and six machine learning models (Decision Tree, GBDT, AdaBoost, LGBM, Logistic Regression, Random Forest), and the SHAP algorithm was used to explain the model's decision process.

**Results:** Of the six machine learning models, the LGBM model has the best predictive performance, with an AUROC of 0.964 on the test set. SHAP analysis showed that LE8, body mass index ("BMI"), diet, Cadmium ("Cd"), Cesium ("Cs"), Molybdenum ("Mo"), Antimony ("Sb"), Tin ("Sn"), education level and pregnancy history were significantly associated with the risk of female infertility. Cd, BMI and LE8 are the variables that contribute most to the prediction of infertility risk. Among them, BMI and LE8 have a negative predictive effect on female infertility in the model, while Cd has a positive contribution to the prediction of female infertility. Further analysis showed that there was a significant interaction between heavy metals and LE8, which may have a synergistic effect on the risk of female infertility.

**Conclusions:** This study used LE8 and heavy metal exposure to create a machine learning model that predicts the risk of female infertility. The model identified ten key factors. The model demonstrated high predictive accuracy and good clinical interpretability. In the future, LE8 and heavy metal exposure can be used to screen for female infertility early on.

KEYWORDS

**infertility, Life's Essential 8, heavy metal exposure, machine learning, SHAP**

# 1 Introduction

Female infertility is the failure to achieve pregnancy after 12 months or more of regular unprotected sex with the same sexual partner in women of childbearing age (1). The World Health Organization (WHO) has recognized infertility as a global public health problem. Approximately 15% of couples of reproductive age worldwide are affected by infertility. Among these, the infertility rate among women of reproductive age in the United States is approximately 15.5% and is increasing at a rate of 0.37% per year (2). Common causes of female infertility include male factor infertility (3), endometriosis (4), and fallopian tube damage (5). As society modernizes, the impact of external environmental and personal health factors on women's reproductive health cannot be ignored. Exposure to environmental factors, unhealthy lifestyles (such as smoking and sedentary lifestyles), and obesity-related metabolic disorders can also increase the risk of infertility in women (6, 7). According to statistics from the NHANES, nearly every pregnant U.S. woman is exposed to at least 43 different potentially harmful chemicals during her pregnancy (8). Female infertility is not only a huge financial burden for patients and the healthcare system, it can also lead to an increase in mental disorders such as depression and anxiety in women (9). In addition, a number of studies have suggested that infertile women may be at increased risk for gynecologic malignancies (10–12). Predicting and identifying risk factors for female infertility is important for restoring the overall fertility rate in the United States, reducing the medical and economic burden, and optimizing the allocation of healthcare resources. Currently, most diagnoses of female infertility are made using imaging tests such as Hysterosalpingography, Magnetic Resonance Imaging and Computed Tomography. Although these examinations can provide professional and effective pathological diagnoses, a comprehensive assessment of the causes of infertility usually requires a combination of multiple examinations, which places high demands on equipment and technology. In addition, the cost of the examination is relatively high, which cannot meet the needs of universal medical care for a wider range of people.

LE8 is a comprehensive cardiovascular health assessment proposed by the American Heart Association. The assessment indicators include 4 health behaviors and 4 health factors, namely diet, physical activity, nicotine exposure, and sleep health, BMI, blood lipids, blood glucose, and blood pressure (13). Currently, researchers are using the LE8 score for risk prediction studies of all-cause mortality risk (14–16), adult cardiovascular health (17, 18), and cardiovascular disease risk (19, 20). Previous studies have applied the LE8 score to research on female infertility, based on its association with cardiovascular health (21, 22). The results indicate that various LE8 indicators are associated with female infertility. Polycystic ovary syndrome is one of the primary causes of female infertility and exhibits complex interactions with obesity. This provides further evidence of the significant association between obesity and female infertility (23). Studies have shown that women with a BMI over 25 kg/m$^2$ before pregnancy are more likely to have difficulty conceiving than women with an ideal BMI (between 20 and 24.99 kg/m$^2$) (24). Specifically, the impact of obesity on female fertility involves physiological, endocrine, and metabolic factors (25), such as abnormal high-density lipoprotein metabolism and elevated levels of unesterified cholesterol. These factors may interfere with oocyte meiosis arrest and developmental potential, thereby causing female infertility (26). Not only is physical activity an effective way to lose weight, but it can also improve reproductive function in women with polycystic ovary syndrome. This includes increasing menstrual regularity and ovulation rate, improving sexual function, and reducing infertility (27). A weight reduction of 2.5% to 5% has been demonstrated to enhance blood sugar, triglycerides, and other indicators (28), good blood sugar control has been shown to possess the potential to improve reproductive health (22). Consequently, interventions such as the adoption of a low-GI diet or a Mediterranean diet have been shown to be effective in the treatment of polycystic ovary syndrome and the promotion of reproductive health (29). Furthermore, extant research has demonstrated a significant correlation between sleep disturbances, tobacco use, and elevated blood pressure with female infertility. Sleep disorders are more prevalent among women experiencing fertility issues, primarily influenced by mechanisms such as disruption of circadian rhythms, abnormalities in melatonin and hormone regulation, and oxidative stress (30). The correlation between smoking and infertility risk is particularly salient among Mexican Americans and women aged 25 to 38 years (31). In women under the age of 30, infertility has been observed to be associated with elevated systolic and diastolic blood pressure. However, no such association has been demonstrated in older women. Consequently, the monitoring of weight and blood pressure in women with a history of infertility is of particular importance in clinical

practice (32). In light of the aforementioned research results, it has been determined that all factors included in the LE8 score exert varying degrees of influence on the incidence of female infertility. The LE8 score offers a novel approach to the screening of female infertility by indirectly predicting the risk of female infertility.

Heavy metals are defined as inorganic elements that possess a density greater than 5 g/cm3 (33). Heavy metals are divided into two groups based on their toxicity: essential and non-essential. Essential heavy metals such as Zn, Cu, Fe and Co are harmless or relatively harmless at low concentrations, while non-essential heavy metals such as Cd, Pb, Hg, As and Cr are highly toxic even at low concentrations (34). The mechanism of health effects induced by oxidative stress after heavy metal exposure (35)has been demonstrated to increase the risk of diseases such as neurological diseases (36), cardiovascular diseases (37), immune system diseases (38), and metabolic syndrome (39). Nevertheless, the marked rise in environmental pollutants over the past few decades has resulted in deleterious physiological effects. Heavy metals are among the most significant pollutants impacting our environment (40). Previous studies have shown an association between heavy metal exposure and female infertility. Increased exposure to environmental heavy metals may affect the regulation and function of the female reproductive system, such as causing polycystic ovary syndrome in women (41), which in turn may increase the risk of female infertility (42). Furthermore, the findings of numerous studies have demonstrated a negative correlation between the presence of Cu, Cr, Co, Cd, Rb, Hg, and Pb and fertility levels. For instance, Hg levels that exceed safe thresholds have been observed to disrupt menstrual cycles in women, potentially contributing to infertility (43, 44). Heavy metals can accumulate in the blood, urine, liver, and other tissues of the human body and adversely affect the body. An earlier cross-sectional study indicated that the blood levels of Cd and Pb in U.S. women were positively correlated with infertility (45). Subsequent studies have found that urine may better reflect the reproductive toxicity of long-term heavy metal exposure than blood, and have confirmed that urinary As and Cd are potential risk factors for female infertility. Urinary As is significantly and positively correlated with female infertility, and Pb exposure has a more pronounced effect on the risk of infertility in older (35–44 years) and obese (BMI≧30 kg/m²) women (46). Based on the above research, we found that independent exposure to heavy metals is significantly associated with the risk of female infertility, and the indirect prediction of the risk of female infertility by the content of a single heavy metal in women's urine may provide new opportunities for the initial screening of female infertility.

Recent research has discussed the relationship between environmental exposure and the combined effect of LE8 on cardiovascular health (47), liver disease (48), and increased risk of death (49). Due to advancements in exposomics testing technologies and individual differences in reproductive health, such as endocrine metabolic heterogeneity and ovarian reserve function, the relationship between various LE8 indicators and heavy metal exposure, as well as their potential mechanisms related to infertility risk, remains unclear. The extant literature on the subject is predominantly confined to linear association analyses of individual factors and reproductive health. There is a paucity of systematic exploration of the synergistic effects of multiple exposure factors and the construction of predictive models. The incorporation of LE8 and heavy metal exposure into risk prediction models has the potential to provide a more comprehensive assessment of the multidimensional causes of female infertility. In principle, this broadens the scope of single-dimensional analysis, while methodologically improving the accuracy and adaptability of prediction models. Consequently, this demonstrates significant practical value.

In the course of our investigation into the nonlinear associations and temporal interactions between LE8, multiple biological indicators, and heavy metal exposure factors, it became evident that traditional linear regression models are encumbered by significant limitations, particularly when confronted with multiple collinearity or confounding factors in the data. These limitations can readily result in model overfitting and a decline in predictive performance. Machine learning (ML) has demonstrated remarkable proficiency in the identification and analysis of intricate correlation patterns within high-dimensional data sets. The program's advanced feature selection and nonlinear modeling capabilities enable the identification of potential variable interaction effects and non-explicit feature structures. At present, the application has been successfully implemented in the prediction of disease risks, including coronary artery disease (50) and male infertility (51). SHapley Additive exPlanations (SHAP) is an advanced visualization technique designed to improve the interpretability of ML decision making. It significantly overcomes the limitations of the traditional "black box" nature of the model by intuitively quantifying the marginal contribution of each feature to the model prediction (52, 53). Furthermore, the integration of model outputs with epidemiological and toxicological evidence pertaining to female infertility has the potential to enhance the interpretability and biological plausibility of research findings, thereby underscoring the methodological potential of interpretable machine learning in studies investigating female reproductive health and infertility risk prediction. NHANES is a nationally representative program of the National Center for Health Statistics (NCHS) that assesses the health and nutritional status of the U.S. population (54). The database integrates input from global experts across over 400 domains, covering demographic information, dietary intake, physical examinations, laboratory measurements, and questionnaire data. To ensure national representativeness, NHANES applies complex statistical methodologies, including stratified and multistage probability sampling.

Therefore, the present study utilized a substantial sample population from the NHANES (2013-2018) as a foundation, incorporated LE8 and heavy metal exposure indicators, and constructed a composite risk prediction model for female infertility. The specific objectives of this study are as follows: (1) The identification of key variables influencing infertility in LE8 and indicators of heavy metal exposure is imperative. (2) The utilization of multiple machine learning algorithms, including GBDT, AdaBoost, and LGBM, in conjunction with the SHAP explainability framework, enabled the development of a risk prediction model for female infertility that is both clinically interpretable and actionable. (3) The present study aims to elucidate the interactive mechanisms between lifestyle and environmental exposure, thereby providing a theoretical basis and

methodological support for the early identification and intervention of female infertility risk.

# 2 Methods

## 2.1 Research subjects

The NHANES study was approved by the NCHS Ethics Committee, which obtained written informed consent from each participant. All NHANES data are openly accessible and have been de-identified (https://www.cdc.gov/nchs/nhanes/about/erb.html). NHANES only measured heavy metal exposure in urine from 2013 to 2018. Therefore, this study used data from this database from 2013 to 2018. The study focused on female participants between the ages of 20 and 45. From the 29,400 participants in the 2013–2018 cycle, the study excluded male participants (n=14,452), female participants younger than 20 years and older than 45 years (n=11,093), and participants with missing or incomplete LE8 indicator and heavy metal exposure urine test results (n=2,982). Missing data for all other variables was less than 20%, so multiple imputation was used to fill in the missing values. The final sample size was 873 female participants. The specific screening and research process is shown in Figure 1.

## 2.2 Assessment of infertility

The present study employs female infertility as the primary outcome variable. In accordance with the prevailing clinical definition of infertility, which is defined as "a reproductive-age woman who has not conceived after 12 months or more of regular unprotected sexual intercourse with the same sexual partner" (1), the present study employs items from the Reproductive Health Questionnaire (RHQ) in the NHANES database that are highly consistent with this definition. "{Have you/Has SP} ever attempted

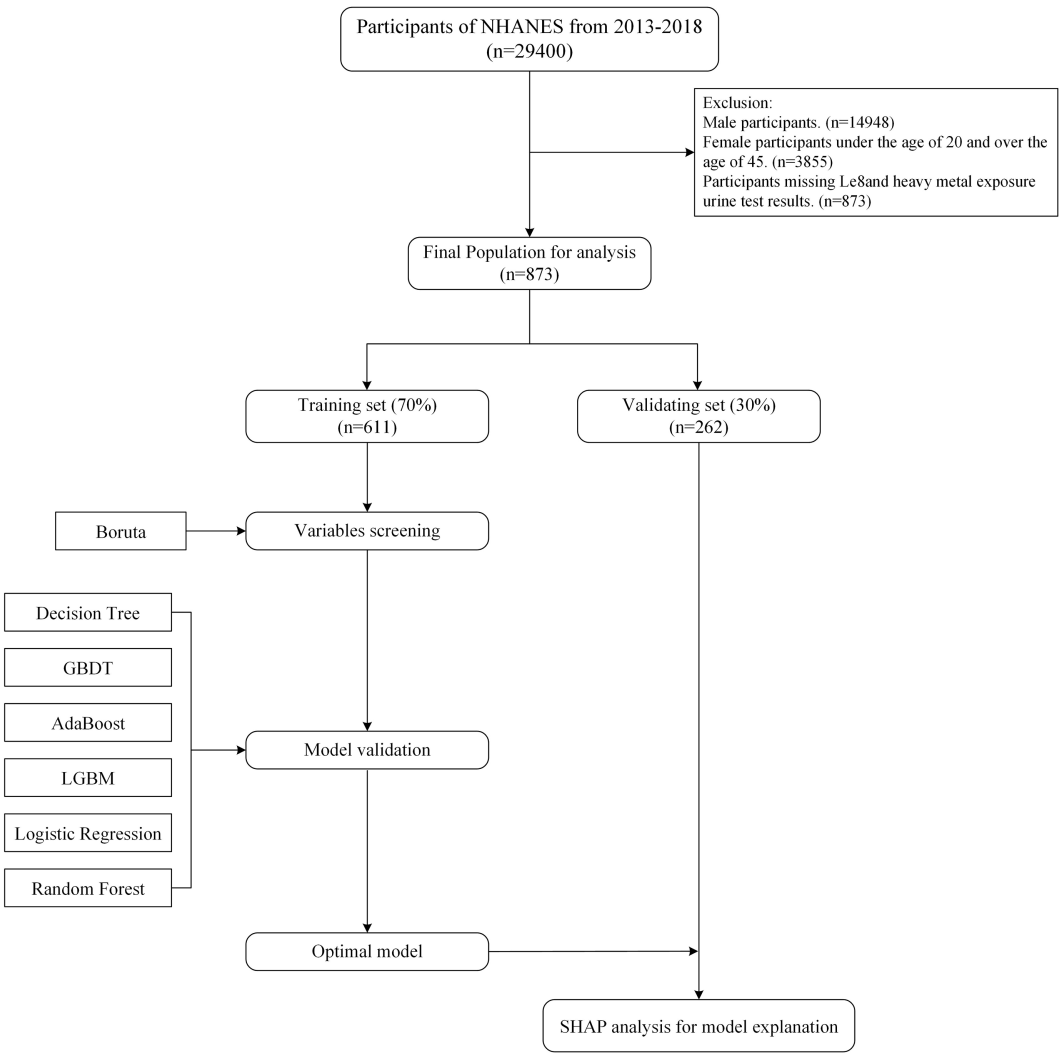


FIGURE 1
Flow chart of sample selection from the NHANES 2013–2018.

to become pregnant over a period of at least a year without becoming pregnant?" Infertile respondents were defined as those who answered "yes" to the aforementioned question, while fertile respondents were defined as those who answered "no". The data were collected by professional interviewers using a computer-assisted personal interview standard procedure, ensuring high consistency and reliability. The relevant methods have been widely applied in reproductive epidemiological studies.

## 2.3 Review of LE8

The LE8 scoring algorithm encompasses four categories of healthy behaviors (diet, physical activity, nicotine exposure, and sleep health) and four categories of health factors (BMI, blood lipids, blood glucose, and blood pressure). The eight cardiovascular health (CVH) indicators are scored in accordance with the American Heart Association's established criteria, with a scale ranging from 0 to 100 points for each indicator. Scores ranging from 0 to 49 are classified as low level, 50 to 79 are considered moderate level, and 80 to 100 are designated as high level (13). The LE8 score is the arithmetic mean of these eight indicators.

## 2.4 Evaluation of heavy metal exposure

In the present study, an exhaustive analysis was conducted on all heavy metals detectable in urine samples from participants in the NHANES database, a total of 11 heavy metals: Barium (Ba), Cadmium (Cd), Cobalt (Co), Cesium (Cs), Manganese (Mn), Molybdenum (Mo), Lead (Pb), Antimony (Sb), Tin (Sn), Thallium (Tl), Titanium (Tu). All measurements were performed using Inductively Coupled Plasma Mass Spectrometry (ICP-DRCMS) at the National Environmental Health Laboratory, ensuring the accuracy of the heavy metal exposure data and providing an important basis for the subsequent relationship between heavy metal exposure and female infertility rates. Further information regarding standard laboratory procedures can be found on the NHANES website.

## 2.5 Covariate

Covariates included age, ethnicity, education level, work situation, pregnancy history, and chronic disease status. Among them, age is a continuous variable and the remaining 5 items are categorical variables that need to be processed numerically. The assignment of the ethnicity variable is divided into five levels corresponding to Mexican American, Other Hispanic, Non-Hispanic White, Non-Hispanic Black, and Other Ethnic Group - Including Multiracial, from 1 to 5. Assign values from 1 to 6 to the work statuses an employee of a private company, business, or individual for wages, salary, or commission; a federal government employee; a state government employee; a local government employee; self-employed in own business, professional practice or farm; working without pay in family business or farm, respectively. The education level variable is assigned a value from 1 to 5, corresponding to less than 9th grade, 9th-11th grade (including 12th grade without a diploma), high school graduate/GED or equivalent, some college or AA degree, and college graduate or higher. The pregnancy history variable assigns a value of 1 to those who have ever been pregnant and 0 to those who have never been pregnant, and the chronic disease status variable assigns a value of 1 to those who have a chronic disease and 0 to those who do not. These covariates were selected based on their established relevance in reproductive medicine research and their potential confounding effect on research results.

## 2.6 Statistical analysis

### 2.6.1 Baseline regression analysis and logistic regression analysis

In the baseline analysis, the demographic characteristics are expressed as the median and its interquartile range (IQR), while the categorical variables are expressed as counts and percentages. The heavy metal factor was transformed by natural logarithm (log10) and further divided into four parts (Q1-Q4). The Pearson correlation coefficient was used to evaluate the relationship between the levels of 11 heavy metals. The overall score and scores for the eight dimensions in LE8 were assigned values of Q1, Q2, and Q3 according to low, medium, and high levels, respectively. In order to preliminarily assess the association between heavy metal exposure, LE8, and female infertility, univariate logistic regression was used. Among these factors, heavy metal exposure and LE8 factors were analyzed using continuous (log-transformed) and categorical variables. Heavy metal exposure factors were divided into four quartiles (55), and LE8 factors were divided into three tertiles (13), with Q1 as the reference category. This approach was used to preliminarily assess the association between these factors and infertility. Concurrently, categorical variables were converted to integer values p for trend tests. To control for confounding factors, two models were constructed for analysis: one unadjusted for any covariates, and another adjusted for age, ethnicity, education level, employment status, pregnancy history, and chronic disease.

### 2.6.2 Model development

This study employs the general classification method, which involves the division of data into a training set comprising 70% of the total and a test set constituting the remaining 30% (56). First, based on the training set and test set, and considering that high-dimensional complex data may lead to classification performance degradation and overfitting in machine learning algorithms, the Boruta feature selection algorithm based on random forest classifiers is introduced before constructing the machine learning model to assess the feature importance of LE8, heavy metals, and other confounding factors, thereby screening out the most critical variables. The Boruta algorithm is a feature selection method based on random forests that can identify the most important features

related to the dependent variable in a data set through multiple dynamic iterations (57).

Secondly, a combination of Synthetic Minority Over-sampling Technique (SMOTE) and undersampling techniques was employed to achieve a balanced distribution between infertile and non-infertile populations. Subsequently, six machine learning methods —Decision Tree, GBDT, AdaBoost, LGBM, Logistic Regression, and Random Forest—were employed to construct models for the core variables to assess the predictive ability of LE8 and heavy metal exposure on female infertility. In the context of model validation, a range of metrics, including Accuracy, Recall, F1 score, and the Matthews Correlation Coefficient (MCC), were employed to assess the predictive capabilities of six machine learning (ML) models. The objective of this assessment was to identify the ML model that demonstrated the most optimal predictive performance. The range of AUROC values is from 0.5 to 1.0 (58). It is evident that as the value within this range increases, the predictive ability concomitantly improves. Following the selection of the optimal model, the overall performance of the model is evaluated using the K-fold cross-validation method. In this study, we employed one part of K as the test set, repeated the model establishment and evaluation cycles K times, and calculated the average (59). This approach has been demonstrated to effectively mitigate performance estimation errors arising from disparate data partitioning methodologies, thereby ensuring a more stable and reliable model performance evaluation.

In addition, the SHAP algorithm is employed to elucidate the decision-making process of the machine learning model, as predicted by the optimal model. The specific contribution of each feature to the final prediction is detailed through its Shapley value and presented visually. Subsequently, through subgroup interaction analysis, we further explored the interaction between heavy metals and the various dimensions of LE8. In the specific analysis process, each factor was analyzed using categorical variables, and confounding factors were controlled. Finally, to further assess the robustness of the model results and the potential impact of confounding biases, this study employed propensity score matching (PSM) for sensitivity analysis. Using infertility as the matching criterion, propensity scores were calculated based on LE8, heavy metal exposure, and covariates. A 1:8 nearest neighbor matching method was employed to construct a matched sample set with balanced characteristics. The construction of the model and the subsequent evaluation of its predictive performance were repeated on the matched samples. This process was undertaken to assess the predictive stability of the original model under various sample structures.

# 3 Results

## 3.1 Baseline characteristics

A total of 103 infertile patients were selected for baseline analysis compared to 770 individuals without infertility, and baseline characteristics are shown in Tables 1–3. Among the 873 participants in this study (mean age: 33.10), the infertility group had a higher mean age (35.09 vs. 32.84, $p<0.05$). The two groups exhibited significant differences in age, pregnancy history, prevalence of chronic diseases (asthma, overweight, and arthritis), heavy metal exposure (cadmium), and LE8-related indicators (LE8, BMI, sleep health, and blood pressure) ($p<0.05$).

## 3.2 Logistic regression

The results of logistic regression analysis (Tables 4, 5) show that LE8, BMI, sleep health, blood pressure and heavy metal Cd are correlated with female infertility through multiple model validation. In Model 1, which did not adjust for covariates, LE8, BMI, blood pressure, sleep health, and heavy metal Cd were found to be significantly associated with the risk of female infertility when considered continuous variables. In Model 2, which was adjusted for age, ethnicity, education level, employment status, pregnancy history, and five chronic diseases as covariates, only LE8, BMI, blood pressure, and sleep health remained significantly associated with the risk of female infertility. As categorical variables, in model 1, LE8 (Q2, Q3), BMI (Q2, Q3), blood pressure (Q3), sleep health (Q3), and heavy metal Cd (Q3) were significantly associated with the risk of female infertility. LE8 (Q2, Q3), BMI (Q2, Q3), blood pressure (Q2, Q3), and sleep health (Q3) in model 2 were associated with the risk of female infertility.

## 3.3 Model variable selection

In this study, the Boruta algorithm with shading was used, the confidence interval was set to 0.01, the source of maximum importance was run 300 times, and the Bonferroni method for multiple comparison adjustment was used to identify 10 potentially effective predictor variables (Figure 2). These characteristic variables with shading were used to train and construct the machine learning model, which included education level, pregnancy history, LE8, diet, BMI, Cd, Mo, Sb, Cs and Sn.

## 3.4 Model evaluation and comparison

As illustrated in Table 6, the model evaluation results, including Accuracy, Recall, F1 score, and the Matthews Correlation Coefficient (MCC), are presented for both the training set and the test set. Figures 3A, C illustrate the DCA curves and ROC curves of the six machine learning models (Decision Tree, GBDT, AdaBoost, LGBM, Logistic Regression, and Random Forest) for the training set, while Figures 3B, D depict the DCA curves and ROC curves for the test set. A comprehensive evaluation of the training set and test set using the two curves was conducted, resulting in the identification of satisfactory model evaluation outcomes. LGBM exhibited the optimal prediction performance and pattern.

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TABLE 1 Comparison of Covariates between Infertility and Non-infertility groups.

| Variable | Overall | None Infertility | Infertility | *p*-value |
|---|---|---|---|---|
| | N 873 | N 770 | N 103 | |
| Age | 33.10 (7.52) | 32.84 (7.54) | 35.09 (7.14) | 0.003 |
| Ethnicity | | | | 0.573 |
| Mexican American | 164 (18.79%) | 140 (18.18%) | 24 (23.30%) | |
| Other Hispanic | 90 (10.31%) | 83 (10.78%) | 7 (6.80%) | |
| Non-Hispanic White | 297 (34.02%) | 264 (34.29%) | 33 (32.04%) | |
| Non-Hispanic Black | 175 (20.05%) | 153 (19.87%) | 22 (21.36%) | |
| Other Ethnic Group - Including Multiracial | 147 (16.84%) | 130 (16.88%) | 17 (16.50%) | |
| Education | | | | 0.643 |
| Less than 9th grade | 40 (4.58%) | 36 (4.68%) | 4 (3.88%) | |
| 9-11th grade (Includes 12th grade with no diploma) | 89 (10.19%) | 79 (10.26%) | 10 (9.71%) | |
| High school graduate/GED or equivalent | 182 (20.85%) | 166 (21.56%) | 16 (15.53%) | |
| Some college or AA degree | 326 (37.34%) | 284 (36.88%) | 42 (40.78%) | |
| College graduate or above | 236 (27.03%) | 205 (26.62%) | 31 (30.10%) | |
| Work situation | | | | 0.825 |
| An employee of a private company, business, or individual for wages, salary, or commission | 745 (85.34%) | 657 (85.32%) | 88 (85.44%) | |
| A federal government employee | 9 (1.03%) | 9 (1.17%) | 0 (0.00%) | |
| A state government employee | 52 (5.96%) | 46 (5.97%) | 6 (5.83%) | |
| A local government employee | 40 (4.58%) | 35 (4.55%) | 5 (4.85%) | |
| A state government employee | 24 (2.75%) | 20 (2.60%) | 4 (3.88%) | |
| Working without pay in family business or farm | 3 (0.34%) | 3 (0.39%) | 0 (0.00%) | |
| Ever pregnant | | | | 0.003 |
| No | 225 (25.77%) | 211 (27.40%) | 14 (13.59%) | |
| Yes | 648 (74.23%) | 559 (72.60%) | 89 (86.41%) | |
| Hypertension | | | | 0.103 |
| No | 742 (84.99%) | 660 (85.71%) | 82 (79.61%) | |
| Yes | 131 (15.01%) | 110 (14.29%) | 21 (20.39%) | |
| Diabetes | | | | 0.284 |
| No | 832 (95.30%) | 736 (95.58%) | 96 (93.20%) | |
| Yes | 41 (4.70%) | 34 (4.42%) | 7 (6.80%) | |
| Asthma | | | | <0.001 |
| No | 711 (81.44%) | 640 (83.12%) | 71 (68.93%) | |
| Yes | 162 (18.56%) | 130 (16.88%) | 32 (31.07%) | |
| Overweight | | | | 0.004 |
| No | 504 (57.73%) | 458 (59.48%) | 46 (44.66%) | |
| Yes | 369 (42.27%) | 312 (40.52%) | 57 (55.34%) | |

*(Continued)*

TABLE 1 Continued

| Variable | Overall | None Infertility | Infertility | *p*-value |
|---|---|---|---|---|
| | N 873 | N 770 | N 103 | |
| Arthritis | | | | 0.030 |
| No | 789 (90.38%) | 702 (91.17%) | 87 (84.47%) | |
| Yes | 84 (9.62%) | 68 (8.83%) | 16 (15.53%) | |
| Heart failure | | | | 0.463 |
| No | 869 (99.54%) | 766 (99.48%) | 103 (100.00%) | |
| Yes | 4 (0.46%) | 4 (0.52%) | 0 (0.00%) | |
| Coronary heart disease | | | | 0.247 |
| No | 870 (99.66%) | 768 (99.74%) | 102 (99.03%) | |
| Yes | 3 (0.34%) | 2 (0.26%) | 1 (0.97%) | |
| Anginapectoris | | | | 0.711 |
| No | 867 (99.31%) | 765 (99.35%) | 102 (99.03%) | |
| Yes | 6 (0.69%) | 5 (0.65%) | 1 (0.97%) | |
| Heart attack | | | | 0.463 |
| No | 869 (99.54%) | 766 (99.48%) | 103 (100.00%) | |
| Yes | 4 (0.46%) | 4 (0.52%) | 0 (0.00%) | |
| Stroke | | | | 0.412 |
| No | 868 (99.43%) | 765 (99.35%) | 103 (100.00%) | |
| Yes | 5 (0.57%) | 5 (0.65%) | 0 (0.00%) | |
| Emphysema | | | | 0.463 |
| No | 869 (99.54%) | 766 (99.48%) | 103 (100.00%) | |
| Yes | 4 (0.46%) | 4 (0.52%) | 0 (0.00%) | |
| Chronic bronchitis | | | | 0.520 |
| No | 833 (95.42%) | 736 (95.58%) | 97 (94.17%) | |
| Yes | 40 (4.58%) | 34 (4.42%) | 6 (5.83%) | |
| Liver condition | | | | 0.140 |
| No | 857 (98.17%) | 754 (97.92%) | 103 (100.00%) | |
| Yes | 16 (1.83%) | 16 (2.08%) | 0 (0.00%) | |
| Chronic disease | | | | <0.001 |
| No | 366 (41.92%) | 339 (44.03%) | 27 (26.21%) | |
| Yes (one kind) | 281 (32.19%) | 246 (31.95%) | 35 (33.98%) | |
| Yes (two or more) | 226 (25.89%) | 185 (24.03%) | 41 (39.81%) | |

In order to reduce model selection bias and variance and accurately evaluate the performance of machine learning models, the study further utilized 5-fold cross-validation to test the optimal LGBM model. The validation results demonstrate that the LGBM model exhibits excellent performance in prediction, thereby substantiating its high predictive accuracy (Figure 4).

## 3.5 Visual analysis of the importance of features

In this study, SHAP analysis was used to evaluate the contribution and importance of each feature variable in the LGBM model in model prediction. The results are shown in

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TABLE 2 Comparison of LE8 between Infertility and Non-infertility groups.

| Variable | Overall | None Infertility | Infertility | *p*-value |
|---|---|---|---|---|
| | N 873 | N 770 | N 103 | |
| LE8 | | | | 0.002 |
| 49≥x≥0 | 69 (7.90%) | 52 (6.75%) | 17 (16.50%) | |
| 79≥x≥50 | 409 (46.85%) | 363 (47.14%) | 46 (44.66%) | |
| 100≥x≥80 | 395 (45.25%) | 355 (46.10%) | 40 (38.83%) | |
| Diet | | | | 0.659 |
| 49≥x≥0 | 458 (52.46%) | 400 (51.95%) | 58 (56.31%) | |
| 79≥x≥50 | 210 (24.05%) | 186 (24.16%) | 24 (23.30%) | |
| 100≥x≥80 | 205 (23.48%) | 184 (23.90%) | 21 (20.39%) | |
| Physical activity | | | | 0.179 |
| 49≥x≥0 | 213 (24.40%) | 185 (24.03%) | 28 (27.18%) | |
| 79≥x≥50 | 38 (4.35%) | 37 (4.81%) | 1 (0.97%) | |
| 100≥x≥80 | 622 (71.25%) | 548 (71.17%) | 74 (71.84%) | |
| Nicotine exposure | | | | 0.420 |
| 49≥x≥0 | 176 (20.16%) | 152 (19.74%) | 24 (23.30%) | |
| 79≥x≥50 | 34 (3.89%) | 32 (4.16%) | 2 (1.94%) | |
| 100≥x≥80 | 663 (75.95%) | 586 (76.10%) | 77 (74.76%) | |
| Sleep health | | | | 0.031 |
| 49≥x≥0 | 155 (17.75%) | 131 (17.01%) | 24 (23.30%) | |
| 79≥x≥50 | 135 (15.46%) | 113 (14.68%) | 22 (21.36%) | |
| 100≥x≥80 | 583 (66.78%) | 526 (68.31%) | 57 (55.34%) | |
| BMI | | | | <0.001 |
| 49≥x≥0 | 393 (45.02%) | 327 (42.47%) | 66 (64.08%) | |
| 79≥x≥50 | 211 (24.17%) | 199 (25.84%) | 12 (11.65%) | |
| 100≥x≥80 | 269 (30.81%) | 244 (31.69%) | 25 (24.27%) | |
| Blood lipids | | | | 0.544 |
| 49≥x≥0 | 136 (15.58%) | 118 (15.32%) | 18 (17.48%) | |
| 79≥x≥50 | 202 (23.14%) | 175 (22.73%) | 27 (26.21%) | |
| 100≥x≥80 | 535 (61.28%) | 477 (61.95%) | 58 (56.31%) | |
| Blood glucose | | | | 0.446 |
| 49≥x≥0 | 39 (4.47%) | 32 (4.16%) | 7 (6.80%) | |
| 79≥x≥50 | 95 (10.88%) | 83 (10.78%) | 12 (11.65%) | |
| 100≥x≥80 | 739 (84.65%) | 655 (85.06%) | 84 (81.55%) | |
| Blood pressure | | | | 0.004 |
| 49≥x≥0 | 61 (6.99%) | 46 (5.97%) | 15 (14.56%) | |
| 79≥x≥50 | 115 (13.17%) | 100 (12.99%) | 15 (14.56%) | |
| 100≥x≥80 | 697 (79.84%) | 624 (81.04%) | 73 (70.87%) | |

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TABLE 3 Comparison of Heavy Metals between Infertility and Non-infertility groups.

| Variable | Overall | None Infertility | Infertility | *p*-value |
|---|---|---|---|---|
| | N 873 | N 770 | N 103 | |
| Ba | | | | 0.591 |
| Q1 | 218 (24.97%) | 196 (25.45%) | 22 (21.36%) | |
| Q2 | 218 (24.97%) | 194 (25.19%) | 24 (23.30%) | |
| Q3 | 218 (24.97%) | 192 (24.94%) | 26 (25.24%) | |
| Q4 | 219 (25.09%) | 188 (24.42%) | 31 (30.10%) | |
| Cd | | | | 0.017 |
| Q1 | 218 (24.97%) | 198 (25.71%) | 20 (19.42%) | |
| Q2 | 218 (24.97%) | 201 (26.10%) | 17 (16.50%) | |
| Q3 | 218 (24.97%) | 182 (23.64%) | 36 (34.95%) | |
| Q4 | 219 (25.09%) | 189 (24.55%) | 30 (29.13%) | |
| Co | | | | 0.433 |
| Q1 | 218 (24.97%) | 199 (25.84%) | 19 (18.45%) | |
| Q2 | 218 (24.97%) | 191 (24.81%) | 27 (26.21%) | |
| Q3 | 218 (24.97%) | 189 (24.55%) | 29 (28.16%) | |
| Q4 | 219 (25.09%) | 191 (24.81%) | 28 (27.18%) | |
| Cs | | | | 0.804 |
| Q1 | 218 (24.97%) | 195 (25.32%) | 23 (22.33%) | |
| Q2 | 218 (24.97%) | 194 (25.19%) | 24 (23.30%) | |
| Q3 | 218 (24.97%) | 189 (24.55%) | 29 (28.16%) | |
| Q4 | 219 (25.09%) | 192 (24.94%) | 27 (26.21%) | |
| Mn | | | | 0.505 |
| Q1 | 218 (24.97%) | 193 (25.06%) | 25 (24.27%) | |
| Q2 | 218 (24.97%) | 195 (25.32%) | 23 (22.33%) | |
| Q3 | 218 (24.97%) | 195 (25.32%) | 23 (22.33%) | |
| Q4 | 219 (25.09%) | 187 (24.29%) | 32 (31.07%) | |
| Mo | | | | 0.942 |
| Q1 | 218 (24.97%) | 194 (25.19%) | 24 (23.30%) | |
| Q2 | 218 (24.97%) | 192 (24.94%) | 26 (25.24%) | |
| Q3 | 218 (24.97%) | 190 (24.68%) | 28 (27.18%) | |
| Q4 | 219 (25.09%) | 194 (25.19%) | 25 (24.27%) | |
| Pb | | | | 0.828 |
| Q1 | 218 (24.97%) | 194 (25.19%) | 24 (23.30%) | |
| Q2 | 218 (24.97%) | 195 (25.32%) | 23 (22.33%) | |
| Q3 | 218 (24.97%) | 190 (24.68%) | 28 (27.18%) | |
| Q4 | 219 (25.09%) | 191 (24.81%) | 28 (27.18%) | |
| Sb | | | | 0.484 |
| Q1 | 218 (24.97%) | 198 (25.71%) | 20 (19.42%) | |

*(Continued)*

TABLE 3 Continued

| Variable | Overall | None Infertility | Infertility | *p*-value |
|---|---|---|---|---|
| | N 873 | N 770 | N 103 | |
| Q2 | 218 (24.97%) | 193 (25.06%) | 25 (24.27%) | |
| Q3 | 218 (24.97%) | 190 (24.68%) | 28 (27.18%) | |
| Q4 | 219 (25.09%) | 189 (24.55%) | 30 (29.13%) | |
| Sn | | | | 0.719 |
| Q1 | 218 (24.97%) | 191 (24.81%) | 27 (26.21%) | |
| Q2 | 218 (24.97%) | 197 (25.58%) | 21 (20.39%) | |
| Q3 | 218 (24.97%) | 190 (24.68%) | 28 (27.18%) | |
| Q4 | 219 (25.09%) | 192 (24.94%) | 27 (26.21%) | |
| Tl | | | | 0.505 |
| Q1 | 218 (24.97%) | 195 (25.32%) | 23 (22.33%) | |
| Q2 | 218 (24.97%) | 193 (25.06%) | 25 (24.27%) | |
| Q3 | 218 (24.97%) | 195 (25.32%) | 23 (22.33%) | |
| Q4 | 219 (25.09%) | 187 (24.29%) | 32 (31.07%) | |
| Tu | | | | 0.696 |
| Q1 | 218 (24.97%) | 190 (24.68%) | 28 (27.18%) | |
| Q2 | 218 (24.97%) | 197 (25.58%) | 21 (20.39%) | |
| Q3 | 218 (24.97%) | 190 (24.68%) | 28 (27.18%) | |
| Q4 | 219 (25.09%) | 193 (25.06%) | 26 (25.24%) | |

Figures 5 and 6. As can be seen in Figure 5, BMI, Cd and LE8 have the dominant effect on predicting the risk of female infertility and have the largest SHAP values. The SHAP values are proportional to the effect on the model output, and the importance decreases from top to bottom. In addition, Cs, Mo, Sb, Sn, diet, education level, and pregnancy history also showed significant predictive ability.

Figure 6 presents the SHAP values distribution of each LE8 indicator, heavy metal exposure, and covariates, as well as their influence on the model output. The findings of the study suggest a negative correlation between BMI, LE8, and Cs with female infertility, while Cd, education level, and pregnancy history exhibit a positive association with infertility. While other variables

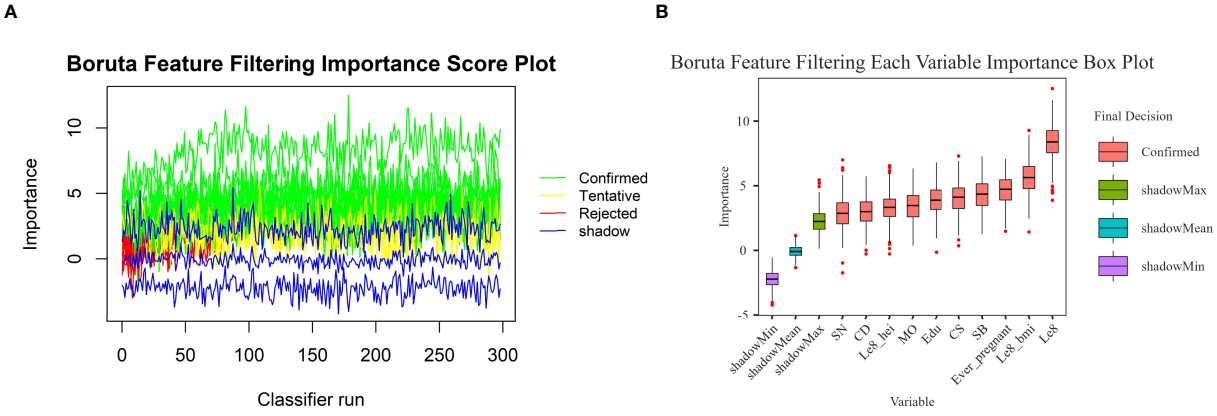


FIGURE 2
Boruta feature screening plot. **(A)** Importance score plot. **(B)** Importance box plot for each variable.

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TABLE 4 Correlation between LE8 and Infertility.

| Variable | Model1 | | Model2 | |
|---|---|---|---|---|
| | OR(95%CI) | Pvalue | OR(95%CI) | *p*-value |
| LE8 | 0.977(0.963-0.990) | 0.001 | 0.971(0.955-0.986) | 0.000 |
| Q1 | | | | |
| Q2 | 0.388(0.210-0.741) | 0.003 | 0.354(0.185-0.694) | 0.002 |
| Q3 | 0.345(0.184-0.664) | 0.001 | 0.293(0.145-0.605) | 0.001 |
| P for trend | | 0.010 | | 0.005 |
| BMI | 0.990(0.984-0.995) | 0.000 | 0.989(0.983-0.995) | 0.001 |
| Q1 | | | | |
| Q2 | 0.299(0.150-0.547) | 0.000 | 0.278(0.138-0.518) | 0.000 |
| Q3 | 0.508(0.306-0.818) | 0.007 | 0.492(0.283-0.832) | 0.010 |
| P for trend | | 0.001 | | 0.002 |
| Blood pressure | 0.988(0.981-0.996) | 0.003 | 0.988(0.980-0.997) | 0.007 |
| Q1 | | | | |
| Q2 | 0.460(0.206-1.024) | 0.056 | 0.397(0.172-0.913) | 0.029 |
| Q3 | 0.359(0.195-0.693) | 0.001 | 0.331(0.166-0.681) | 0.002 |
| P for trend | | 0.003 | | 0.005 |
| Blood glucose | 0.994(0.984-1.005) | 0.274 | 0.996(0.986-1.008) | 0.520 |
| Q1 | | | | |
| Q2 | 0.661(0.243-1.913) | 0.425 | 0.783(0.279-2.327) | 0.646 |
| Q3 | 0.586(0.265-1.484) | 0.218 | 0.723(0.313-1.888) | 0.473 |
| P for trend | | 0.241 | | 0.489 |
| Blood lipids | 0.995(0.988-1.003) | 0.185 | 0.996(0.989-1.004) | 0.324 |
| Q1 | | | | |
| Q2 | 1.011(0.537-1.946) | 0.972 | 0.997(0.522-1.940) | 0.992 |
| Q3 | 0.797(0.461-1.438) | 0.432 | 0.841(0.478-1.542) | 0.561 |
| P for trend | | 0.322 | | 0.476 |
| Diet | 0.997(0.990-1.004) | 0.385 | 0.996(0.989-1.003) | 0.241 |
| Q1 | | | | |
| Q2 | 0.890(0.528-1.460) | 0.652 | 0.826(0.483-1.375) | 0.471 |
| Q3 | 0.787(0.455-1.316) | 0.375 | 0.729(0.409-1.258) | 0.268 |
| P for trend | | 0.362 | | 0.243 |
| Physical activity | 0.998(0.994-1.003) | 0.522 | 0.998(0.993-1.004) | 0.515 |
| Q1 | | | | |
| Q2 | 0.179(0.010-0.879) | 0.096 | 0.161(0.009-0.805) | 0.079 |
| Q3 | 0.892(0.566-1.441) | 0.631 | 0.884(0.552-1.450) | 0.617 |
| P for trend | | 0.783 | | 0.787 |
| Sleep health | 0.991(0.983-0.999) | 0.017 | 0.989(0.981-0.997) | 0.006 |
| Q1 | | | | |

*(Continued)*

TABLE 4 Continued

| Variable | Model1 | | Model2 | |
|---|---|---|---|---|
| | OR(95%CI) | Pvalue | OR(95%CI) | *p*-value |
| Q2 | 1.063(0.563-1.999) | 0.850 | 0.815(0.420-1.572) | 0.542 |
| Q3 | 0.591(0.358-1.004) | 0.045 | 0.498(0.290-0.872) | 0.013 |
| P for trend | | 0.019 | | 0.007 |
| Nicotine exposure | 0.998(0.993-1.003) | 0.464 | 0.996(0.990-1.002) | 0.194 |
| Q1 | | | | |
| Q2 | 0.396(0.062-1.428) | 0.223 | 0.321(0.049-1.199) | 0.142 |
| Q3 | 0.832(0.516-1.385) | 0.464 | 0.687(0.398-1.209) | 0.183 |
| P for trend | | 0.562 | | 0.251 |

demonstrate high contribution rates, the mechanisms through which they act remain opaque, and their actions may be related to interactions with other variables.

Figure 7 presents the heat maps of each indicator, which clearly show the predictive direction of each factor on infertility. The findings suggest that the predictive directions of each factor align with the results of the honeycomb diagram.

## 3.6 Subgroup analysis of characteristics

Given the investigation's objective to assess the joint predictive effect of LE8 and heavy metal exposure on the risk of female infertility, a decision was made to proceed with the execution of subgroup analysis and interaction tests on the identified core predictive variables. The aforementioned core predictive variables

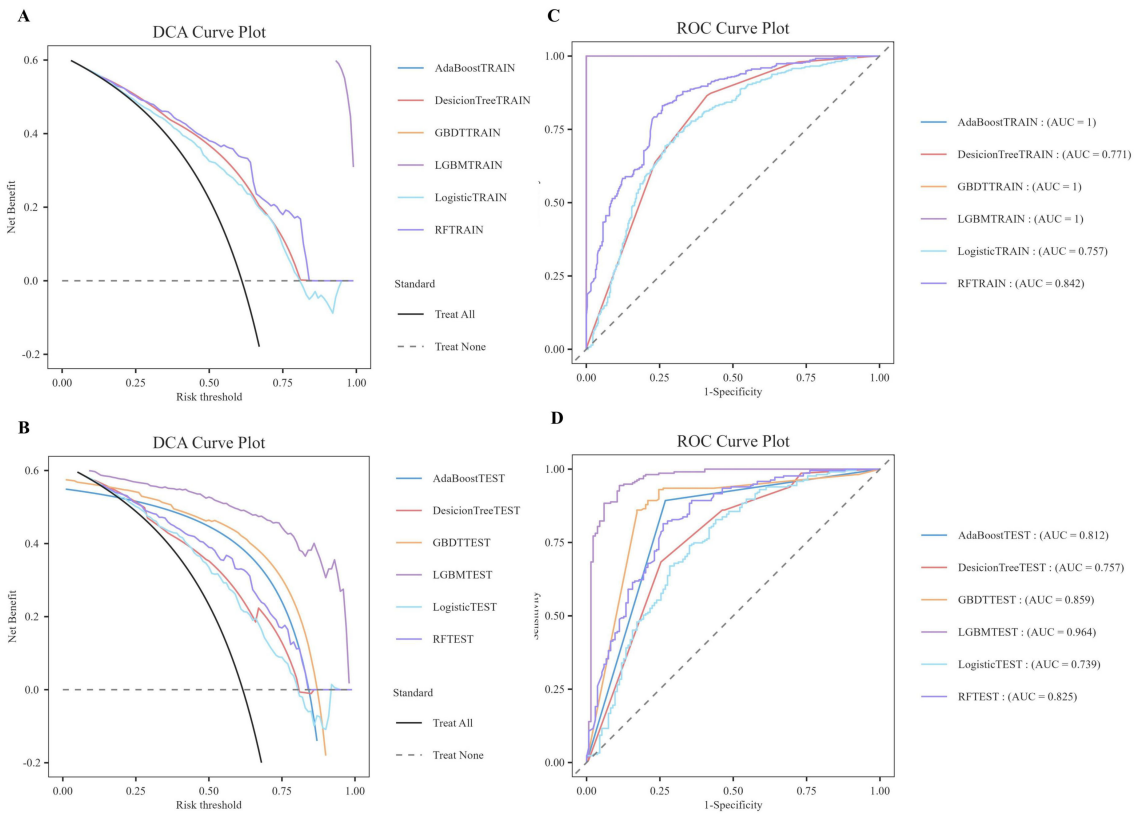


FIGURE 3
DCA/ROC curves of the train and test sets of 6 ML models. **(A)** DCA curves of the train set. **(B)** DCA curves of the test sets. **(C)** ROC curves of the train set. **(D)** ROC curves of the test sets. A DCA curve that is close to the upper-right corner of the coordinate diagram and a ROC curve that is close to the upper-left corner of the coordinate diagram both indicate good prediction performance.

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TABLE 5 Correlation between Heavy metal exposure and Infertility.

| Variable | Model1 | | Model2 | |
|---|---|---|---|---|
| | OR(95%CI) | Pvalue | OR(95%CI) | *p*-value |
| Ba | 1.287(0.827-2.005) | 0.263 | 1.349(0.857-2.127) | 0.196 |
| Q1 | | | | |
| Q2 | 1.051(0.565-1.957) | 0.875 | 1.139(0.608-2.142) | 0.684 |
| Q3 | 1.259(0.694-2.307) | 0.449 | 1.352(0.730-2.527) | 0.338 |
| Q4 | 1.469(0.825-2.656) | 0.195 | 1.566(0.865-2.878) | 0.142 |
| P for trend | | 0.152 | | 0.115 |
| Cd | 1.603(1.009-2.561) | 0.047 | 1.386(0.842-2.295) | 0.201 |
| Q1 | | | | |
| Q2 | 0.743(0.371-1.460) | 0.392 | 0.788(0.391-1.571) | 0.499 |
| Q3 | 1.856(1.053-3.343) | 0.035 | 1.752(0.962-3.267) | 0.071 |
| Q4 | 1.489(0.828-2.723) | 0.188 | 1.325(0.701-2.544) | 0.390 |
| P for trend | | 0.029 | | 0.110 |
| Co | 1.172(0.693-1.990) | 0.555 | 1.138(0.668-1.948) | 0.636 |
| Q1 | | | | |
| Q2 | 1.481(0.801-2.786) | 0.214 | 1.584(0.844-3.027) | 0.156 |
| Q3 | 1.607(0.878-3.005) | 0.129 | 1.607(0.866-3.044) | 0.137 |
| Q4 | 1.535(0.835-2.880) | 0.172 | 1.550(0.825-2.966) | 0.177 |
| P for trend | | 0.180 | | 0.207 |
| Cs | 1.047(0.550-2.015) | 0.890 | 0.948(0.496-1.834) | 0.873 |
| Q1 | | | | |
| Q2 | 0.953(0.518-1.751) | 0.877 | 1.117(0.603-2.076) | 0.723 |
| Q3 | 1.240(0.698-2.223) | 0.464 | 1.304(0.720-2.385) | 0.383 |
| Q4 | 1.137(0.633-2.052) | 0.668 | 1.109(0.605-2.045) | 0.738 |
| P for trend | | 0.492 | | 0.645 |
| Mn | 1.452(0.612-3.097) | 0.360 | 1.412(0.579-3.112) | 0.415 |
| Q1 | | | | |
| Q2 | 1.206(0.662-2.218) | 0.541 | 0.854(0.460-1.578) | 0.614 |
| Q3 | 1.154(0.629-2.130) | 0.643 | 0.901(0.485-1.666) | 0.738 |
| Q4 | 1.414(0.791-2.564) | 0.246 | 1.279(0.722-2.288) | 0.401 |
| P for trend | | 0.289 | | 0.366 |
| Mo | 1.145(0.693-1.909) | 0.599 | 1.164(0.698-1.961) | 0.563 |
| Q1 | | | | |
| Q2 | 1.000(0.553-1.808) | 1.000 | 1.095(0.600-2.005) | 0.767 |
| Q3 | 1.138(0.640-2.033) | 0.660 | 1.233(0.680-2.253) | 0.491 |
| Q4 | 0.995(0.550-1.798) | 0.986 | 1.023(0.555-1.890) | 0.942 |
| P for trend | | 0.901 | | 0.850 |
| Pb | 1.262(0.748-2.130) | 0.383 | 1.221(0.715-2.088) | 0.464 |

*(Continued)*

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TABLE 5 Continued

| Variable | Model1 | | Model2 | |
|---|---|---|---|---|
| | OR(95%CI) | Pvalue | OR(95%CI) | *p*-value |
| Q1 | | | | |
| Q2 | 1.049(0.571-1.930) | 0.877 | 0.945(0.508-1.756) | 0.858 |
| Q3 | 1.249(0.696-2.263) | 0.457 | 1.207(0.665-2.206) | 0.536 |
| Q4 | 1.243(0.692-2.251) | 0.467 | 1.123(0.617-2.058) | 0.704 |
| P for trend | | 0.382 | | 0.545 |
| Sb | 1.179(0.671-2.029) | 0.559 | 1.186(0.648-2.130) | 0.573 |
| Q1 | | | | |
| Q2 | 1.051(0.565-1.957) | 0.875 | 1.314(0.697-2.504) | 0.401 |
| Q3 | 1.313(0.727-2.396) | 0.368 | 1.534(0.820-2.917) | 0.184 |
| Q4 | 1.414(0.791-2.564) | 0.246 | 1.651(0.877-3.163) | 0.123 |
| P for trend | | 0.180 | | 0.111 |
| Sn | 1.231(0.837-1.796) | 0.285 | 1.231(0.810-1.857) | 0.325 |
| Q1 | | | | |
| Q2 | 0.829(0.451-1.512) | 0.541 | 0.772(0.411-1.432) | 0.414 |
| Q3 | 1.088(0.615-1.933) | 0.771 | 1.014(0.559-1.843) | 0.962 |
| Q4 | 1.038(0.584-1.851) | 0.898 | 0.938(0.500-1.753) | 0.841 |
| P for trend | | 0.685 | | 0.944 |
| Tl | 1.391(0.731-2.687) | 0.320 | 1.352(0.701-2.646) | 0.373 |
| Q1 | | | | |
| Q2 | 1.098(0.602-2.012) | 0.760 | 1.153(0.624-2.142) | 0.649 |
| Q3 | 1.049(0.571-1.930) | 0.877 | 0.972(0.517-1.824) | 0.928 |
| Q4 | 1.398(0.789-2.508) | 0.254 | 1.468(0.816-2.677) | 0.203 |
| P for trend | | 0.289 | | 0.282 |
| Tu | 1.176(0.757-1.813) | 0.467 | 1.265(0.801-1.986) | 0.310 |
| Q1 | | | | |
| Q2 | 0.723(0.393-1.313) | 0.290 | 0.694(0.371-1.277) | 0.243 |
| Q3 | 0.959(0.543-1.692) | 0.885 | 0.986(0.551-1.761) | 0.961 |
| Q4 | 0.954(0.540-1.683) | 0.871 | 0.970(0.536-1.753) | 0.920 |
| P for trend | | 0.901 | | 0.810 |

Model 1 does not adjust for any factors; Model 2 includes the covariates of age, ethnicity, education level, work situation, ever pregnant, and chronic diseases.

consist of the four heavy metal variables (Cd, Mo, Sb, Cs, Sn) and the three LE8 indicators (LE8, diet, BMI). The dataset generated using Synthetic Minority Over-sampling Technique combined with undersampling was utilized for the aforementioned analyses. This investigation was undertaken to ascertain whether LE8 and heavy metal exposure interact to influence female infertility (Figures 8–12). Preliminary findings indicate that the overall LE8 score demonstrates a substantial degree of interaction with Cd, Cs, Mo, and Sn, as indicated by key indicators in LE8. When the levels of these heavy metals are at the Q1 and Q4 quartiles, LE8 is at a moderate level, which is often associated with infertility risk. However, when LE8 is at a high level, it can reduce infertility risk to some extent. The present study demonstrates that dietary intake exhibits significant interactions with Cd, Cs, Mo, and Sb. When the concentrations of these heavy metals reach the Q2, Q3, and Q4 quartiles, moderate dietary intake is frequently linked to an elevated risk of infertility, while high dietary intake has been observed to reduce the risk of infertility. A significant interaction effect was observed between BMI

TABLE 6 Model evaluation for training and test sets.

| Model Name | Accuracy | Recall | F1-Score | MCC |
|---|---|---|---|---|
| Decision Tree TRAIN | 0.75739 | 0.86492 | 0.81327 | 0.47744 |
| GBDT TRAIN | 1.00000 | 1.00000 | 1.00000 | 1.00000 |
| AdaBoost TRAIN | 1.00000 | 1.00000 | 1.00000 | 1.00000 |
| LGBM TRAIN | 1.00000 | 1.00000 | 1.00000 | 1.00000 |
| Logistic TRAIN | 0.71429 | 0.84879 | 0.78399 | 0.37888 |
| RF TRAIN | 0.76970 | 0.92137 | 0.83015 | 0.50680 |
| Decision Tree TEST | 0.73639 | 0.86047 | 0.80087 | 0.42537 |
| GBDT TEST | 0.84814 | 0.89302 | 0.87872 | 0.67641 |
| AdaBoost TEST | 0.83095 | 0.89302 | 0.86682 | 0.63804 |
| LGBM TEST | 0.91117 | 0.96744 | 0.93065 | 0.81217 |
| Logistic TEST | 0.71920 | 0.86977 | 0.79237 | 0.38346 |
| RF TEST | 0.77364 | 0.93488 | 0.83576 | 0.51383 |

and Cd. When Cd is at the Q3 percentile, the risk of infertility increases in the group with moderate BMI levels. When Cd is at the Q4 percentile, the risk of infertility increases in the group with high BMI levels.

## 3.7 Sensitivity analysis

It must be acknowledged that extant research findings may be subject to data bias. Furthermore, given that the NHANES database employs self-reported data to measure female infertility symptoms, it is possible that some women may be infertile without realizing it. Therefore, to further validate the robustness of the research findings, propensity score matching (PSM) was used to balance the sample for sensitivity analysis. To ensure a balanced data structure without a significant loss of samples, a 1:8 matching method was employed, with the sample size of the non-infertility group being adjusted to 488. Following a thorough examination of the results, it was determined that the standardized mean differences (SMDs) for the majority of key variables underwent a substantial reduction, falling below 0.25. Furthermore, the intervention and control groups exhibited no substantial disparities following matching, suggesting that the features were well-balanced (Table 7).

Subsequent to the initial matching process, the Boruta algorithm was re-implemented, employing the identical parameter settings as previously utilized. The results of the study indicated the presence of LE8, BMI, diet, Cd, Sb, Mn, and Cs. The salient factors identified prior to and following the matching process exhibited substantial consistency, with LE8, BMI, diet, Cd, Sb, and Cs being identified in both instances (Figure 13).

Subsequently, the same six models were used to evaluate the samples. Preliminary findings indicate that, based on the evaluation results, LGBM continues to demonstrate superior predictive capabilities (Table 8; Figure 14).

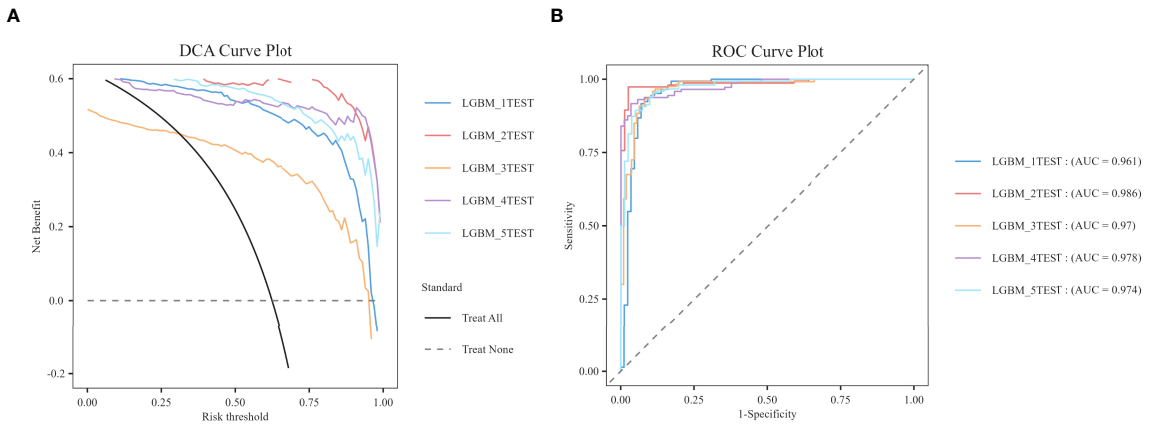


FIGURE 4
5-fold cross-validation. **(A)** DCA curve. **(B)** ROC curve.

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TABLE 7 Balance test.

| Variable name | Matching status | SMD | Intergroup *p*-value |
|---|---|---|---|
| Age | Before matching | 0.307 | 0.003 |
| | After matching | 0.166 | 0.123 |
| Ethnicity | Before matching | 0.183 | 0.573 |
| | After matching | 0.120 | 0.881 |
| Education | Before matching | 0.172 | 0.643 |
| | After matching | 0.101 | 0.933 |
| Work situation | Before matching | 0.192 | 0.825 |
| | After matching | 0.063 | 0.950 |
| Ever pregnant | Before matching | 0.347 | 0.003 |
| | After matching | 0.214 | 0.062 |
| Ba | Before matching | 0.116 | 0.591 |
| | After matching | 0.054 | 0.821 |
| Cd | Before matching | 0.212 | 0.017 |
| | After matching | 0.117 | 0.481 |
| Co | Before matching | 0.063 | 0.433 |
| | After matching | 0.053 | 0.413 |
| Cs | Before matching | 0.015 | 0.804 |
| | After matching | 0.011 | 0.110 |
| Mn | Before matching | 0.099 | 0.505 |
| | After matching | 0.063 | 0.766 |
| Mo | Before matching | 0.053 | 0.942 |
| | After matching | 0.058 | 0.947 |
| Pb | Before matching | 0.089 | 0.828 |
| | After matching | 0.055 | 0.966 |
| Sb | Before matching | 0.064 | 0.484 |
| | After matching | 0.045 | 0.593 |
| Sn | Before matching | 0.107 | 0.719 |
| | After matching | 0.050 | 0.435 |
| Tl | Before matching | 0.105 | 0.505 |
| | After matching | 0.073 | 0.789 |
| Tu | Before matching | 0.071 | 0.696 |
| | After matching | 0.035 | 0.886 |
| Chronic disease | Before matching | 0.421 | <0.001 |
| | After matching | 0.219 | 0.129 |
| LE8 | Before matching | 0.313 | 0.002 |
| | After matching | 0.245 | 0.048 |
| Diet | Before matching | 0.097 | 0.659 |
| | After matching | 0.057 | 0.872 |
| Physical activity | Before matching | 0.236 | 0.179 |
| | After matching | 0.124 | 0.589 |
| Nicotine exposure | Before matching | 0.149 | 0.420 |
| | After matching | 0.088 | 0.730 |
| Sleep health | Before matching | 0.270 | 0.031 |
| | After matching | 0.173 | 0.275 |
| BMI | Before matching | 0.476 | <0.001 |
| | After matching | 0.239 | 0.098 |
| Blood lipids | Before matching | 0.115 | 0.544 |
| | After matching | 0.101 | 0.645 |
| Blood glucose | Before matching | 0.122 | 0.446 |
| | After matching | 0.110 | 0.552 |
| Blood pressure | Before matching | 0.299 | 0.004 |
| | After matching | 0.221 | 0.088 |

Finally, further SHAP explainability analysis was conducted (Figure 15), and the results showed that the effects of the six factors (LE8, BMI, diet, Sb, Cd, and Cs) remained consistent with the aforementioned analysis, indicating that the results of this study are robust.

## 4 Discussion

This study utilized data from the US population in the NHANES between 2013 and 2018, in conjunction with the LE8 scoring dimensions, heavy metal exposure, and covariates such as age, education level, work situation, pregnancy history, and chronic disease status, to predict the risk of infertility in U.S. women. The Boruta algorithm was employed to identify ten core variables that significantly contribute to the prediction of infertility: educational level, pregnancy history, LE8 overall, diet, BMI, Cd, Mo, Sb, Cs, and Sn. In light of the aforementioned variables, the training and construction of six machine learning models was undertaken. A comparison of models indicates that the LGBM model exhibits the optimal predictive capacity, suggesting that the LGBM model demonstrates remarkably high precision in forecasting the likelihood of female infertility. In the subsequent SHAP interpretability analysis based on the LGBM model, we found that BMI, LE8, and Cd were the variables that contributed most to the model in terms of LE8 scores and heavy metal exposure, with BMI leading to a higher risk of female infertility. The ensuing discourse will concentrate on the 10 feature variables identified and screened by the Boruta algorithm. It will further argue the potential association between each core variable and infertility risk based on existing epidemiological and toxicological studies.

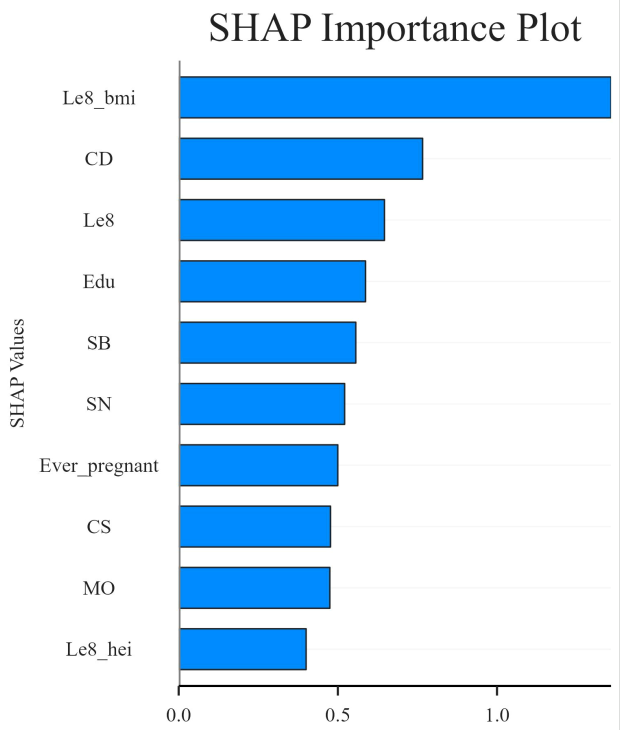


FIGURE 5
SHAP column chart.

BMI is a commonly used indicator for assessing an individual's weight status and degree of obesity, based on the LE8 score. Previous studies have confirmed its close association with female reproductive health, such as ovarian function and the menstrual cycle. Overweight and obese women (BMI≧25kg/m$^2$) are more likely to face the risk of infertility, and the risk increases with increasing BMI (23). Obesity has been demonstrated to induce systemic oxidative stress and to precipitate the development of complications through mechanisms that affect the insulin signaling pathway, adipocyte function, and inflammatory response (26). Reproductive organ dysfunction, including decreased ovarian reserve function, diminished egg quality, reduced fertilization capacity, and abnormal embryonic development, has been observed to be associated with obesity (60). Studies have long confirmed that polycystic ovary syndrome is the most common cause of anovulatory infertility in women (23). Obesity exacerbates the metabolic abnormalities of polycystic ovary syndrome, and the prevalence rate among overweight and obese women is as high as 80% (61). Concurrently, obese women demonstrate inferior outcomes when undergoing assisted reproductive technology treatment. Evidence of this includes a diminished response to ovulation-inducing medications, a reduced number of oocytes retrieved, and diminished embryo quality.

Diet is a common indicator used to assess an individual's health status, nutritional level, and lifestyle characteristics, and is of

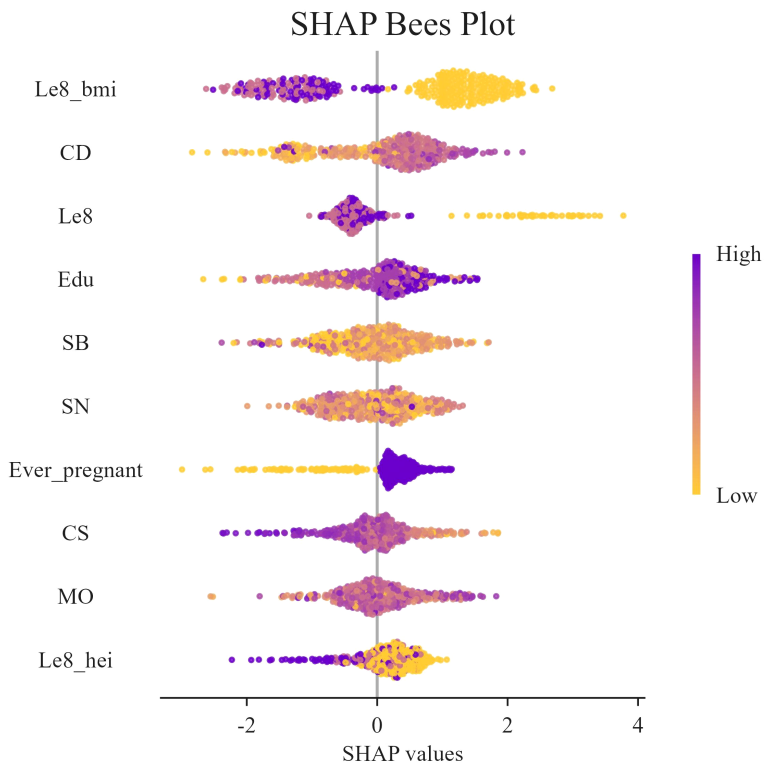


FIGURE 6
SHAP swarm plot. In the honeycomb diagram, the length of each indicator represents its contribution to infertility prediction, and the color of each point indicates its value magnitude. The 0 coordinate serves as a dividing line; values greater than 0 indicate a positive predictive value, and values less than 0 indicate a negative predictive value.

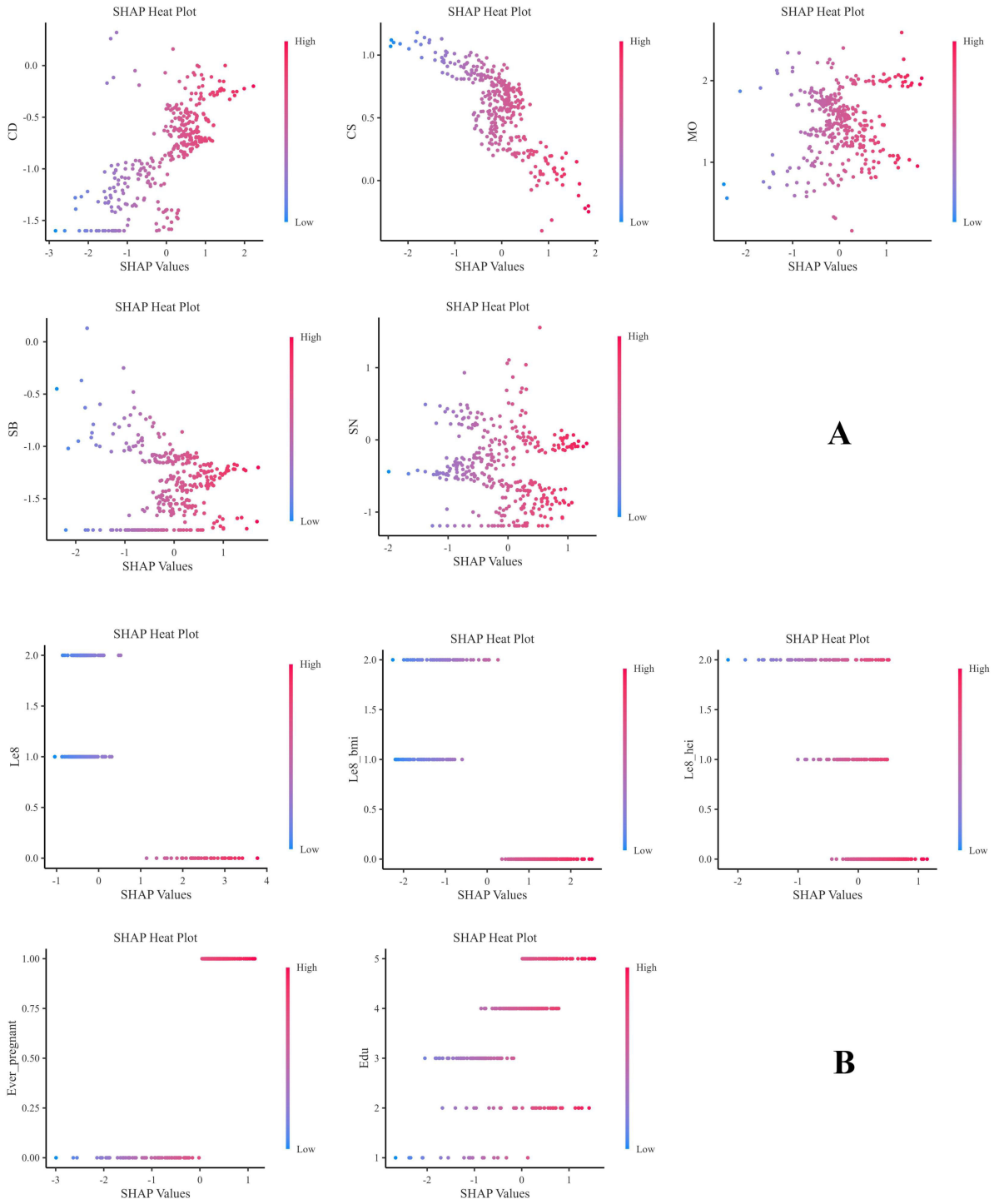


FIGURE 7
SHAP thermal dispersion diagram **(A, B)**.

great importance in research related to women's reproductive health. Previous studies also support the influence of dietary patterns on women's reproductive health. On the one hand, a pro-inflammatory diet may affect women's reproductive health by increasing systemic inflammation (62). For instance, a diet high in sweet beverages, desserts, caffeinated drinks, potatoes, fast food, and other similar items may result in inflammatory responses, obesity, and insulin resistance. Moreover, such dietary habits may increase the risk of fertility in women, particularly in patients diagnosed with polycystic ovary syndrome (63–65). Studies have confirmed that a light fasting diet combined with flaxseed powder supplementation can significantly improve obesity, blood lipid and blood sugar

TABLE 8 Model evaluation for training and test sets.

| Model Name | Accuracy | Recall | F1-Score | MCC |
|---|---|---|---|---|
| Decision Tree TRAIN | 0.75682 | 0.85135 | 0.79412 | 0.50720 |
| GBDT TRAIN | 1.00000 | 1.00000 | 1.00000 | 1.00000 |
| AdaBoost TRAIN | 1.00000 | 1.00000 | 1.00000 | 1.00000 |
| LGBM TRAIN | 1.00000 | 1.00000 | 1.00000 | 1.00000 |
| Logistic TRAIN | 0.64764 | 0.78378 | 0.71020 | 0.27869 |
| RF TRAIN | 0.77419 | 0.81532 | 0.79912 | 0.54212 |
| Decision Tree TEST | 0.76879 | 0.89216 | 0.81982 | 0.51616 |
| GBDT TEST | 0.80925 | 0.90196 | 0.84793 | 0.60229 |
| AdaBoost TEST | 0.80347 | 0.90196 | 0.84404 | 0.59019 |
| LGBM TEST | 0.89017 | 0.92157 | 0.90821 | 0.77210 |
| Logistic TEST | 0.65896 | 0.76471 | 0.72558 | 0.28085 |
| RF TEST | 0.74566 | 0.81373 | 0.79048 | 0.46886 |

metabolic disorders in patients with polycystic ovary syndrome and combined infertility (66). On the other hand, there is growing evidence that a healthy diet has a positive effect on fertility. A healthy diet, such as the Mediterranean diet, which is rich in whole grains, monounsaturated or polyunsaturated fats, vegetables, fruits, fish, and olive oil, can improve egg quality and quantity through multiple mechanisms, including increasing antioxidant capacity, improving hormone levels, and reducing inflammatory responses, thereby improving fertility success rates (67, 68). At the same time, a healthy diet can help alleviate depressive symptoms, improve physical health, and indirectly improve fertility success rates (69, 70). In addition, the total LE8 score has been validated in multiple studies as a negative predictor of female infertility. There is a significant negative correlation between overall LE8 score and female infertility. Women with better cardiovascular health are 41% less likely to suffer from infertility than those with poorer cardiovascular health (22, 71), Infertility at a young age may have a greater impact on long-term cardiovascular health (21). As the LE8 score increases, there is a concomitant improvement in the cardiovascular health of the woman, as well as an enhancement in her overall lifestyle, both of which have a beneficial effect on her reproductive health. The synergistic effect of multiple factors has been demonstrated to engender improvements in fertility and to reduce the risk of infertility.

In this model, we found an association between Cd, Mo, Sb, Cs and Sn exposure and the risk of female infertility. Cd showed a positive contribution to the prediction of the risk of female infertility. Previous research has provided the first evidence of a positive association between cadmium exposure and the risk of primary ovarian insufficiency in the Chinese population (72). Cd exposure not only induces oxidative stress in the reproductive organs, but also interferes with the synthesis and secretion of reproductive hormones, thereby affecting reproductive function (73–75). Some research even shows that even low levels of cadmium in the blood can significantly affect women's reproductive health and increase the risk of infertility (76). The findings of this study offer convergent evidence in support of previous findings and extend them by providing an exotic validation. That is to say, they demonstrate a positive association between cadmium exposure and the risk of female infertility in a U.S. population.

Mo, Sb and Sn demonstrate a relatively intricate nonlinear relationship, with both exhibiting a two-way risk prediction for the risk of female infertility. Existing studies have shown a non-linear relationship between Mo concentration and infertility. Previous studies have shown that urinary molybdenum concentrations significantly affect clinical *in vitro* fertilization outcomes. Higher concentrations of molybdenum are associated with lower implantation rates, clinical pregnancy rates, and live birth rates (77). Later studies found that the concentration of trace elements such as Mo in the urine of women with premature ovarian failure was significantly lower than that of healthy controls, and that these elements may be related to the development of premature ovarian failure (78). The two studies show very different results. A further interpretation of the results shows that the excess Mo in the first study had a toxic effect on the reproductive system, affecting the ovarian response and embryonic development, and thus the outcome of assisted reproduction. Mo in the latter study is one of the essential trace elements in the human body, involved in the activity of various enzymes and metabolic processes. If Mo levels are low, it can negatively affect fertility by interfering with the activity of key enzymes. Therefore, the association between Mo and infertility needs to be further explored and verified with larger sample sizes and more mechanistic studies. Previous studies have suggested that Sb exposure may contribute to the development of polycystic ovary syndrome by inducing oxidative stress and disrupting redox

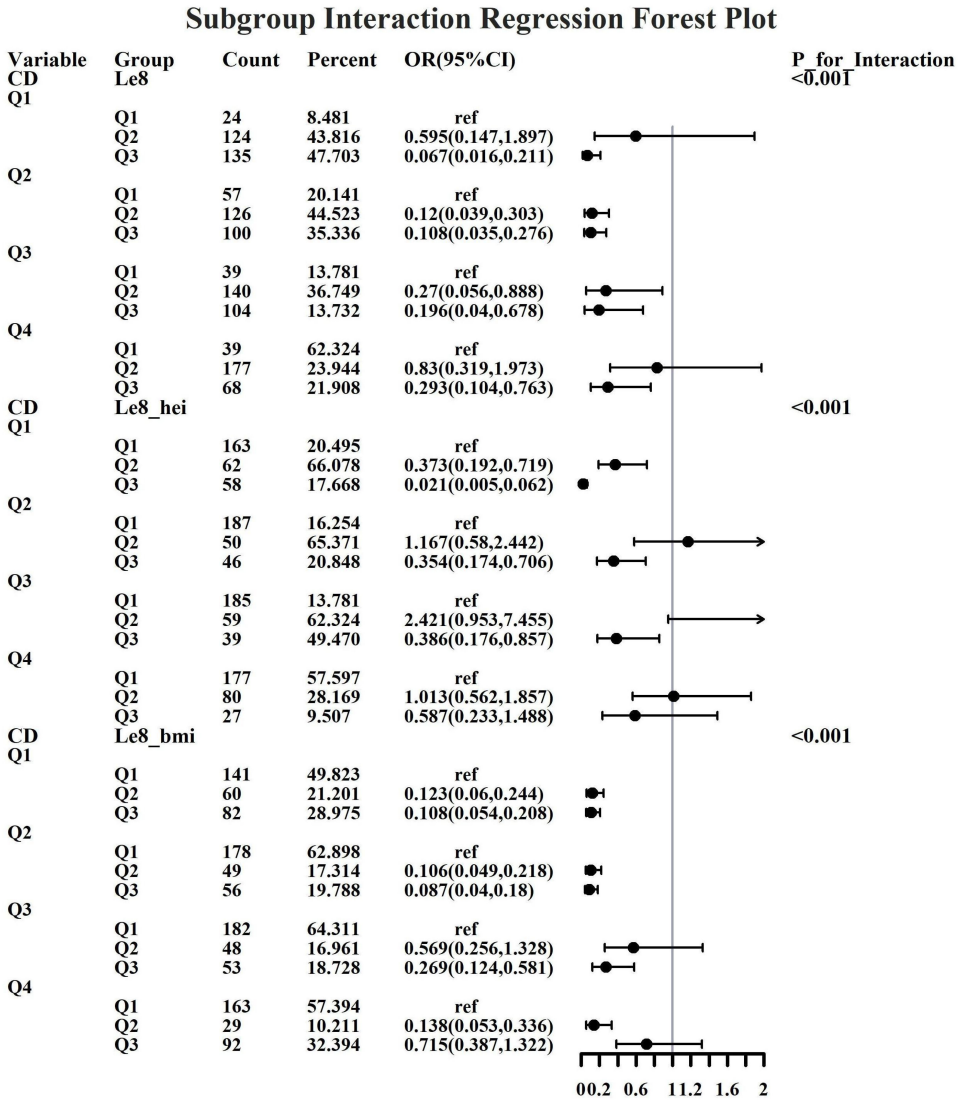


FIGURE 8
Forest plot of logistic regression based on Cd exposure.

homeostasis, thereby increasing the risk of infertility (79). Subsequent studies have also shown a significant non-linear relationship ($p$<0.05) between Ba, Mo, and Sb and infertility (80). However, no studies have systematically investigated the relationship between Sb content and the risk of infertility. Research has demonstrated that tin accumulation in the human body may have deleterious effects on reproductive health, given its classification as a non-essential trace element. Epidemiological studies have demonstrated that women with long-term exposure to or intake of higher doses of tin exhibit significantly increased tin concentrations in urine. This is closely associated with reduced ovarian response, diminished embryonic development potential, and lower live birth rates (81). Additionally, Chi et al. (2025) utilized NHANES database data to further discover that for every increase of one quartile range in urinary tin concentration, the risk of female infertility correspondingly increased by 8% (80).

Finally, although the mechanism between Cs and female infertility is not yet clear, the SHAP heat map (Figure 7) of this study clearly shows that Cs content in the fourth quartile has a negative correlation with female infertility. However, a subsequent analysis of subgroups and interaction testing (Figure 9) revealed that within the fourth quartile, where LE8 exhibited a moderate score indicative of a general state of health, an elevated Cs content was found to be associated with a heightened risk of infertility. This finding suggests that the effect of Cs on female fertility may not be a simple linear relationship, but may be modulated by an individual's overall health status or other unknown factors. Therefore, future research needs to further explore the complex relationship between

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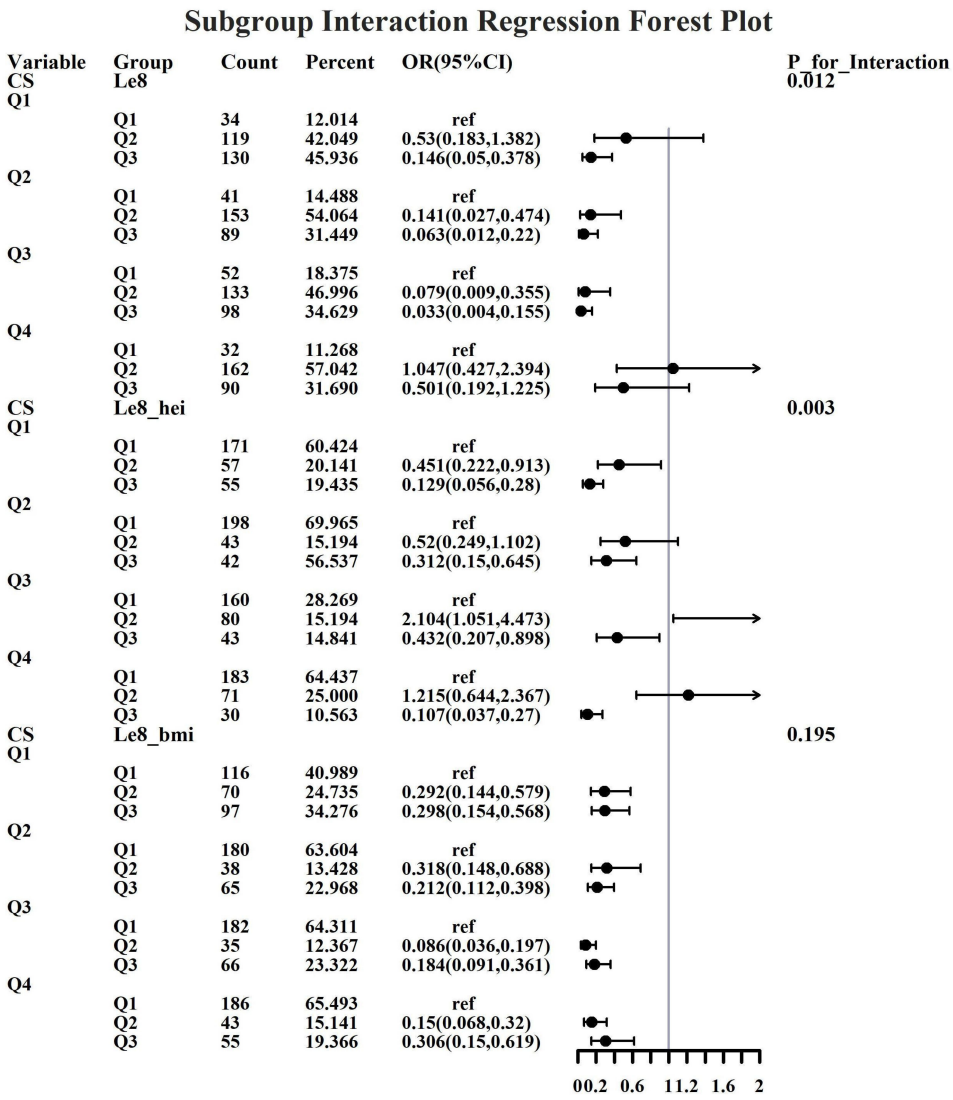


FIGURE 9
Forest plot of logistic regression based on Cs exposure.

Cs and female infertility and whether this relationship is disrupted or influenced by other factors. In addition, when assessing reproductive health risks, it is important to consider the effects of individual and combined exposures to multiple heavy metals and their specific mechanisms of action under different conditions.

Educational level and pregnancy history are important covariates for predicting the risk of female infertility. However, the current research results show that both are confounding factors. Combined with previous research, education level may be negatively correlated with the risk of female infertility. A correlation has been observed between a higher level of education and a lower risk of infertility in women. This phenomenon may be attributed to the fact that women with a higher level of education have a deeper understanding of health issues and are more inclined to seek medical help (82). A correlation has been demonstrated between a lower level of education and an increased prevalence of female sexual dysfunction, which can impact fertility. This phenomenon may be attributed, at least in part, to a deficiency in knowledge regarding sexual health. The enhancement of educational initiatives and the dissemination of information pertaining to sexual health may prove effective in the amelioration of sexual dysfunction (83). A history of pregnancy may be positively associated with the risk of female infertility. A number of studies have indicated that patients with a medical history of ectopic pregnancy are 62% more likely to experience implantation window displacement compared to those without such a history. This heightened risk can be attributed to the fact that implantation window displacement can impede the successful implantation of the embryo in the endometrium at the optimal time, consequently resulting in implantation failure and subsequent infertility (84). In addition, a history of ectopic pregnancy, polycystic ovary syndrome, and primary infertility are important risk factors for secondary infertility. However, further research is necessary

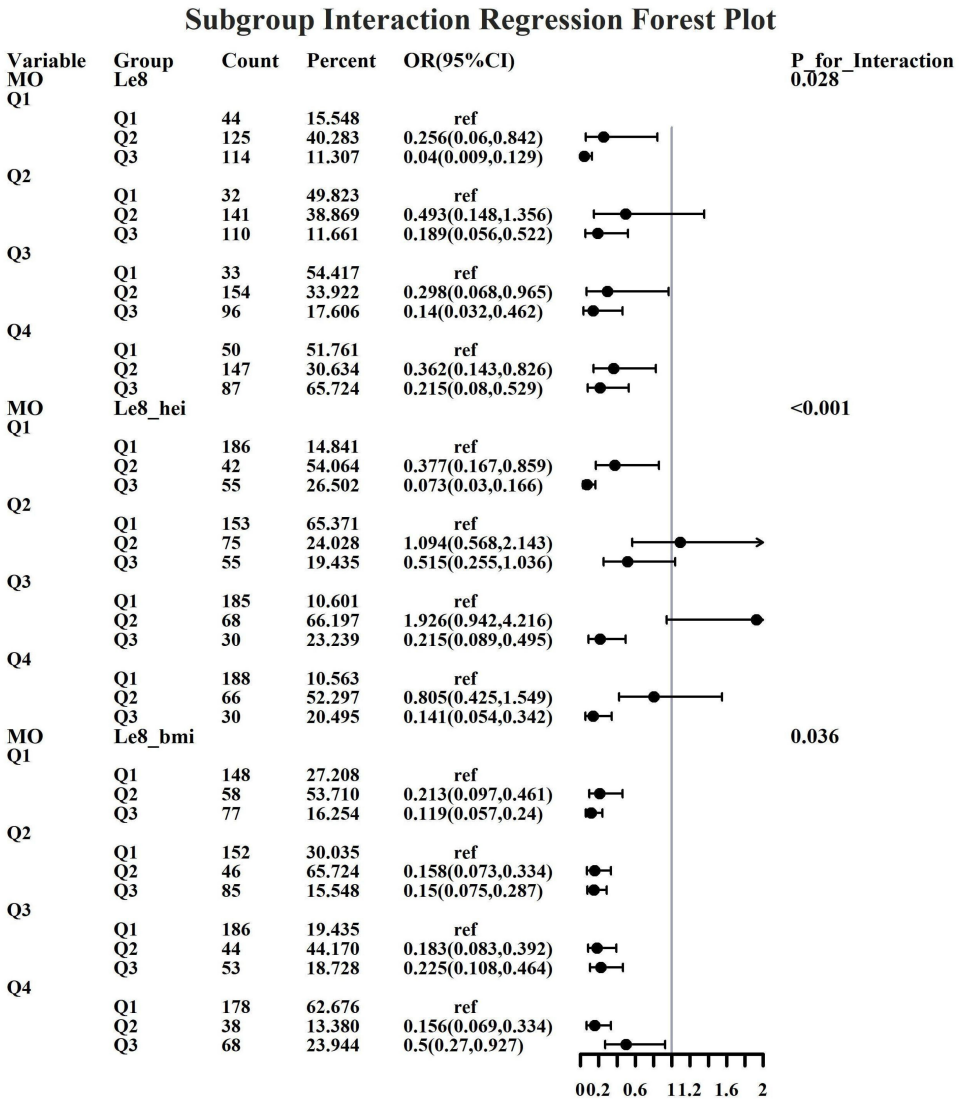


FIGURE 10
Forest plot of logistic regression based on Mo exposure.

to elucidate the intricate relationship and specific mechanisms between these covariates and the risk of infertility. Such studies are crucial for comprehending the multifaceted influences on female infertility and for developing effective clinical prediction and prevention strategies.

This study employed data from the NHANES to investigate the association between LE8 and heavy metal exposure and female infertility, with a sample that has clear regional representativeness. Despite the absence of specific information on the causes of infertility, the study population includes women aged 20–45 years, thereby enabling the generalizability of the findings to U.S. women of a similar age group. While dietary patterns, BMI, and heavy metal exposure vary across countries, these factors are globally prevalent, and their associations with female reproductive health have been validated in multiple countries and ethnic groups, suggesting a degree of cross-cultural applicability. Meanwhile, the female infertility risk prediction model developed by this institute utilizes publicly available and easily accessible NHANES data, offering an economical and efficient solution for the initial screening and early risk warning of female infertility, thereby realizing a health management model of "proactive identification and precise intervention". Additionally, the controllable factors identified by the model, such as BMI, dietary habits, and heavy metal exposure, provide data support and guidance for policy-making and personalized reproductive health education. Consequently, these findings provide novel insights that could inform future public health initiatives aimed at the early detection and prevention of female infertility.

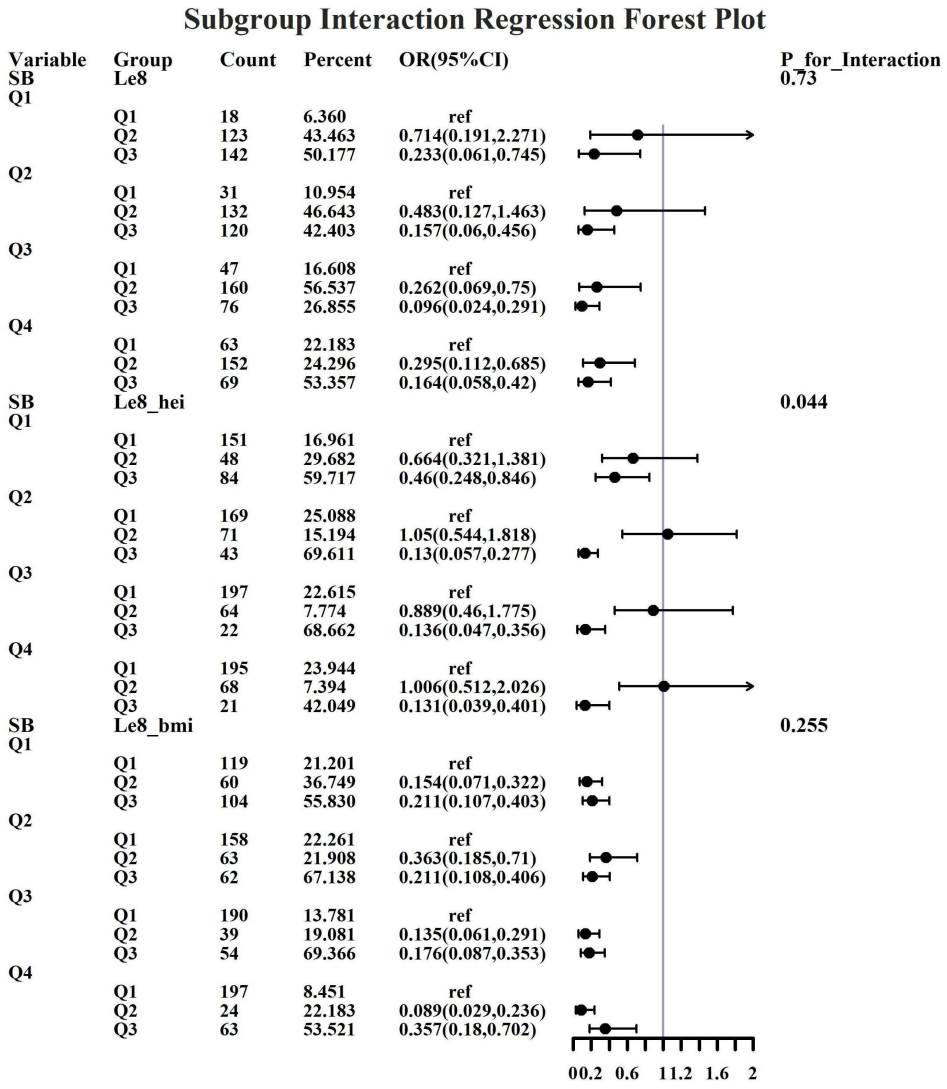


FIGURE 11
Forest plot of logistic regression based on Sb exposure.

## 5 Conclusion

In our study, we analyzed data from the NHANES database, combined the LE8 score and heavy metal exposure indicators, and used a stepwise machine learning strategy to construct a joint prediction model for the risk of female infertility. The conclusions of this study must be interpreted in light of its limitations. Firstly, the NHANES database is characterized by a retrospective and observational design, which precludes the capacity to observe temporal changes in variables. Further prospective validation and supplementation are necessary to enhance the robustness and comprehensiveness of the developed model. Secondly, the aetiology of female infertility is predicated on self-reported data, and the underlying causes remain to be elucidated. Despite the utilization of sensitivity analysis in the study to evaluate the model's performance, additional external validation is necessary to ensure its robustness and reliability. Moreover, the NHANES database does not currently encompass specific surveys concerning women's exposure to heavy metals. Subsequent analyses could be conducted by combining hospital data. While the LGBM model has been shown to demonstrate efficacy in predicting infertility risk using NHANES data, its effectiveness in other datasets or application scenarios remains to be fully validated. In subsequent research, we anticipate implementing model transfer learning and external validation based on multi-country datasets to enhance the model's generalization capability and prediction accuracy. Concurrently, we will enhance the application of various machine learning technologies to improve the interpretability and robustness

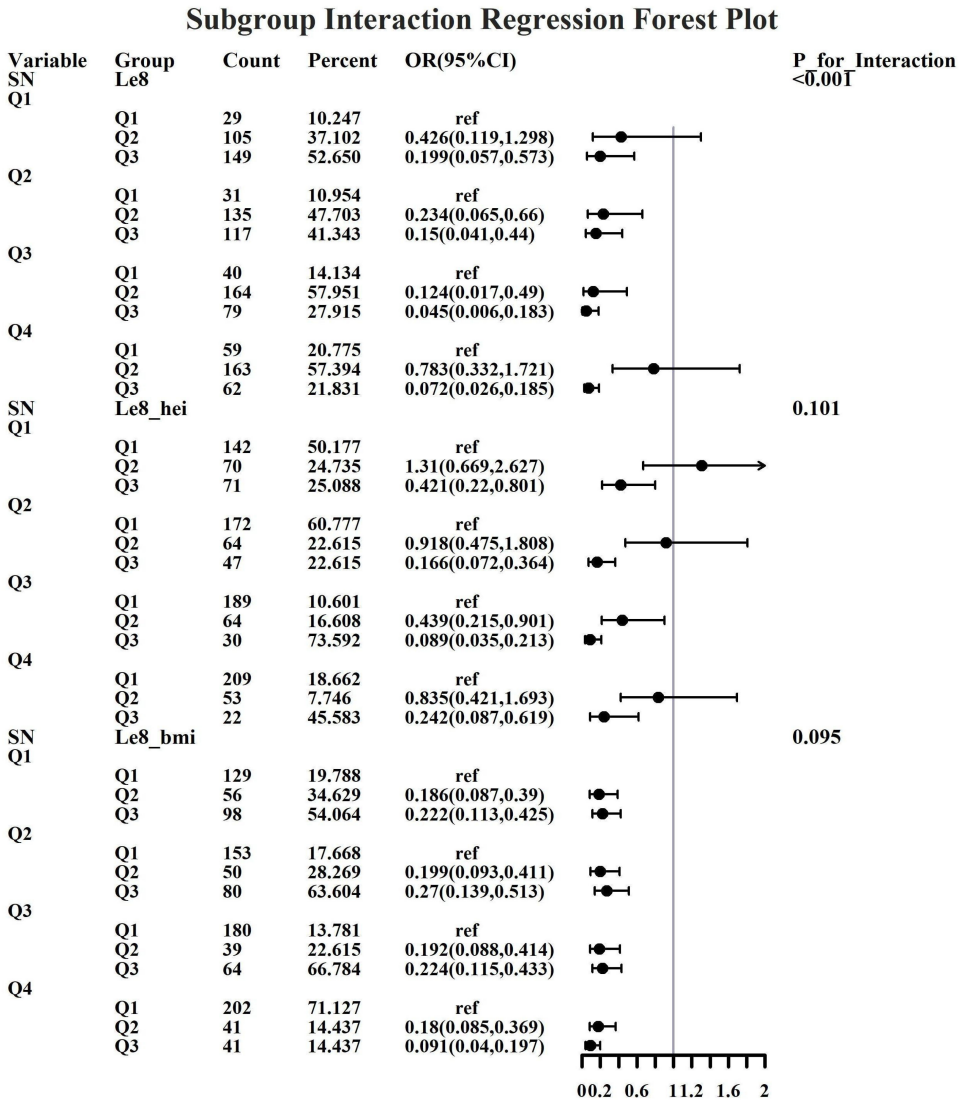


FIGURE 12
Forest plot of logistic regression based on Sn exposure.

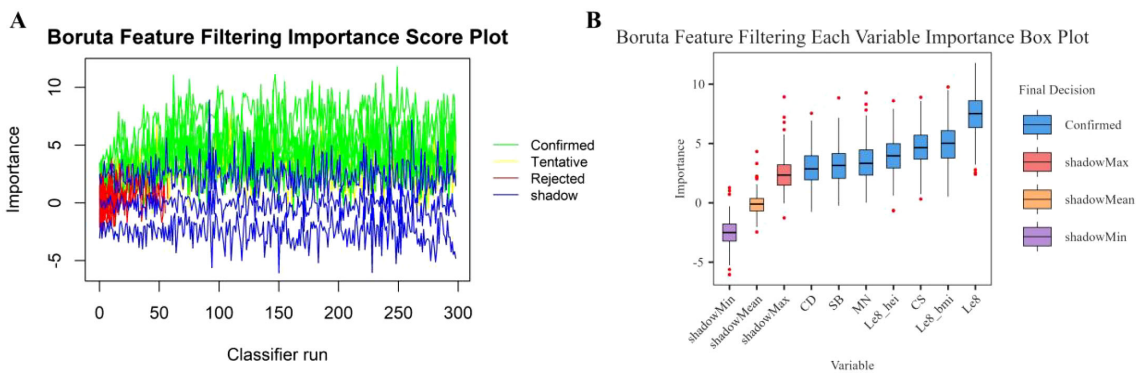


FIGURE 13
Boruta feature screening plot. **(A)** Importance score plot. **(B)** Importance box plot for each variable.

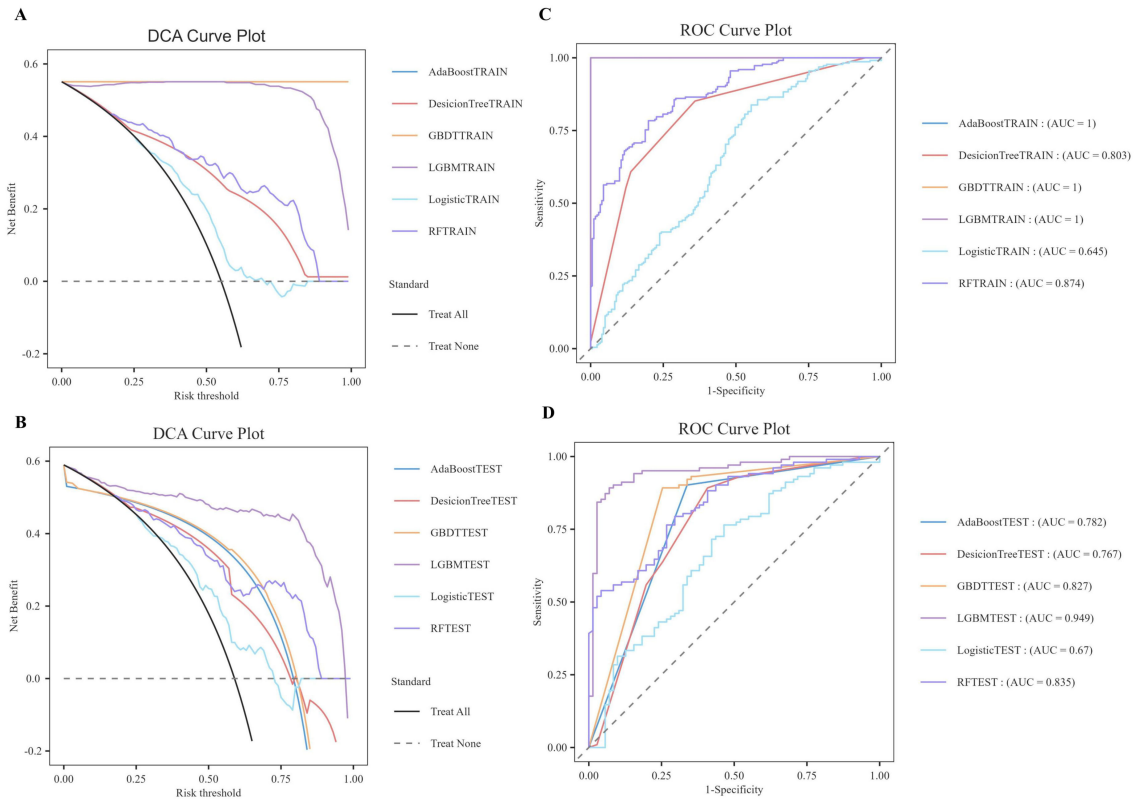


FIGURE 14
DCA/ROC curves of the train and test sets of 6 ML models. **(A)** DCA curves of the train set. **(B)** DCA curves of the test sets. **(C)** ROC curves of the train set. **(D)** ROC curves of the test sets.

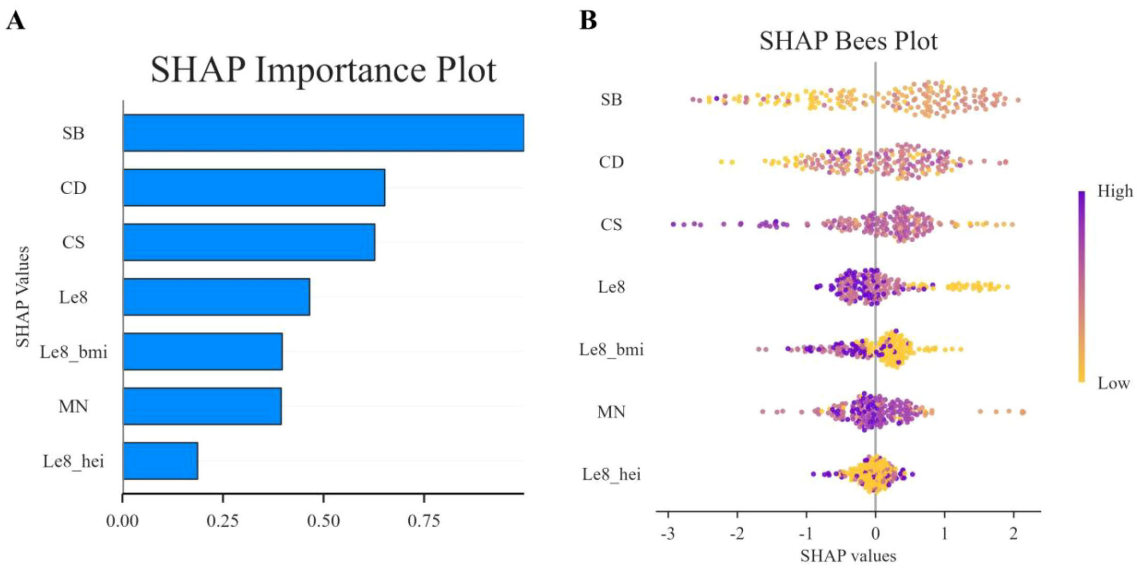


FIGURE 15
**(A)** SHAP column chart. **(B)** SHAP swarm plot.

of the model, ensuring its reliability in diverse datasets and application scenarios.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

## Ethics statement

The studies involving humans were approved by NCHS Ethics Review Board. The parent institution of the NCHS ERB is the National Center for Health Statistics (NCHS), which is part of the Centers for Disease Control and Prevention (CDC). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

## Author contributions

XG: Writing – original draft, Writing – review & editing. QL: Data curation, Methodology, Writing – review & editing. XW: Supervision, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Generative AI statement

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# Appendix

| Abbreviations | Designation |
|---|---|
| LE8 | Life's essential 8 |
| AdaBoost | Adaptive Boosting |
| GBDT | Gradient Boosting Decision Tree |
| LGBM | Light Gradient Boosting Machine |
| AUC | Area Under the Receiver Operating Characteristic Curve |
| MCC | Matthews Correlation Coefficient |
| SHAP | SHapley Additive exPlanations |
| ML | Machine Learning |
| BMI | Body Mass Index |
| NHANES | National Health and Nutrition Examination Survey |

| Indicators description | Designation |
|---|---|
| Le8 bmi | Body Mass Index, BMI |
| Le8 pa | Physical Activity |
| Le8 hei | Diet |
| Le8 sleep | Sleep Health |
| Le8 smoke | Nicotine Exposure |
| Le8 bp | Blood Pressure |
| Le8 glucose | Blood Glucose |
| Le8 non_hdl | Blood Lipids(Non-High-Density Lipoproteinemia) |
| BA | Barium |
| CD | Cadmium |
| CO | Cobalt |
| CS | Cesium |
| MN | Manganese |
| MO | Molybdenum |
| PB | Lead |
| SB | Antimony |
| SN | Tin |
| TL | Thallium |
| TU | Titanium |

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# Machine learning algorithm based on combined clinical indicators for the prediction of infertility and pregnancy loss

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**Background and objectives:** Diagnosis and treatment of infertility and pregnancy loss are complicated by various factors. We aimed to develop a simpler, more efficient system for diagnosing infertility and pregnancy loss.

**Methods:** This study included 333 female patients with infertility and 319 female patients with pregnancy loss, as well as 327 healthy individuals for modeling; 1264 female patients with infertility and 1030 female patients with pregnancy loss, as well as 1059 healthy individuals for validating the models. The average age and basic information were matched between the groups. Three methods were used for screening 100+ clinical indicators, and five machine learning algorithms were used to develop and evaluate diagnostic models based on the most relevant indicators.

**Results:** Multivariate analysis revealed significant differences in several factors between the patients and the control group. 25-hydroxy vitamin D3 (25OHVD3) was the factor exhibiting the most prominent difference, and most patients presented deficiency in the levels of this vitamin. 25OHVD3 is associated with blood lipids, hormones, thyroid function, human papillomavirus infection, hepatitis B infection, sedimentation rate, renal function, coagulation function, and amino acids in patients with infertility. The model for infertility diagnosis included eleven factors and exhibited area under the curve (AUC), sensitivity, and specificity values higher than 0.958, 86.52%, and 91.23%, respectively. The model for potential pregnancy loss was also developed using five machine learning algorithms and was based on 7 indicators. According to the results obtained from the testing set, the sensitivity was higher than 92.02%, the specificity was higher than 95.18%, the accuracy was higher than 94.34%, and the AUC was higher than 0.972.

**Conclusion:** The simplicity, good diagnostic performance, and high sensitivity of the models presented here may facilitate early detection, treatment, and prevention of infertility and pregnancy loss.

KEYWORDS

**infertility, pregnancy loss, machine learning, 25OHVD3, diagnosis**

# 1 Introduction

Infertility is a condition defined as being unable to conceive after having regular unprotected sex for at least one year (1). Previous reports have indicated that 17% of women and 9.4% of men in the USA have used an infertility service (2). Infertility not only affects the mental health of the afflicted individual but is also a potential risk factor for many cancers (3). The causes of female infertility are varied, including physiological, psychological, behavioral, and genetic factors (4). The process of diagnosing female infertility is therefore complex and time-consuming (5). The first step is to evaluate the medical history of the patient and to perform a physical examination, followed by laboratory tests (6). The function of the ovaries, fallopian tubes, and uterus is then evaluated, and the clinician finally makes a diagnosis by combining all of this information and relying on his or her experience (7). It takes at least 1–2 years from trying to conceive for about 1 year to confirm the diagnosis of infertility in the hospital. Therefore, early diagnosis of the condition is essential to shorten the time to successful conception. While imaging plays an indispensable role in assessing anatomic abnormalities, tubal obstruction, and ovarian reserve, it is not suitable for large-scale screening (8). Moreover, the diagnosis and treatment of infertility are complicated by various factors that must be considered (9). Therefore, there is a need to establish a simpler clinical screening index to be used for early prevention and intervention in cases of female infertility.

Pregnancy loss is defined as the natural termination of a pregnancy prior to fetal viability, and it includes spontaneous, missed, and incomplete abortions, as well as a molar pregnancy (10). Pregnancy loss is a common problem among women of childbearing age, with a reported incidence of 10-30% out of all detected pregnancies (11). A failed pregnancy can occur at any stage of pregnancy for a variety of reasons and may be associated with key physiological changes in the embryo, the uterine environment, and the ovaries (12, 13). Women with a history of pregnancy loss have higher rates of psychological conditions and chronic diseases (14). Although most cases of miscarriage are sporadic, some couples experience repeated miscarriages, which is a challenging situation to be addressed clinically (15). The causes of sporadic and recurrent miscarriage are multiple, but the risk of adverse pregnancy outcomes in almost all cases is influenced by prior obstetric history (16). Ultrasound is critical in the assessment and management of pregnancies of unknown location and can be of help in the differential diagnosis of early miscarriage, pregnancy of unknown location, and ectopic pregnancy (17). However, it cannot be used to assess the causes of unexplained pregnancy loss. The diagnostic criteria, treatment, preventive measures, and prediction methods for recurrent pregnancy loss vary globally as there is no international consensus on a definition (11, 18). Although the effects on couples are well documented, pregnancy loss is an understudied disorder with no precise diagnostic model or obvious treatment. Therefore, it is important to develop a precise and simple model to predict pregnancy loss in advance.

In general, the risk of recurrence of sporadic early pregnancy loss is low (approximately 12% to 14%) (15). However, pregnancy outcomes affect the proportion of recurrent miscarriages (19). Women affected by pregnancy loss have a 60% to 70% chance of a successful pregnancy; therefore, pregnancy loss is not the same as infertility (20). However, how to distinguish between these two conditions has rarely been addressed in the literature.

The current application of machine learning (ML) in healthcare highlights the potential to enhance disease diagnosis and clinical care, thus achieving early warning, improving patient outcomes, and increasing the diagnostic efficiency of clinicians (21, 22). The detection of laboratory indicators has made a significant contribution to disease diagnosis, and their synergy with ML algorithms can provide superior diagnostic accuracy and reduce false positives (23). However, its adoption in clinical practice for the diagnosis of infertility and pregnancy loss has not yet been realized, and the evaluation of ML-based diagnostic technologies in terms of infertility and pregnancy loss outcomes remains an ongoing endeavor.

The current definition of infertility acknowledges the importance of the total amount of time during which the patient has sought to become pregnant and the negative impact of age. To reduce the time to intervention and improve prognosis, the present study aimed to establish a simple and efficient method that provides early warning of infertility and potential pregnancy loss. We also systematically investigated the effect of 25-hydroxy vitamin D3 (25OHVD3) on infertility and pregnancy loss and whether the combination of 25OHVD3 and other clinical indicators can aid in the diagnosis of these conditions.

# 2 Materials and methods

## 2.1 Sample collection

In this study, we collected data from female patients who visited Xijing Hospital (Xi'an, China) from January 1, 2022, to June 1, 2023. All patients underwent medical history evaluation and physical examination, as well as clinical laboratory and ultrasound tests. All the included patients were diagnosed by gynecologists and infertility specialists and had a clear diagnosis that followed the appropriate guidelines (24–26). The participants were divided into two groups (333 patients diagnosed with infertility and 319 patients diagnosed with failed pregnancy). In addition, a third group (control) of 327 age-matched healthy women was included in the study. The first group of patients included cases of infertility related to conditions in the fallopian tubes, cervix, uterus, and ovaries, as well as cases in which the cause of infertility was unknown. The second group included patients with a history of abortion or ectopic pregnancy but who had not been diagnosed with infertility. The inclusion and exclusion criteria, as well as a flow chart for patient recruitment, are shown in Figure 1. To assess the validity of our model, we also collected data from 2,294 female patients treated at the Fertility and Infertility Center of Xijing Hospital from January 1,

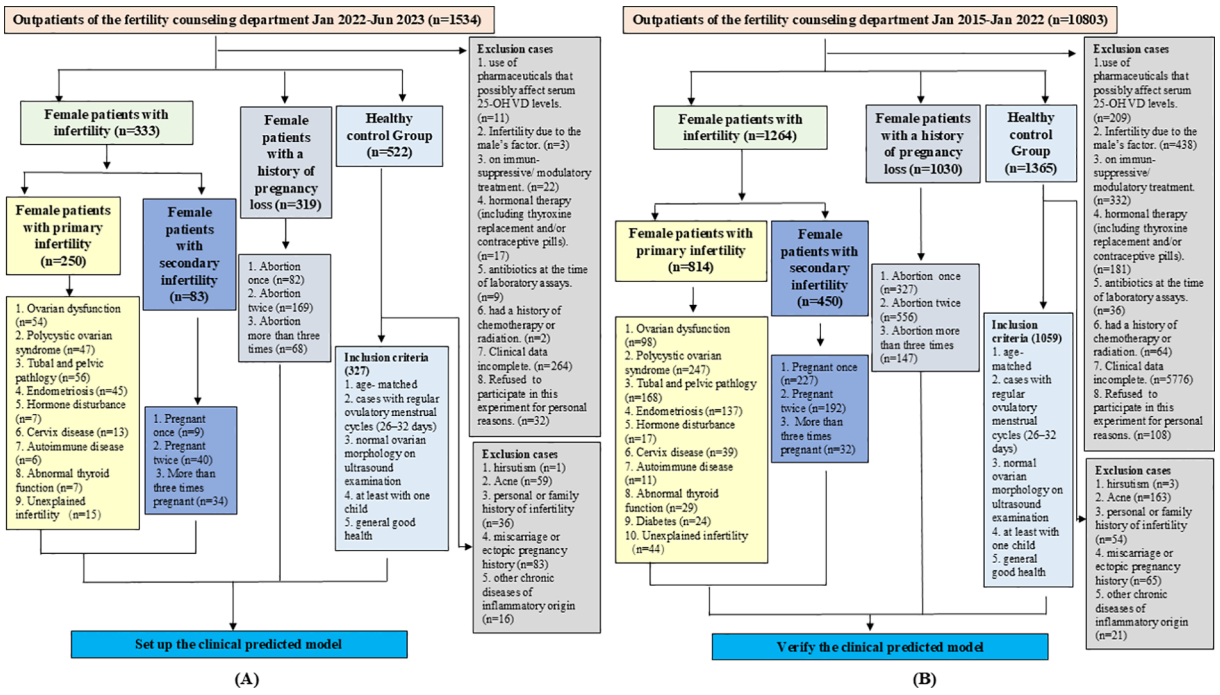


FIGURE 1
Patient inclusion and exclusion criteria, and flowchart for recruitment. **(A)** Flowchart for recruiting patients to establish diagnostic models. **(B)** Flowchart for recruiting patients to verify the diagnostic models.

2015, to January 1, 2022, with a clear diagnosis of infertility (1264) and pregnancy loss (1030), as well as from 1059 age-matched healthy women. The research protocol was approved by the Ethics Committee of Xijing Hospital (KY20212027-C-1), and informed consent was obtained via telephone interviews because of the retrospective nature of the study.

## 2.2 Data collection

Serum levels of 25OHVD3 and 25-hydroxy vitamin D2 (25OHVD2) were analyzed using high performance liquid chromatography-mass spectrometry (HPLC-MS/MS). All the laboratory tests were analyzed by the Clinical Laboratory Department of Xijing Hospital, and the results were stored in the Laboratory Information System (LIS). All consultation and basic information were obtained from the Hospital Information System, LIS, and follow-up telephone interviews. All the data were collected by more than three independent technicians and statisticians in accordance with the standardization requirements. All electronic data involved in this study were stored on independent USB drives. The USB flash drive and paper document information were stored in a confidential cabinet, which had dual locks and the keys were independently kept by two fixed individuals. USB flash drives could only be used on designated, protected, and confidential computers. The confidential cabinet and confidential computer were managed and protected by dedicated personnel to ensure that the data and information of participants were not leaked. The data included basic patient information, demographic information, physical examination results, diagnosis, infertility period, smoking status, alcohol consumption, and other information (Table 1).

## 2.3 Sample pretreatment for 25OHVD2 and 25OHVD3 detection

For HPLC-MS/MS detection of 25OHVD2 and 25OHVD3, 500 µL of internal standard solution was added to 100 µL of serum, following which the homogeneous solution was shaken and mixed for 1 min and centrifuged at 15,000 rpm for 10 min. The supernatant was then transferred for N2 drying. For the derivatization reaction, a 4-phenyl-1,2,4-triazoline-3,5-dione solution was added to the drying sample and incubated at 25°C for 30 min. The derivatization solution was subjected to N2 drying, following which 50 µL of methanol was added, mixed for 1 min, and centrifuged at 15,000 rpm for 10 min at 25°C. The supernatant was prepared for HPLC-MS/MS detection.

## 2.4 HPLC-MS/MS analysis of 25OHVD2 and 25OHVD3

In this study, 25OHVD2 and 25OHVD3 levels were analyzed using an HPLC-MS/MS system equipped with an Agilent 1200 HPLC system (Agilent1200, Waldbronn, Germany) and an API 3200 QTRAP MS/MS system (Sciex, Darmstadt, Germany). Mobile phase A consisted of an aqueous solution containing 1% formic acid and 1% ammonium formate, while phase B consisted of a methanol solution containing 1% formic acid and 1% ammonium formate.

TABLE 1 Patient characteristics.

| Variables | Female controls (n=327) | Female patients with pregnancy loss (n=319) | Female patients with infertility (n=333) |
|---|---|---|---|
| Body Mass Index ($kg/m^2$) | 24.75 ± 4.12 | 25.37 ± 3.61 | 24.13 ± 3.26 |
| Smoking History (%) | 3.77 | 6.06 | 6.73 |
| Alcohol History (%) | 1.89 | 3.13 | 3.00 |
| Diabetic (%) | 0.00 | 6.06 | 5.77 |
| Hypertension (%) | 0.00 | 6.67 | 11.54 |
| Regular Sporting (%) | 7.55 | 27.27 | 9.62 |

The optimized gradient elution was operated at a flow rate of 0.6 ml/min: 0 to 0.1 min, 70% B; 0.1 to 0.6 min, 70–95% B; 0.6 to 3.1 min, 95% B; and 3.1 to 4.0 min, 95–70% B. An autosampler was set to inject 20 µL at each step. MS data were detected via electrospray ionization (ESI) in a positive ion mode, and the remaining parameters were as follows: multiple reaction monitor scan type; collection ion pair: 25(OH)$VD_2$: 619.3/298.3, d3-25(OH)$VD_2$: 622.3/301.3, 25(OH)$VD_3$: 607.3/298.3, d3-25(OH)$VD_3$: 610.3/301.3; ion spray voltage, 5.5kV; ion source temperature, 600°C; curtain gas (CUR), 40.0 psi; nebulizer gas (GS1), 55.0 psi; declustering potential (DP), 40 V; entrance potential (EP), 4.0 V; collision energy (CE), 27; and collision cell exit potential (CXP), 3.0.

## 2.5 Feature selection and establishment of the diagnostic models

100+ clinical indicators were listed in Supplementary Table S1. All the missing values were supplemented by mean values. All data had not been normalized. Spearman correlation, recursive feature elimination (REF), and mutual information (MI)) were selected as methods of feature selection for the model of diagnosis. Spearman correlation analysis is used to assess the monotonic relationship between two continuous or ordered variables (27). It is used to characterize the correlation between two variables that have ordinal or distributional characteristics that cannot be described in terms of mean and standard deviation. MI is a metric that quantifies the dependence and relationship between two variables and represents the amount of information provided by one probabilistic variable about the other (28). RFE is the main representative of wrap-around feature selection, which brings classification algorithms into the process of feature selection to eliminate redundancy between features and output the best combination of features (29). To establish effective diagnostic models, thirty indicators with the highest contribution values were screened and cross-validated using these three methods. Common indicators were used to build the model. The selected features will vary with the number of samples and different screening methods, so we use multiple ML algorithms and metrics to simultaneously establish, validate, and evaluate our diagnostic model. Gaussian naive bayes (GNB), K-nearest neighbors (KNN), decision tree (DT), logistic regression (LR), and eXtreme gradient boosting (XGBoost) were used to develop and evaluate diagnostic models based on the common indicators. The performance of our model was improved by using a ten-fold crossover (train: 9, test:1, random allocation). External datasets were independently validated to enhance the generalizability of the models.

## 2.6 Statistical analysis

SPSS 23.0 (IBM, Armonk, NY, USA) was used for data analysis. Quantitative data are expressed as mean ± standard deviation. Group comparisons were made using the chi-square test for categorical variables and one-way analysis of variance (ANOVA), and the Kruskal-Wallis test for continuous variables. The multiple comparisons using the Bonferroni correction to reduce the risk of Type I error, and statistical significance was set at $p<0.05/N$, where N represents the number of comparisons. R Software (version 3.6.2, R Statistical Computing Project) was used for data visualization. The Python language was used for indicator screening and for diagnostic model building and evaluation. Orthogonal partial least squares discriminant analysis (OPLS-DA) was performed to determine overall differences between the groups. Risk factors for infertility and pregnancy loss were evaluated using logistic regression analysis. The diagnostic performance of the model was analyzed using receiver operating characteristic (ROC) curves.

# 3 Results

## 3.1 Patient characteristics

As shown in Figure 1, a total of 1534 patients were initially included in this study for modeling, and 360 were initially excluded according to the exclusion criteria. A total of 522 women were initially included in the healthy control group, with 195 excluded according to the telephone follow-up results, and the remaining 327 were finally enrolled. The average ages of patients with infertility (n=333), those with pregnancy loss (n=319), and healthy individuals (n=327) were 30.40 ± 14.72, 30.71 ± 14.10, and 31.15 ± 14.83 years, respectively, with no significant differences among the groups. In the infertility group, 75.08% of the patients had primary infertility and 24.92% had secondary infertility. In the secondary infertility group, 10.85% of the patients had been pregnant once, 48.19% had been pregnant twice, and 40.96% had been pregnant three times or more. In the pregnancy loss group, 25.71% of the patients had experienced an abortion once, 52.98% had experienced it twice, and 21.31% had experienced it three times or more. The main characteristics of each group are presented in Figure 1A and Table 1. Drinking and smoking were more common in the infertility group compared to the control group. The clinical indicators of each group are also presented in

Supplementary Table S1. The basic information, inclusion and exclusion criteria for the 2294 patients and 1059 healthy individuals included in the validation set were also shown in Figure 1B.

## 3.2 Distribution of measured indicators in each group

As shown in Supplementary Figure S1A, the overall difference in 100+ clinical indicators clearly distinguished the infertility group from the normal control group. Among female patients, significant differences in the levels of the following indicators were observed between the group of patients with infertility and the control group: 25OHVD2, 25OHVD3, prothrombin time (PT), luteinizing hormone (LH), erythrocyte sedimentation rate (ESR), hyaline cast (Hy. CAST), high-density lipoprotein (HDL-C), thrombin time (TT), anticardiolipin antibodies (ACA), creatinine (CRE), homocysteine (HCY), mucous strands (MUCUS), NonSEC, mean corpuscular hemoglobin concentration (MCHC), triglyceride (TG), progesterone (PROG), estradiol (E2), urea (BUN), urinary epithelial cells (EC), gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST), red blood cells (RBC), cystatin C (CysC), and pathocast (Path CAST) (VIP>1.00; Supplementary Figure S1B). Notably, 25OHVD3 was the indicator exhibiting the biggest differences between these two groups.

Similarly, an overall difference of 100+ clinical markers clearly distinguished the pregnancy loss group from the normal control group (Supplementary Figure S1C). Fourteen clinical indicators, including anti-thyroid peroxidase antibody (TPOAb), monocytes (MONO), neutrophilic granulocytes (NEUT), eosinophils (EO), human papillomavirus 59 (HPV59), red blood cell distribution width (RDW), Path CAST, red blood cell specific volume (HCT), free thyroxine 4 (FT4), human papillomavirus 81 (HPV 81), urine potential of hydrogen (UPH), albumin (ALB), basophils (BASO), and alanine aminotransferase (ALT), were significantly different between the group of patients with pregnancy loss and the control group (Supplementary Figure S1D).

## 3.3 Distribution of 25OHVD levels in each group

Levels above 30, 20–30, and below 20 ng/ml are regarded as normal, inadequate, and deficient, respectively (30). The study participants were divided into three groups according to their 25OHVD levels. The percentage of patients included in each group is shown in Figure 2. Deficiency, insufficiency, and sufficiency in 25OHVD were observed in 75.68%, 18.32%, and 6.01% of the patients with pregnancy loss, respectively (Figure 2A). Among the patients with infertility, the rates of 25OHVD deficiency, insufficiency, and sufficiency were 81.19%, 14.42%, and 4.39%, respectively (Figure 2B). Remarkably, we found that although 85% of the patients with infertility, 75% of the patients with pregnancy loss, and 61% of the healthy women in the control group had been supplemented with vitamin D (Figure 2C), the concentration of 25OHVD3 analyzed using

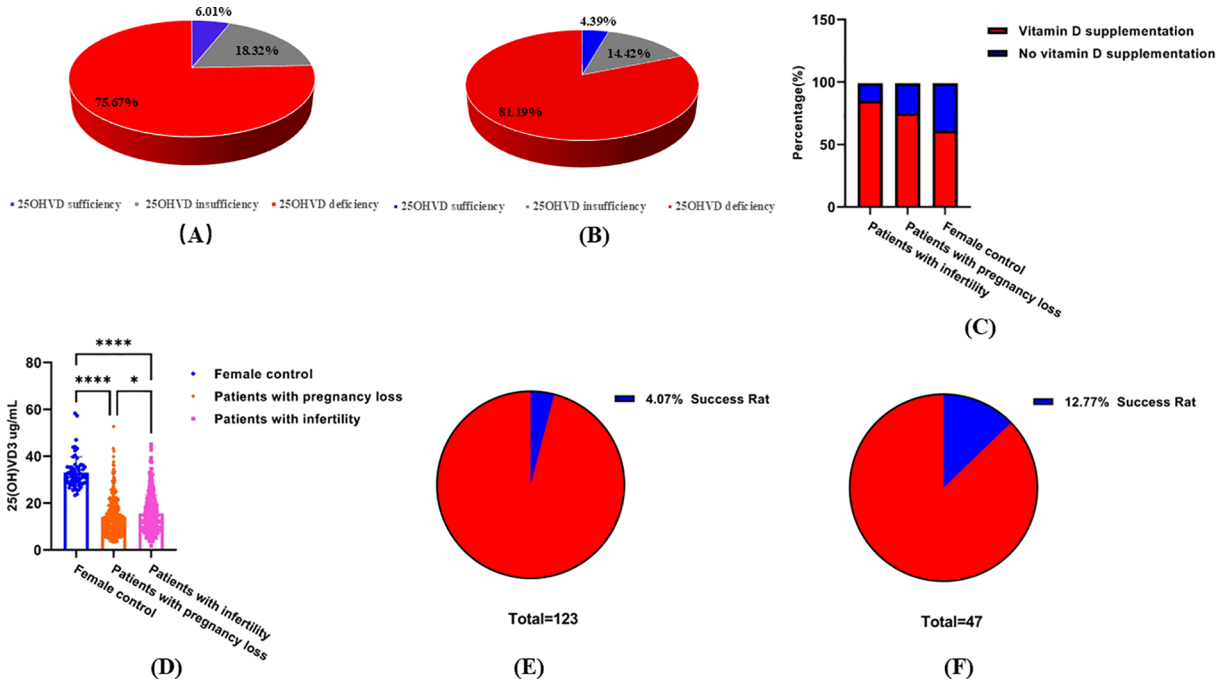


FIGURE 2
Serum 25OHVD3 levels. **(A)** Deficiency, insufficiency, and sufficiency percentage in 25OHVD of the patients with infertility. **(B)** Deficiency, insufficiency, and sufficiency percentage in 25OHVD of the patients with pregnancy loss. **(C)** supplementation of female groups. **(D)** Vitamin D concentration in different study groups. *, $p < 0.05$; ****, $p < 0.0001$. **(E)** successful pregnancy rate of patients with infertility (25OHVD3<20ng/mL) choosing *in vitro* fertilization technology. **(F)** successful pregnancy rate of patients with infertility (25OHVD3>20ng/mL) choosing *in vitro* fertilization technology.

ANOVA in the first two groups was much lower than that observed in healthy individuals (Figure 2D). Although we did not find a dose-response relationship between vitamin D level categories and infertility risk, our results showed that 48.84% of patients with infertility (25OHVD3<20ng/mL) chose *in vitro* fertilization technology, but only 4.07% of the patients successfully became pregnant (Figure 2E). In contrast, 57.32% of patients with infertility (25OHVD3>20ng/mL) chose *in vitro* fertilization technology, but 12.77% of the patients successfully became pregnant (Figure 2F).

## 3.4 Model development and diagnostic performance

Eleven indicators were selected via three methods (Spearman, REF, MI) as candidates for the model of infertility diagnosis: High-density lipoprotein (HDL), TG, 25OHVD3, PT, ACA, 25OHVD, HCY, urine bacterial count (BACT), TPOAb, E2, and hepatitis B core antibody (Anti-HBc) (Figure 3A). Five ML algorithms were used to establish and evaluate the model based on these 11 indicators. The results showed that the sensitivity for the training set was higher than 86.52%, the specificity was higher than 91.23%, the accuracy was higher than 89.70%, and the area under the curve (AUC) of the ROC was higher than 0.958 (Figure 3B; Table 2. The sensitivity for the testing set was higher than 81.81%, the specificity was higher than 88.08%, the accuracy was higher than 84.70%, and the AUC of the ROC was higher than 0.928 (Figure 3C; Table 2). The sensitivity for the validation set was higher than 74.81%, the specificity was higher than 84.00%, the accuracy was higher than 79.00%, and the AUC of the ROC was higher than 0.825 (Figure 3D; Table 2). XGboost performed the best by comparing the results of training sets, test sets, and validation sets in these five ML. Learning curve could illustrate the impact of the number of training samples on the model performance (Figure 3E). The results indicated that the ML algorithm used in this study did not exhibit overfitting or underfitting. The model has basically reached the performance platform and does not require additional data for further training. The SHAP model of the eleven indicators in the diagnostic model revealed that 25OHVD and 25OHVD3 contributed the most, followed by E2, Anti-HBc, TPOAb, HCY, TG, HDL, BACT, ACA, and PT (Figure 3F).

Seven indicators were selected via the three methods as candidates for the model of pregnancy loss: EC, TPOAb, HDL, testosterone (TESTO), 25OHVD3, PT, and 25OHVD (Figure 4A). Five ML algorithms were used to evaluate the model based on these seven indicators. The results showed that the sensitivity for the training set was higher than 92.02%, the specificity was higher than 95.18%, the accuracy was higher than 94.34%, and the AUC of the ROC curve was higher than 0.972 (Figure 4B; Table 2). The sensitivity for the testing set was higher than 90.78%, the specificity was higher than 88.02%, the accuracy was higher than 92.88%, and the AUC of the ROC curve was higher than 0.948 (Figure 4C; Table 2). The sensitivity for the validation set was higher than 87.25%, the specificity was higher than 86.88%, the accuracy was higher than 90.65%, and the AUC of the ROC was higher than 0.900 (Figure 4D; Table 2). XGboost performed the best by

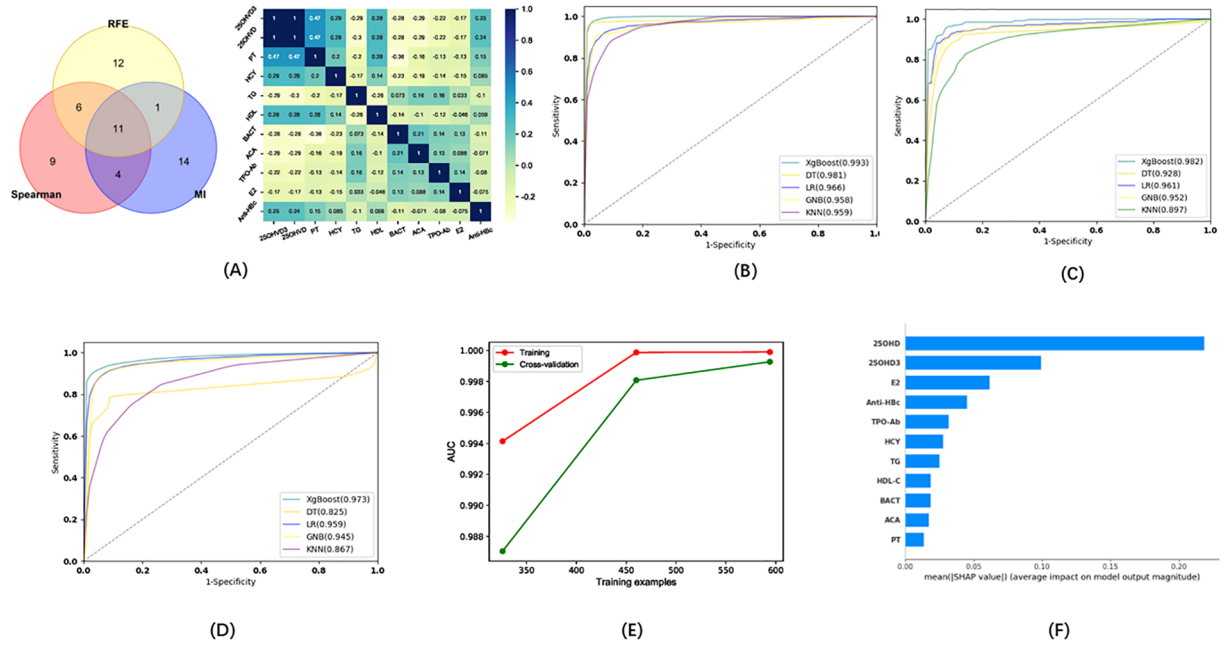


FIGURE 3
**(A)** Wayne diagrams for the MI, REF, and Spearman methods used to screen candidates for differentiating patients with infertility from healthy individuals. Hotspot map of candidate indicators for this differentiation. **(B)** Receiver operating characteristic (ROC) curve for the training set in the model for infertility diagnosis. **(C)** ROC curve for the test set in the model for infertility diagnosis. **(D)** ROC curve for the validation set in the model for infertility diagnosis. **(E)** Learning curve for the training set in the model for infertility diagnosis. **(F)** SHAP model for 11 indicators of the model for infertility diagnosis.

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TABLE 2 Evaluation of the model for infertility and control, pregnancy loss and control, infertility and pregnancy loss.

| Model | Algorithms | Set | Sensitivity | Specificity | Accuracy | Area under the curve |
|---|---|---|---|---|---|---|
| Infertility and Control | XgBoost | Train | 96.60% (95.19%-98.01%) | 94.41% (92.60%-96.22%) | 92.33% (90.20%-94.46%) | 0.993 (0.987-0.999) |
| | | Test | 92.33% (90.20%-94.46%) | 95.84% (94.28%-97.40%) | 94.09% (92.22%-95.96%) | 0.982 (0.972-0.992) |
| | | Verify | 90.22% (88.97%-91.47%) | 96.92% (96.23%-97.61%) | 93.28% (92.25%-94.31%) | 0.973 (0.967-0.979) |
| | DT | Train | 96.03% (94.50%-97.56%) | 98.54% (97.62%-99.46%) | 97.27% (96.01%-98.53%) | 0.981 (0.970-0.992) |
| | | Test | 91.07% (88.77%-93.37%) | 94.01% (92.13%-95.89%) | 92.27% (90.13%-94.41%) | 0.928 (0.907-0.949) |
| | | Verify | 79.04% (77.23%-80.85%) | 89.89% (88.62%-91.16%) | 83.99% (82.40%-85.58%) | 0.825 (0.808-0.842) |
| | Knn | Train | 88.18% (85.54%-90.82%) | 91.23% (88.96%-93.50%) | 89.70% (87.24%-92.16%) | 0.959 (0.943-0.975) |
| | | Test | 81.81% (78.58%-85.04%) | 88.08% (85.43%-90.73%) | 84.70% (81.72%-87.68%) | 0.987 (0.872-0.922) |
| | | Verify | 74.81% (72.85%-76.77%) | 84.00% (82.41%-85.59%) | 79.00% (77.19%-80.81%) | 0.867 (0.852-0.882) |
| | LR | Train | 90.36% (87.98%-92.74%) | 94.32% (92.49%-96.15%) | 92.32% (90.19%-94.45%) | 0.966 (0.952-0.980) |
| | | Test | 89.93% (87.49%-92.37%) | 93.44% (91.47%-95.41%) | 91.52% (89.28%-93.76%) | 0.961 (0.946-0.976) |
| | | Verify | 89.66% (88.38%-90.94%) | 93.27% (92.24%-94.30%) | 91.30% (90.12%-92.48%) | 0.959 (0.951-0.967) |
| | GNB | Train | 86.52% (83.71%-89.33%) | 96.94% (95.60%-98.28%) | 91.68% (89.46%-93.90%) | 0.958 (0.942-0.974) |
| | | Test | 86.24% (83.40%-89.08%) | 96.68% (95.28%-98.08%) | 91.36% (89.10%-93.62%) | 0.952 (0.935-0.969) |
| | | Verify | 83.81% (82.21%-85.41%) | 96.38% (95.63%-97.13%) | 89.54% (88.25%-90.83%) | 0.945 (0.936-0.954) |
| Pregnancy Loss and Control | XgBoost | Train | 96.45% (94.98%-97.92%) | 98.20% (97.15%-99.25%) | 97.33% (96.05%-98.61%) | 0.993 (0.986-1.000) |
| | | Test | 94.42% (92.57%-96.27%) | 96.83% (95.44%-98.22%) | 95.81% (94.21%-97.41%) | 0.987 (0.978-0.996) |
| | | Verify | 94.02% (92.96%-95.08%) | 95.13% (94.17%-96.09%) | 94.58% (93.57%-95.59%) | 0.973 (0.966-0.980) |
| | DT | Train | 97.04% (95.70%-98.38%) | 99.29% (98.63%-99.95%) | 98.18% (97.13%-99.23%) | 0.985 (0.975-0.995) |
| | | Test | 94.53% (92.70%-96.36%) | 96.62% (95.18%-98.06%) | 95.66% (94.03%-97.29%) | 0.959 (0.943-0.975) |
| | | Verify | 94.51% (93.49%-95.53%) | 86.88% (85.31%-88.45%) | 90.65% (89.32%-91.98%) | 0.900 (0.886-0.914) |
| | Knn | Train | 93.34% (91.32%-95.36%) | 95.31% (93.62%-97.00%) | 94.34% (92.48%-96.20%) | 0.987 (0.978-0.996) |
| | | Test | 90.78% (88.41%-93.15%) | 94.95% (93.19%-96.71%) | 92.88% (90.79%-94.97%) | 0.964 (0.949-0.979) |
| | | Verify | 87.25% (85.70%-88.80%) | 94.65% (93.64%-95.66%) | 91.00% (89.70%-92.30%) | 0.948 (0.938-0.958) |
| | LR | Train | 92.02% (89.81%-94.23%) | 96.94% (95.57%-98.31%) | 94.51% (92.68%-96.34%) | 0.972 (0.959-0.985) |
| | | Test | 98.55% (97.61%-99.49%) | 88.02% (85.32%-90.72%) | 96.89% (95.51%-98.27%) | 0.966 (0.952-0.980) |
| | | Verify | 91.15% (89.86%-92.44%) | 96.14% (95.29%-96.99%) | 93.96% (92.89%-95.03%) | 0.931 (0.920-0.942) |
| | GNB | Train | 93.87% (91.93%-95.81%) | 95.18% (93.46%-96.90%) | 94.53% (92.70%-96.36%) | 0.977 (0.965-0.989) |
| | | Test | 93.73% (91.77%-95.69%) | 94.90% (93.13%-96.67%) | 94.43% (92.58%-96.28%) | 0.973 (0.960-0.986) |
| | | Verify | 90.68% (89.35%-92.01%) | 93.05% (91.90%-94.20%) | 91.88% (90.64%-93.12%) | 0.959 (0.950-0.968) |
| Infertility and Pregnancy Loss | XgBoost | Train | 88.58% (85.98%-91.18%) | 91.91% (89.72%-94.10%) | 90.22% (87.81%-92.63%) | 0.956(0.940-0.972) |
| | | Test | 84.85% (81.87%-87.83%) | 86.09% (83.23%-88.95%) | 85.28% (82.34%-88.22%) | 0.925(0.904-0.946) |
| | | Verify | 85.32% (83.79%-86.85%) | 85.51% (83.99%-87.03%) | 85.41% (83.88%-86.94%) | 0.928 (0.917-0.939) |
| | DT | Train | 77.05% (73.46%-80.64%) | 91.52% (89.28%-93.76%) | 84.12% (81.08%-87.16%) | 0.903(0.879-0.927) |
| | | Test | 69.74% (65.75%-73.73%) | 86.38% (83.55%-89.21%) | 77.93% (74.40%-81.46%) | 0.810(0.777-0.843) |
| | | Verify | 69.30% (67.18%-71.42%) | 82.63% (80.97%-84.29%) | 75.29% (73.34%-77.24%) | 0.795 (0.917-0.939) |
| | Knn | Train | 70.83% (66.89%-74.77%) | 91.11% (88.82%-93.40%) | 80.76% (77.44%-84.08%) | 0.905(0.881-0.929) |
| | | Test | 58.74% (54.39%-63.09%) | 83.19% (80.06%-86.32%) | 70.56% (66.61%-74.51%) | 0.767(0.731-0.803) |

*(Continued)*

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TABLE 2 Continued

| Model | Algorithms | Set | Sensitivity | Specificity | Accuracy | Area under the curve |
|---|---|---|---|---|---|---|
| | | Verify | 55.14% (52.79%-57.49%) | 83.71% (82.10%-85.32%) | 67.97% (65.81%-70.13%) | 0.761 (0.742-0.780) |
| | LR | Train | 74.93% (71.21%-78.65%) | 80.6% (77.26%-83.94%) | 77.71% (74.17%-81.25%) | 0.846 (0.816-0.876) |
| | | Test | 73.90% (70.12%-77.68%) | 80.10% (76.73%-83.47%) | 76.85% (73.25%-80.45%) | 0.838 (0.807-0.869) |
| | | Verify | 76.05% (74.12%-77.98%) | 79.92% (78.14%-81.70%) | 77.79% (75.93%-79.65%) | 0.852 (0.837-0.867) |
| | GNB | Train | 54.05% (49.63%-58.47%) | 96.86% (95.50%-98.22%) | 75% (71.28%-78.72%) | 0.806 (0.773-0.839) |
| | | Test | 52.58% (48.15%-57.01%) | 96.02% (94.49%-97.55%) | 73.77% (69.98%-77.56%) | 0.798(0.764-0.832) |
| | | Verify | 52.93% (50.56%-55.30%) | 91.99% (90.86%-93.12%) | 70.47% (68.38%-72.56%) | 0.796 (0.778-0.814) |

comparing the results of training sets, test sets, and validation sets in these five ML algorithms. The results of the learning curve indicated that the ML algorithm used in this study did not exhibit overfitting or underfitting (Figure 4E). The model had basically reached the performance platform and did not require additional data for further training. The SHAP model of the 7 indicators in the diagnostic model revealed that 25OHVD3 and 25OHVD contributed the most, followed by TESTO, HDL, PT, TPOAb, and EC (Figure 4F).

In addition, we tried to develop a model capable of distinguishing between patients with infertility and those with predicted pregnancy loss. Eight indicators (E2, LDL, Non SEC, BU, ACA, Antiβ2-G1, FSH, LH) were selected via the three methods as candidates for this model (Figure 5A). Five ML algorithms were used to evaluate the diagnostic model based on these eight indicators. The results showed that the sensitivity for the training set was higher than 54.05%, the specificity was higher than 80.60%, the accuracy was higher than 75.00%, and the AUC of the ROC was higher than 0.767 (Figure 5B; Table 2). The sensitivity for the testing set was higher than 52.58%, the specificity was higher than 80.10%, the accuracy was higher than 70.56%, and the AUC of the ROC was higher than 0.806 (Figure 5C; Table 2). The sensitivity for the validation set was higher than 52.93%, the specificity was higher than 79.92%, the accuracy was higher than 67.97%, and the AUC of the ROC was higher than 0.761 (Figure 5D; Table 2). XGboost performed the best by comparing the results of training sets, test sets, and validation sets in these five ML algorithms. The results of the learning curve indicated that the ML algorithm used in this study did not exhibit overfitting or underfitting (Figure 5E). The model had basically reached the performance platform and did not require additional data for further training. The SHAP model of the eight indicators in the diagnostic model revealed that LDL and Non SEC contributed the most, followed by FSH, LH, ACA, BU, Antiβ2-G1, and E2 (Figure 5F).

## 3.5 Significantly different indicators in infertility risk factor assessment

To identify potential indicators of infertility risk, we used a binary logistic regression analysis to assess the relationship between these indicators and infertility. As shown in Supplementary Table S2, ESR60M, HDL, PT, 25OHVD3, LH, TT, CysC, ACA, HCY, CRE, MCHC, GGT, and MUCUS were statistically significant risk factors for female infertility.

25OHVD3 exhibited the most marked difference in cases of infertility. To investigate its role in the occurrence and development of infertility, we also looked at its correlation with a variety of clinical indicators. Intriguingly, 25OHVD3 was correlated with HPV31, HPV35, HPV26, ESR, thyroglobulin (TgZ), T4, E2, TG, Anti-HBc, HCY, BASO, CRE, and PROG (Figure 6A). 25OHVD2 was also correlated with HPV45, HPV55, HPV56, platelet (PLT), mean corpuscular hemoglobin (MCH), and prolactin (PRL) (Figure 6A). In patients with pregnancy loss, 25OHVD3 was not significantly associated with any of these clinical markers (Figure 6B). 25OHVD2 was significantly correlated with E2 and PROG (Figure 6B).

# 4 Discussion

Many couples expecting to become pregnant struggle with infertility, the risk of which is reported to be equal for male and female patients (31). The causes of infertility are multiple (32). Previous reports have indicated that the causes of infertility are unknown in approximately 30% of infertile couples (33). The age of the female partner is among the factors that have been associated with unexplained infertility (34). Given the multitude of factors that must be considered, developing a system that can aid in the efficient, early, and accurate diagnosis of infertility remains both necessary and clinically challenging (35).

Our analyses identified positive correlations between the levels of BUN, ACA, HCY, MCHC, GGT, EC, TG, E2, CEA, and AST and female infertility, while negative correlations were observed between those of 25OHVD3, ESR60M, HDL-C, PT, TT, LH, CysC, CRE, MUCUS, and globulin and female infertility. Our results further indicate that HPV infection, abnormal coagulation function, thyroid dysfunction, abnormal blood lipid metabolism, 25OHVD deficiency or insufficiency, anemia, and abnormal liver function were risk factors for miscarriage and infertility. Over the past decade, our understanding regarding the benefits of vitamin D has improved significantly, particularly with regard to its non-

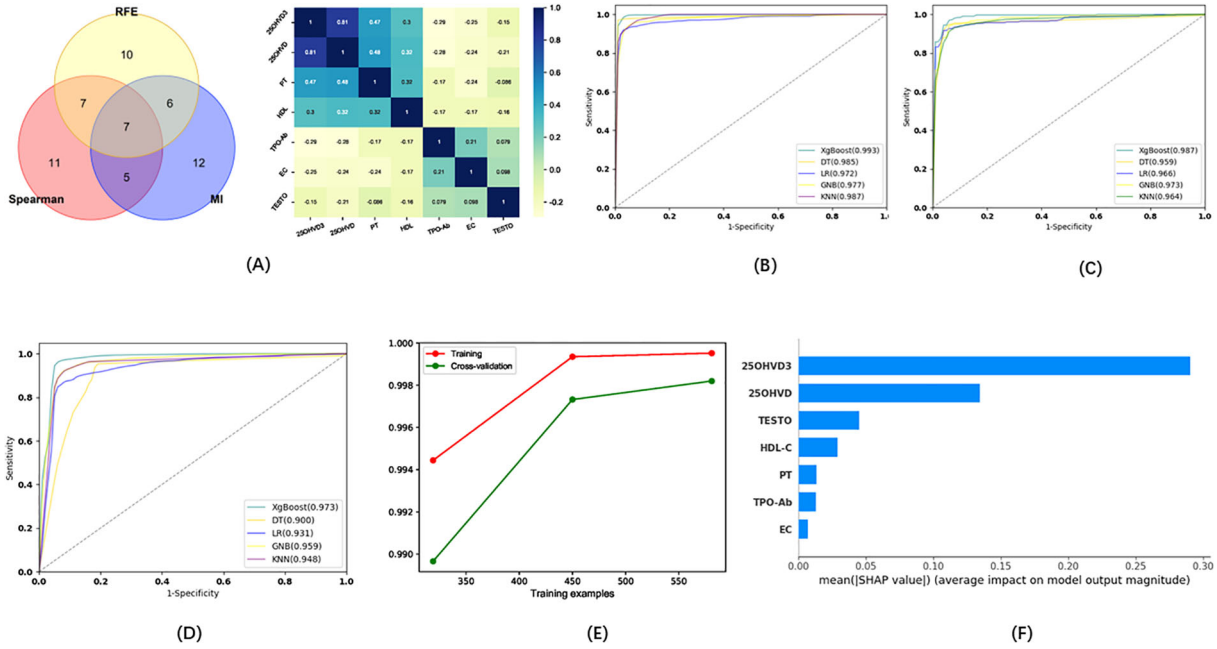


FIGURE 4
**(A)** Wayne diagrams for MI, REF, and SPEARSON methods used to screen candidates for differentiating patients with pregnancy loss from healthy individuals. Hotspot map of candidate indicators for this differentiation. **(B)** ROC curve for the training set in the model for the prediction of pregnancy loss. **(C)** ROC curve for the test set in the model for the prediction of pregnancy loss. **(D)** ROC curve for the validation set in the model for the prediction of pregnancy loss. **(E)** Learning curve for the training set in the model for the prediction of pregnancy loss. **(F)** SHAP model for 7 indicators of the model for the prediction of pregnancy loss.

skeletal functions (36). The vitamin D receptor (VDR) is expressed in most organs, suggesting that the roles of vitamin D extend beyond its functions in regulating calcium homeostasis and bone health (37). Numerous studies have reported associations between poor vitamin D status and cancer, allergies, immune disorders, cardiovascular metabolic diseases, irritable bowel syndrome, autism, muscle function, and brain function (36, 38). Given the numerous reports on the effects of vitamin D on other systems in the body, recent research has also focused on its role in human fertility (39). Vitamin D (cholecalciferol) has no biological activity; it must be activated by 25-hydroxylation in the liver, which converts cholecalciferol to the main circulating metabolite, 25-hydroxyvitamin D (25OHVD) (40, 41). Renal 1a-hydroxylase then converts 25OHVD to an active metabolite, $1,25(OH)_2D$, which binds to and activates VDR (42). The best method for assessing vitamin D status is to measure the serum concentration of 25OHVD, as it has a longer cyclic half-life and higher serum concentration than $1,25(OH)_2D$ (43). The role of immunity in infertility and miscarriage has been demonstrated (44). Vitamin D signaling can regulate a variety of immune responses by regulating the differentiation and cycle of T cells, B cells, neutrophils, DC cells and other immune cells (45, 46). In addition, the enzyme CYP27B1, which produces the vitamin D hormone form $1,25(OH)_2D$, are expressed throughout the immune system. Notably, CYP27B1 expression in immune cells is independent of calcium homeostatic inputs (46). The importance of $1,25(OH)_2D$ signaling in the regulation of the immune system is further emphasized by the numerous signaling pathways that control CYP27B1 expression in various immune cell types (47). In addition, vitamin D activates autophagy in a variety of cell types, including keratinocytes, hepatocytes, and endothelial cells, in response to cellular injury and oxidative stress (48). Therefore, we propose the hypothesis that vitamin D signaling could affect infertility and miscarriage by modulating immunity to influence the number of mature oocytes and the rate of blastocyst formation. In addition, Kinuta et al. found that VDR deficient mutant mice exhibited significant gonadal dysfunction, leading to high gonadotropin-induced hypogonadism and decreased ovarian aromatase activity (49). Therefore, VD is an important factor for the complete function of the gonads. While research regarding the relationship between vitamin D and fertility has yielded promising results, vitamin D deficiency has been associated with numerous diseases, meaning that its specificity for disease diagnosis remains poor (50). However, to the best of our knowledge, few studies have focused on whether the combination of 25OHVD and other clinical indicators can be useful in the diagnosis of infertility.

25OHVD3 was not only one of the indicators that showed the most marked difference in cases of infertility, but was also identified by all three of the methods used. To investigate its role in the occurrence and development of infertility, we also looked at its correlation with a variety of clinical indicators. Intriguingly, 25OHVD3 is associated with blood lipids, hormones, thyroid function, HPV infection, hepatitis B infection, sedimentation rate, renal function, coagulation function, and amino acids. However, in patients with pregnancy loss, although 25OHVD3 was also one of the most prominent indicators and was also identified by all three of

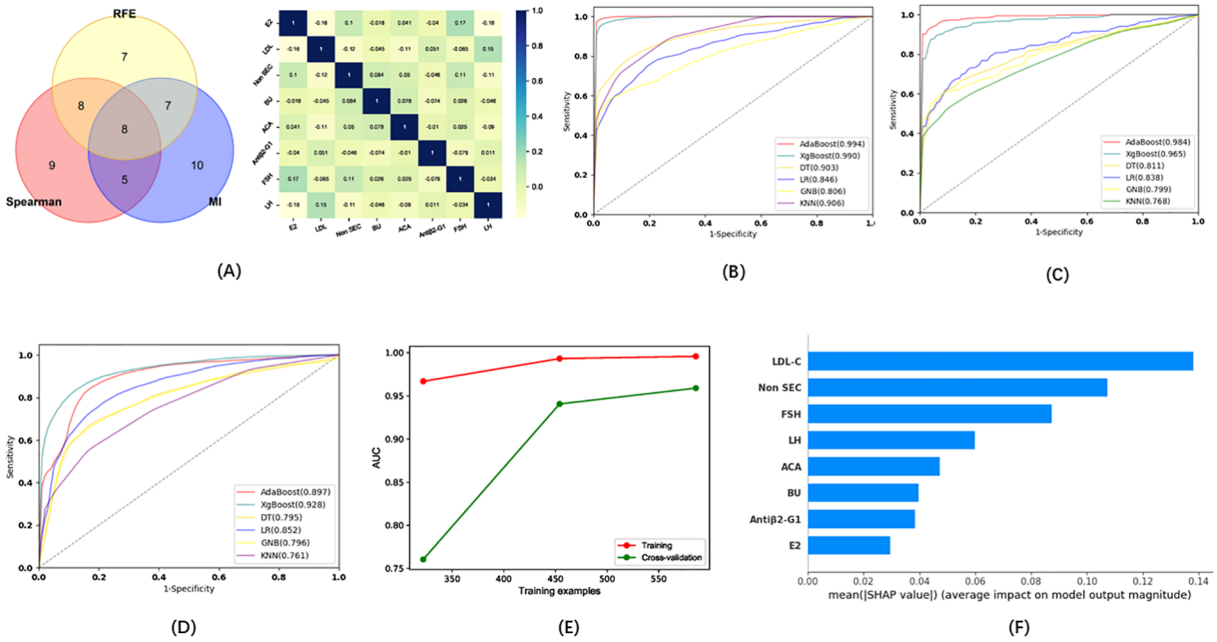


FIGURE 5
**(A)** Wayne diagrams for the MI, REF, and SPEARSON methods used to screen candidates for differentiating patients with infertility from those with pregnancy loss. Hotspot map of candidate indicators for this differentiation. **(B)**. ROC curves for the training set in the model for the differentiation of patients with infertility and with pregnancy loss. **(C)** ROC curve for the test set in the model for the differentiation of patients with infertility and with pregnancy loss. **(D)** ROC curve for the validation set in the model for the differentiation of patients with infertility and with pregnancy loss. **(E)** Learning curve for the training set in the model for the differentiation of patients with infertility and with pregnancy loss. **(F)** SHAP model for 8 indicators of the model for the differentiation of patients with infertility and with pregnancy loss.

the methods used, the correlation with HPV infection, coagulation function, platelet, thyroid function and other indicators disappeared. These results suggest that 25OHVD3 has a unique role in infertility, and its pathogenesis remains to be studied. The pathogenesis of infertility and that of pregnancy loss are indeed different, but the nature of the differences needs to be further studied.

Some authors have also attempted to develop new diagnostic methods for infertility. Cheng et al. established a cardiometabolic index (CMI) for diagnosing infertility (AUC=0.60, 95%CI: 0.56-0.65); the improved CMI index combined with BMI had a better predictive effect on infertility (AUC=0.722, 95%CI: 0.676-0.767) (51). Jiang et al. studied the plasma exosomes of 75 patients with polycystic ovary syndrome (PCOS) and used miR-126-3p, miR-

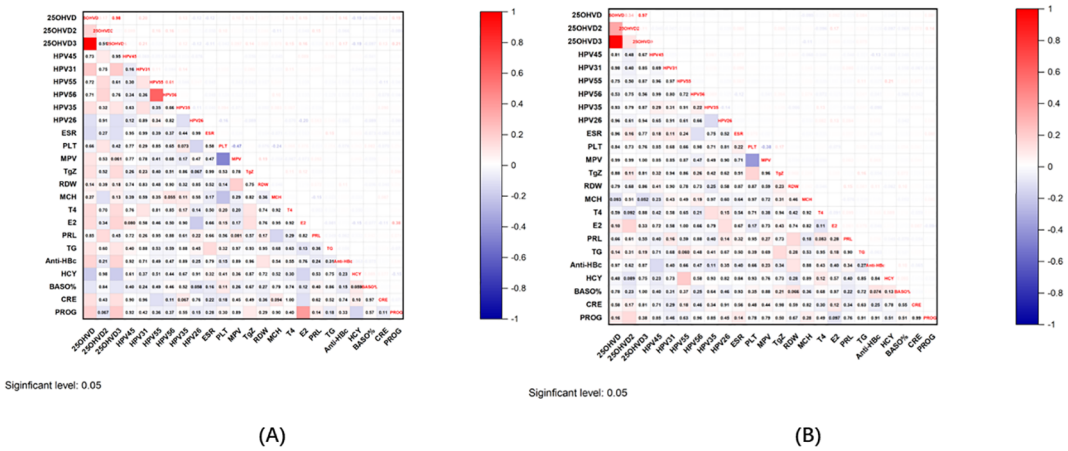


FIGURE 6
**(A)** Correlation graph of 25OHVD2, 25OHVD3, and 25OHVD in infertility with other clinical indicators. **(B)** Correlation graph of 25OHVD2, 25OHVD3, and 25OHVD in pregnancy loss with other clinical indicators.

146a-5p, miR-20b-5p, miR-106a-5p, and miR-18a-3p to distinguish PCOS patients from control individuals. The AUC of the ROC curve was 0.781 (52). However, there is still room for improvement in terms of infertility diagnosis. Since our results demonstrate that 25OHVD3 plays a role in the development of infertility, diagnosis based on multiple factors may have greater clinical significance. Similarly, we observed excellent diagnostic performance for the eleven factors included in our model for the diagnosis of female infertility, with AUC, sensitivity, and specificity values higher than 0.958, 86.52%, and 91.23%, respectively. In addition, we developed a diagnostic model to distinguish between infertility and pregnancy loss. Although the sensitivity of the models established by GNB, KNN, and DT ML in the verification set is not high (52.93%, 55.14%, and 69.30%, respectively), however the sensitivity of the models established by LR, and XgBoost ML in the verification set is 76.05%, and 85.32%, respectively, indicating that the models established by LR, and XgBoost, ML can be used to distinguish fertility from pregnancy loss. To the best of our knowledge, there are currently few diagnostic models for distinguishing infertility from pregnancy loss, and our results can fill in this gap. Moreover, the sensitivity and specificity of our model were markedly higher than those estimated for routine parameters and for most models that have been reported so far. The models we have developed are relatively simple, as the data can be obtained via routine laboratory analyses. Moreover, the present results may aid in the development of new indices for the diagnosis, treatment, and prevention of infertility. In addition, the models are suitable for use in large-scale screening to provide early warning of infertility, which can help ensure that patients do not miss the window of opportunity for treatment. Despite these advantages mentioned above, the performance of the discrimination model between infertility and pregnancy loss is low. The reason may be due to the overlap of clinical manifestations or the similarity of pathologic mechanisms in some of the two diseases, such as hormonal disorders, thyroid abnormalities and other clinical manifestations in patients with the two diseases. The introduction of other more differentiated indicators may be one of the ways to improve the diagnostic model, which needs further research.

## 5 Limitations

This study has some limitations. Firstly, we investigated the medical records from a single hospital located in one of the more developed cities in western China. Most of the patients and healthy individuals came from city. Due to the longer treatment time, most of the patients who came to our hospital for treatment and physical examination had better living conditions and higher education level. Therefore, the data inevitably have a certain bias. Secondly, vitamin D is affected by factors that include dietary intake and sunlight exposure, among others. Although our study considered daily intake, the levels of vitamin D also fluctuate seasonally, and therefore this potential indicator would need to be validated in different populations and during different seasons, something that was beyond our current means. Lastly, since population lifestyles are largely influenced by the region considered, and more than 99% of the participants in this study were from Western China, caution must be exercised while extrapolating the results to other regions of the world. In our future research, we will expand the number of enrolled participants and cooperate with multiple hospitals in China to verify the effect of our diagnostic model.

## 6 Conclusion

We sought to determine whether combining 25OHVD3 with other clinical indicators could increase its value in the diagnosis of infertility and pregnancy loss. Our results demonstrated that 25OHVD3 was the factor exhibiting the most marked difference between patients with infertility and the control group, and between patients affected by pregnancy loss and the control group. 25OHVD3 has a role in the occurrence and development of infertility. Both of the models we developed using five machine learning algorithms exhibited superior performance. These models are advantageous in that they are relatively simple, as the data can be obtained via routine laboratory analyses. Ultimately, the good performance and high sensitivity of the models presented here may facilitate early detection of infertility and pregnancy loss, in turn enabling timely diagnosis and treatment within the optimal reproductive window. Despite our promising findings, further studies involving larger populations are required to verify the practicality of our models and whether they can yield a clear clinical benefit, as well as the most appropriate methods for stratifying candidate patients.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

## Ethics statement

The studies involving humans were approved by Ethics Committee of Xijing Hospital (KY20212027-C-1), and informed consent was obtained via telephone interviews because of the retrospective nature of the study. The studies were conducted in accordance with the local legislation and institutional requirements.

## Author contributions

RZ: Formal Analysis, Writing – original draft. YG: Resources, Writing – review & editing. XZ: Resources, Writing – review & editing. JW: Writing – review & editing, Resources. XH: Data curation, Resources, Writing – review & editing. LY: Resources, Validation, Writing – review & editing. LZ: Investigation, Writing – review & editing. JG: Data curation, Writing – review & editing. JL: Funding acquisition, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2025.1544724/full#supplementary-material

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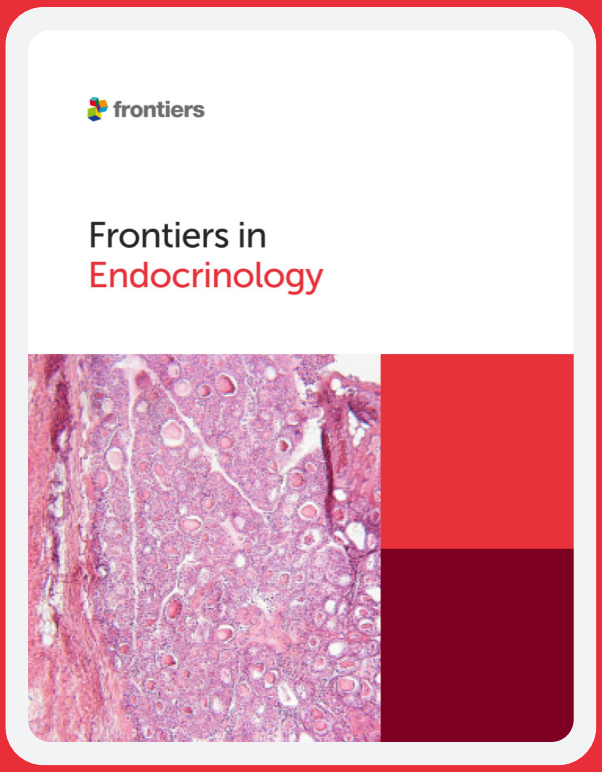


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