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Quantitative determination of vaginal diamines as a biomarker for vaginal dysbiosis and IVF-ET outcome

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Abstract

Background *Lactobacillus* species generally predominate in a healthy vaginal microbiota, and alterations of this population correlate with increased risk of genital infections and poor reproductive outcomes. Composition and functions of the vaginal microbiota are influenced by a variety of factors, among which hormones represent a major player. As therapy with gonadotropins is a key step in controlled ovarian hyperstimulation (COH) before in vitro fertilization and embryo transfer (IVF-ET), in this work the effect of hormone therapy for COH on the vaginal microbiota and embryo implantation outcome was evaluated in a cohort of women undergoing IVF-ET.

Methods A cohort of 108 infertile females were prospectively enrolled in the study. Hormone therapy consisted of recombinant follicle stimulating hormone and recombinant luteinizing hormone in a gonadotropin-releasing hormone antagonist regimen. Two vaginal swabs, before and after therapy, were collected from patients. Vaginal swabs were analyzed by microbiological standard culture methods for the presence of genital pathogens and lactobacilli. Changes in the vaginal microbiota were also assessed by using a vaginal diamine assay for the quantification of diamines, which are considered as a biomarker of the catabolic activity of bacteria involved in bacterial vaginosis.

Results COH significantly impacted on both vaginal diamine levels and microbiota composition. Median concentration of diamines in vaginal fluids increased from 0 µM (IQR = 0–8.96) to 21.86 µM (IQR = 5.11–46.74) before (pre-HT) and after (post-HT) hormone therapy, respectively ($p < 0.0001$). The diamine value of 9.5 µM was found to be the threshold between eubiotic and dysbiotic microbiota. Microbiological analysis of pre-HT and post-HT samples showed a post-HT enrichment of the vaginal population in streptococci, enterococci, enterobacteria, staphylococci and fungi, which are prevalent in aerobic vaginitis. The frequency of vaginal samples positive for genital pathogens and with elevated concentrations of diamines (> 9.5 µM) was significantly increased in post-HT compared to pre-HT specimens ($p < 0.0001$). Levels of diamines were significantly lower in patients with successful IVF-ET compared to those with failed implantation ($p = 0.015$), and 56.25% of patients who achieved embryo implantation had diamine levels below 9.5 µM. Finally, multivariate analysis of factors associated with implantation outcomes showed that

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younger maternal age, absence of genital pathogens and low levels of vaginal diamine post-HT were all associated with positive reproductive outcomes.

Conclusions The data from this study demonstrate that, following COH, an increase in diamine levels and genital pathogens occurred, leading to vaginal dysbiosis. Diamine concentrations higher than 9.5 μM post-HT significantly correlated with embryo implantation failure and may be used as a predictive biomarker of vaginal dysbiosis and IVF-ET outcome.

Keywords Vaginal diamines, Vaginal dysbiosis, Genital pathogens, Lactobacilli, Biomarker, In vitro fertilization and embryo transfer, Controlled ovarian hyperstimulation, Gonadotropin treatment

Introduction

About 30 years after the birth of the first 'test tube' baby, in vitro fertilization (IVF) and embryo transfer (ET) has become a widely available treatment for several causes of infertility. For assisted reproductive technology (ART), gonadotropins and gonadotropin-releasing hormone (GnRH) antagonists are routinely employed to induce controlled ovarian hyperstimulation (COH). Hence, high doses of exogenous gonadotropins [recombinant follicle stimulating hormone (rFSH) plus recombinant luteinizing hormone (rLH)] are administered to stimulate the development of multiple follicles in the pre-ovulatory stage by interfering with the physiological mechanisms which normally ensure the selection of a single dominant follicle [1, 2]. The use of such protocols may enable the selection of one or more embryos for transfer, while supernumerary embryos can be cryopreserved for later cycles [2]. According to the latest global report by the International Committee for Monitoring ART, cumulative delivery rate for fresh and frozen-thawed ET was 33.1% in 2016 [3].

The health status of the female genital tract is largely related to the presence of a normal vaginal microbiota [4, 5]. From puberty to menopause and during normal pregnancy, high levels of estrogens promote the accumulation of vaginal glycogen that favors the establishment of a *Lactobacillus*-dominated microbiota, which is mainly responsible for the preservation of a homeostatic (eubiotic) vaginal microenvironment [5, 6]. Perturbations of the vaginal microbiota may lead to microbial 'dysbiosis', which includes both bacterial vaginosis (BV) and aerobic vaginitis (AV) [7–9]). BV is a common clinical condition of reproductive-aged women and is characterized by an imbalance of the vaginal microbiota with an overgrowth of anaerobic bacteria and a reduction of the normal lactobacillary flora [10–12]. BV is prevalent among infertile women due to tubal/pelvic factors [13, 14] and has been linked to low pregnancy rates in patients subjected to IVF treatments [15]. However, BV may also be found in pregnant women [16, 17], where it has been associated to potentially life-threatening maternal–fetal outcomes, including intrauterine and fetal infections, premature rupture of membranes, miscarriage, and pre-term birth [13, 18, 19]. AV is an inflammatory condition in which the normal vaginal microbiota shifts to a population dominated by enterobacteria, staphylococci, streptococci and enterococci [20, 21]. AV differs from BV in some features, but they both share the displacement of the normal lactobacillary flora with genital pathogens [8] and the association with adverse reproductive outcomes [13, 18, 22–25]. In contrast to AV, a hallmark of BV is the positive reaction to the "whiff" test (fishy amine odor) originally described by Pheifer et al. [26] and still used for BV diagnosis according to Amsel's clinical criteria [27]. The test is based on the detection of diamines by their typical odor upon addition of potassium hydroxide to the vaginal fluid [26]. Diamines are small aliphatic hydrocarbon molecules derived from the decarboxylation of certain amino acids (*i.e.*, L-lysine, L-arginine, L-ornithin, and L-methionine) [28], and in the human vagina, their production is mainly carried out by anaerobic bacteria [29]. Hence, diamines are considered as biomarkers of BV [30–33], while vaginal samples from healthy women do not contain diamines [34, 35]. Recent studies have deepened our understanding on vaginal diamines by showing that diamines not only are BV biomarkers but also play a causal role in the pathogenesis of vaginal dysbiosis, as amino acid decarboxylation leads to proton consumption and pH increase, which can directly jeopardize *Lactobacillus* survival and allow the outgrowth of BV-associated species [29, 36]. To evaluate the feasibility of using diamines as biomarkers of vaginal health/disease, the enzymatic 'vaginal diamine assay' (VADA) was developed, which detects and quantifies cadaverine and putrescine, the most abundant diamines in the vaginal fluid [34], in clinical samples [37]. Its simplicity and low cost suggest that it could be suitable for routine diagnostic tests.

This study aims to evaluate the effect of COH on the vaginal microbiota of women undergoing IVF-ET by concurrently measuring vaginal diamines and characterizing the vaginal flora, and to examine the association of microbiological findings with embryo implantation outcomes. The results obtained demonstrate that a rise in vaginal diamines and genital pathogens occurred after COH. A diamine concentration of 9.5 μM was identified

as a threshold for dysbiotic microbiota and a potential predictive biomarker of embryo implantation failure.

Materials and methods

Patients

A cohort of 108 women aged 25–46 years [mean ± SD: 36.47 ± 4.42; median (IQR): 36 (34–40)], undergoing COH for IVF–ET procedures at the Medically Assisted Reproduction Unit of Siena University Hospital, were consecutively enrolled over a 6-month period. All patients were unable to conceive naturally for at least 1 year before entering the study. The causes of infertility were classified as follows: endocrine/ovulatory disorders (29.5%), tubal occlusion (5.5%), male factor infertility (32.5%), and idiopathic (32.5%). All enrolled women were non-smokers and had no known comorbidities that could affect reproductive outcomes or vaginal health. Median body mass index (BMI) was 22.3 [IQR = 20.2–24.5]. Participants reported no vaginal infections in the two months preceding enrolment. Exclusion criteria were antibiotic therapy a month before the beginning of the study. All participants signed a written informed consent, and the study protocol was approved by the ethic regional commission (CEAVSE, code 20170619).

COH

Recombinant gonadotropins at personalized doses of 150–300 IU were administered starting from the first or second day of spontaneous or induced menstruation. The dose of gonadotropins was adjusted according to ovarian response evaluated by ultrasound examination and hormonal blood profile. As soon as the dominant follicle reached 14 mm in diameter, a GnRH antagonist was administered daily until ovulation was induced. Ovulation triggering was performed by injection of human chorionic gonadotrophin (hCG) (10,000 IU) when at least 3 follicles reached 18 mm in diameter. Oocyte pick-up was scheduled 34–36 h after ovulation triggering. No probiotics were administered to patients during hormone treatment cycles.

IVF-ET

Approximately 2 h after oocyte retrieval, cumulus cells were removed using enzymatic digestion and gentle pipetting, and the denuded oocytes were placed in fertilization medium for at least 1 h before IVF. Oocytes were fertilized by the ICSI procedure, and ET was performed on day 3 after IVF. All transferred embryos were grade 1 or 2, according to the Istanbul consensus criteria [38]. Serum levels of hCG were determined on day 14 post-ET, and the presence of a gestational sac was evaluated by transvaginal ultrasound examination at 7 weeks of gestation.

Clinical samples

Vaginal specimens were collected according to the protocol provided by the Italian Association of Clinical Microbiologists [39]. The first sample was obtained during the screening visit one month before starting the hormone therapy (HT) used for COH (pre-HT), while the second swab was retrieved after COH (post-HT) at the time of oocyte retrieval. Because some pre-HT samples were unavailable, analyses were conducted on approximately 100 pre-HT samples, whereas post-HT analyses included all 108 vaginal swabs. All samples were sent to the laboratory for microbiological analysis.

Detection and identification of cultivable bacteria

Standard bacteriological culture methods were used to identify both genital pathogens and vaginal lactobacilli from vaginal swabs. The rayon swabs (FL Medical, Padova, Italy) were resuspended in 1 ml of sterile 0.9% NaCl solution with 10% glycerol. The wet swab was directly used for plating onto agar plates, while the liquid was employed for assessing the concentration of diamines by VADA. Selective and differential solid culture media (Oxoid, Milan, Italy) were used to detect cultivable bacteria and fungi. Microbial identification was carried out by using a Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) BioTyper (MBT) LT/SH Smart instrument coupled with the software programs FlexControl (v 3.4) and MBT Compass (v 4.1) (Bruker Daltonics, Leipzig, Germany).

Vaginal swabs were regarded as positive for the presence of genital pathogens when viable counts were $\geq 10^4$ colony forming units (CFU) per swabs. Quantitative analysis of lactobacilli in vaginal swabs was carried out on Rogosa agar (Oxoid). The presence of lactobacilli was regarded as 'normal' when viable counts were $\geq 10^3$ CFU/swab, and 'low' at values $< 10^3$ CFU/swab. Assay detection limit was 10^2 CFU/swab.

Vaginal diamine assay (VADA)

The diamine content in 100 µl of vaginal swabs was quantified by VADA. VADA is an enzymatic assay based on diamine oxidase (DAO) which catalyzes the production of H_2O_2 upon reaction with putrescine and cadaverine, as previously described [37]. Clinical samples were tested both with and without the addition of DAO to quantify H_2O_2 physiologically produced by vaginal lactobacilli or human cells (*i.e.*, vaginal macrophages). For each sample, the optical density at 510 nm (OD_{510}) obtained without the addition of DAO was subtracted from that obtained with DAO. The resulting OD_{510} was used to calculate the diamine concentration using a reference standard curve obtained with a mixture of cadaverine and putrescine at

1:1 molar ratio (range 2–512 μM). Results were expressed as μM of diamines per sample.

Statistical analyses

Statistical analyses were performed by using the software GraphPad Prism 9.4.0 (GraphPad Software, San Diego, CA, USA) and R (R Foundation for Statistical Computing, Vienna, Austria). Statistical comparisons between diamine concentrations in pre-HT and post-HT samples, in patients with positive and negative IVF-ET outcomes, as well as comparisons of the age of women with positive or negative implantation results, were performed using the Mann–Whitney test ($p < 0.05$). Results are represented as median with interquartile range (IQR).

The threshold to bisect pre-HT and post-HT diamine concentrations was calculated using the Receiver Operating Characteristic (ROC) method employing the ROCR v1.0.11 R package [40]. In detail, the ROC curve was used to graphically represent the performance of different potential thresholds in diamine concentration, reporting sensitivity and specificity for each threshold. The higher the Area Under the Curve (AUC), the better the overall performance. The best threshold was identified as the point on the curve closest (measured using the Youden's method) to the top-right corner of the graph, where both sensitivity and specificity are equal to 1. The 95% confidence interval (CI) was calculated using the ci.auc function from pROC v1.18.0 package.

To analyze the effects of COH on genital pathogens, vaginal lactobacilli and diamine concentrations, and then their impact on embryo implantation outcomes, the Chi square test with Yates' correction (for sample size ≥ 10) and Fisher' exact test (for sample size < 10) were employed.

Multivariate logistic regression analysis of variables associated with embryo implantation outcome was performed using the glm function, setting family = binomial. Results are reported as odds ratios (ORs) and 95% CI. ORs represent the direction of the association between each variable and outcome. An OR between 0 and 1 indicates a negative association, whereas an OR > 1 indicates a positive association.

Table 1 Prevalence of microbial species in vaginal swabs before and after hormone therapy used for COH

Microbial species[a]	Hormone therapy[b]		
	Pre-HT (%)	Post-HT (%)	Total (%)
Streptococcus spp.	7 (20)	35 (38.5)	42 (33.3)
Enterococcus faecalis	5 (14.3)	13 (14.3)	18 (14.3)
Streptococcus agalactiae	7 (20)	8 (8.8)	15 (11.9)
Escherichia coli	8 (22.9)	7 (7.7)	15 (11.9)
Candida spp.	5 (14.3)	9 (9.9)	14 (11.1)
Other *Enterobacterales*	0	10 (10.9)	10 (7.9)
Staphylococcus spp.	0	9 (9.9)	9 (7.2)
Gardnerella vaginalis	3 (8.5)	0	3 (2.4)
Total clinical strains	35	91	126

[a] *Streptococcus* spp. includes streptococci of groups A, C, F and G, while *S. agalactiae* (group B *Streptococcus*) and *E. faecalis* (group D *Streptococcus*) are indicated as separate units; other *Enterobacterales* comprise *Klebsiella pneumoniae, Klebsiella oxytoca, Proteus mirabilis* and *Citrobacter koserii*; *Staphylococcus* spp. includes *Staphylococcus aureus, Staphylococcus epidermidis* and other coagulase-negative staphylococci; *Candida* spp. comprises *Candida albicans* and *Candida glabrata*

[b] Microbiological analysis was performed on a total of 209 (101 pre-HT and 108 post-HT) vaginal swabs. Percentages of each microbial species/genus relative to the number of clinical isolates retrieved from pre-HT, post-HT, and pre-HT + post-HT (total) are shown in parentheses

Results

Prevalence of vaginal pathogens in patients undergoing IVF-ET

Forty percent (84/209) of the vaginal swabs tested positive for at least one genital tract pathogen. A comparison of the samples before and after hormone therapy used for COH revealed that 27.7% of pre-HT swabs were positive for one or more genital pathogens, while the number of positive swabs significantly increased to 51.9% post-HT ($p = 0.000229$).

A total of 126 clinical isolates belonging to 17 different microbial species were identified across tested samples (Table 1). Group A, C, G and F *Streptococcus* were the most prevalent bacterial isolates accounting for 33.3% (42/126) of strains, followed by *Enterococcus faecalis* (14.3%), *Streptococcus agalactiae* and *Escherichia coli* (11.9%), and *Candida* spp. (*C. albicans* and *C. glabrata*; 11.1%) (Table 1). The microbial genus/species that exhibited the highest increase after therapy were *Streptococcus* spp. (fivefold increase), followed by *E. faecalis* (2.6-fold increase) and *Candida* spp. (1.8-fold increase) (Table 1). Notably, enterobacteria other than *E. coli* (10/108 swabs) and *Staphylococcus* spp. (9/108 swabs) were detected only in post-HT samples (Table 1).

These findings suggest that COH promotes the overgrowth of streptococci, enterococci, enterobacteria, staphylococci and yeasts, contributing to the onset of vaginal dysbiosis.

Influence of COH on vaginal diamine levels

The diamines putrescine and cadaverine were quantified in the vaginal fluids of the patients pre-HT and post-HT. Hormone therapy had a significant impact on diamine levels. Pre-HT specimens ($n = 99$) had low diamine concentrations [median (IQR) = 0 μM (0–8,96)], whereas post-HT samples ($n = 108$) showed a robust increase of diamine levels [median (IQR) = 21,86 μM (5,11–46,74)] ($p < 0.0001$) (Fig. 1A).

As vaginal diamines are considered a marker of BV, a threshold concentration to distinguish eubiotic from dysbiotic microbiota was determined using the ROC method. Each point of the ROC curve represents a potential threshold value with its respective sensitivity and

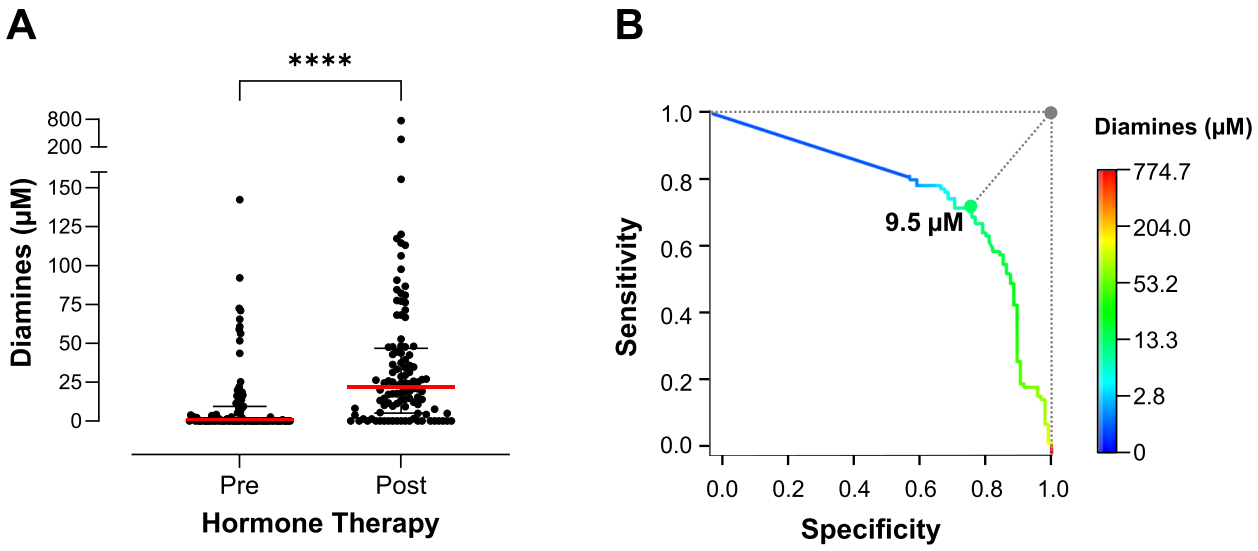

Fig. 1 Concentration of diamines in vaginal fluids before and after COH. A cohort of 108 infertile females was evaluated. Two vaginal swabs were collected from patients, of which the first swab during the screening visit one month before COH, and the second at the time of oocyte pick-up. The diamines putrescine and cadaverine were quantified in vaginal fluids using VADA [37]. **A** Results are represented as median with IQR of diamine concentrations before (Pre) and after (Post) hormone therapy. Each dot represents a single vaginal sample, and red lines show median values. Asterisks indicate statistically significant differences (****, $p < 0.0001$). **B** ROC curve sensitivity versus specificity to identify the best threshold that discriminates between pre- and post-HT diamine concentrations. In the ROC curve, each point represents a potential decisional threshold with its corresponding sensitivity and specificity value. The color of each portion of the curve relates to different threshold values reported in the log2 scale on the right and ranging from 0 μM (blue) to 774.7 μM (red). The best threshold point (green dot) has the lowest distance from the optimal theoretical performance (grey dot, sensitivity = 1, specificity = 1)

specificity. The best threshold value in bisecting low and high diamine concentrations in pre- and post-HT vaginal swabs was calculated as the point of the curve closest to the top-right corner where both sensitivity and specificity have the highest values (equal to 1). Therefore, the best threshold was identified at 9.5 μM (specificity = 0.76, sensitivity = 0.72) (Fig. 1B). The area under the curve was 0.76 (95% CI = 0.70–0.83), indicating a good discriminatory performance.

COH negatively impacts on the vaginal microbiota of IVF-ET patients

To analyze the effects of COH on the vaginal microbiota of IVF-ET patients, genital pathogens, vaginal lactobacilli and diamine levels were concurrently evaluated before (pre-HT) and after (post-HT) hormone therapy. Genital tract pathogens were qualitatively assessed (absence vs. presence of at least one species at counts $\geq 10^4$ CFU/swab), whereas threshold values were set for vaginal lactobacilli ($\geq 10^3$ CFU/swab) and vaginal diamine concentrations (≤ 9.5 μM).

The frequency of patients positive for genital pathogens significantly increased from 28/101 pre-HT to 56/108 post-HT ($p = 0.000229$, OR = 2.79, Fig. 2A). Insight into the subgroup of the 28 pre-HT positive patients showed that, following COH, 21/28 (75%) women remained positive, whereas 7 became negative. The total number of clinical isolates was comparable before and after HT ($n = 35$ and $n = 33$, respectively); however, the number of strains per positive patient increased, as the proportion of women positive for ≥ 2 pathogens post-HT (8/21, 38%) was almost double that observed pre-HT (6/28, 21.4%), (Supplementary Table S1). Analysis of the *Lactobacillus* flora showed that the number of women with low amounts of lactobacilli raised from 17.5% (17/97) pre-HT to 26% (28/108) post-HT, but differences were not significant ($p = 0.17$; OR = 0.6, Fig. 2B). Finally, evaluation of COH impact on diamine levels demonstrated that the majority of pre-HT samples (75/99, 75.8%) had low diamine concentrations (≤ 9.5 μM), whereas such percentage was overturned after hormone therapy when 71.3% (77/108) of post-HT swabs presented diamine concentrations above 9.5 μM. Differences were highly significant ($p < 1.05e\text{-}11$, OR = 7.67; Fig. 2C).

These findings suggest that COH in patients undergoing IVF-ET is associated with the development of vaginal dysbiosis, as evidenced by the increase in genital pathogens, the moderate decrease in lactobacilli and the rise of vaginal diamines.

High concentrations of vaginal diamines, presence of genital pathogens and advanced maternal age are significantly correlated to implantation failure

The outcome of embryo implantation after IVF-ET was assessed in the study population. Out of 108 enrolled patients, 18 did not complete the therapeutic cycle

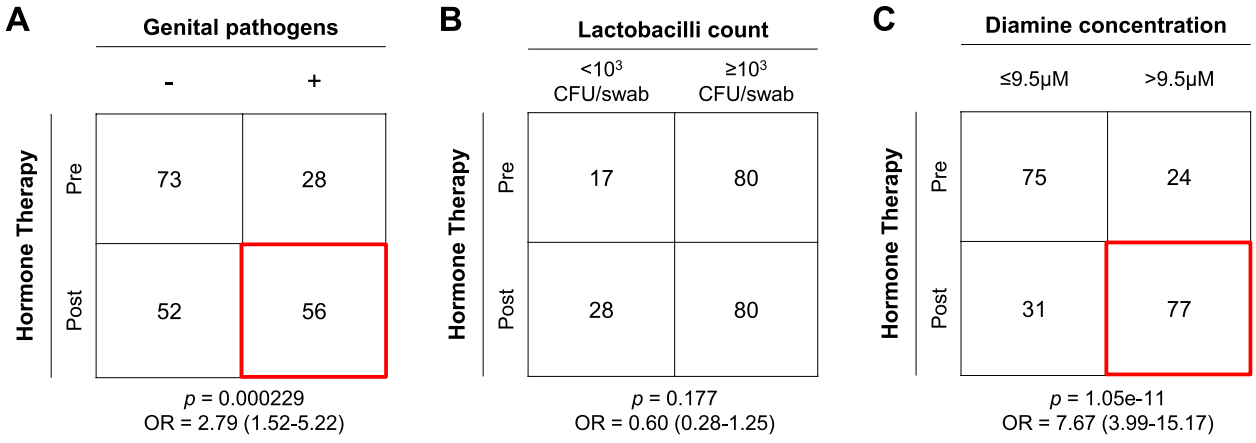

Fig. 2 Impact of COH on the vaginal microbiota of IVF-ET patients. Study population and samples were as described in Fig. 1. Continency tables report: **A** the number of vaginal swabs negative (-) or positive (+) for at least one genital tract pathogen in pre-HT ($n = 101$) and post-HT ($n = 108$) samples, **B** the number of swabs with low ($< 10^3$ CFU/swab) or normal ($\geq 10^3$ CFU/swab) counts of vaginal lactobacilli in pre-HT ($n = 97$) and post-HT ($n = 108$) samples, **C** the number of vaginal samples with low (≤ 9.5 μM) or high (> 9.5 μM) diamine concentrations in pre-HT ($n = 99$) and post-HT ($n = 108$) samples. The χ^2 test with Yates' correction was used

because no oocytes were retrieved ($n = 11$) or no embryos developed ($n = 7$). Thus, a total of 90 women were included in the analyses. Of those, 17.8% (16/90) had a successful implantation.

To evaluate the impact of vaginal diamine levels on IVF-ET outcome, the median diamine concentration of post-HT samples was calculated in patients who achieved a successful embryo implantation compared to those who experienced failure. Diamine concentrations were 5.2 μM (IQR = 0.32–25.49) and 25.14 μM (IQR = 11.89–56.25) in women with successful and adverse IVF-ET outcomes, respectively ($p = 0.015$), (Fig. 3A). Then, the diamine threshold of 9.5 μM, previously identified to separate eubiotic from dysbiotic microbiota, was applied to the IVF-ET results. Out of 74 patients with failed embryo implantation, 78.4% had diamine concentrations above the threshold, while 9/16 (56.25%) women with successful implantation belonged to the group with diamine concentrations below 9.5 μM ($p = 0.011$; OR = 0.21) (Fig. 3B). In agreement with the diamine results, almost 60% (44/74) of patients with IVF-ET failure were positive for genital pathogens after COH, compared to only 18.8% (3/16) of women who obtained a successful implantation despite being positive on microbiological testing ($p = 0.004$; OR = 0.16), (Fig. 3C). In addition to high diamine levels and presence of genital pathogens, advanced maternal age was also significantly associated with implantation failure, as the median age of women with negative and positive IVF-ET outcomes was 36 years (IQR = 34.25–40.75) and 33.5 years (IQR = 31.25–36), respectively ($p = 0.007$; Fig. 3D).

No significant differences were observed with diamine concentrations or presence of genital pathogens pre-HT in relation to implantation outcome. Likewise, no significant associations were also observed for vaginal lactobacilli in either pre-HT ($p = 0.28$; OR = 3.84) or post-HT ($p = 0.37$; OR = 2.06) samples and IVF-ET results. However, 93.3% and 81.25% of patients with positive IVF-ET exhibited vaginal lactobacillary counts $\geq 10^3$ CFU/swab pre-HT and post-HT, respectively (Supplementary Fig. S1).

Based on univariate analyses, variables showing significant associations with implantation outcome or exhibiting odds ratios > 2 or < 0.5 were further evaluated using a multivariate logistic regression model to assess their potential independent effects on IVF-ET results. The model included: patient age, presence of vaginal lactobacilli pre- and post-HT, presence of genital pathogens post-HT, and diamines concentration > 9.5 μM post-HT. Results confirmed that presence of genital pathogens and elevated diamine concentrations as well as older patient age were independently associated with adverse reproductive outcomes. In contrast, no independent association was observed for vaginal lactobacilli in either pre-HT or post-HT vaginal specimens (Supplementary Table S2).

Overall, these findings indicate that vaginal dysbiosis -defined by diamine levels above 9.5 μM and presence of genital pathogens- as well as advanced maternal age are independently associated with poor reproductive outcomes.

Discussion

Few studies have connected COH, the vaginal and endometrial microbiota, and IVF-ET outcomes. There are still substantial gaps in knowledge, including the characterization of microbiota of IVF-ET patients after COH in clinical trials and the identification of microbial biomarkers that can predict embryo implantation outcome. In this study, we provide evidence that gonadotropin

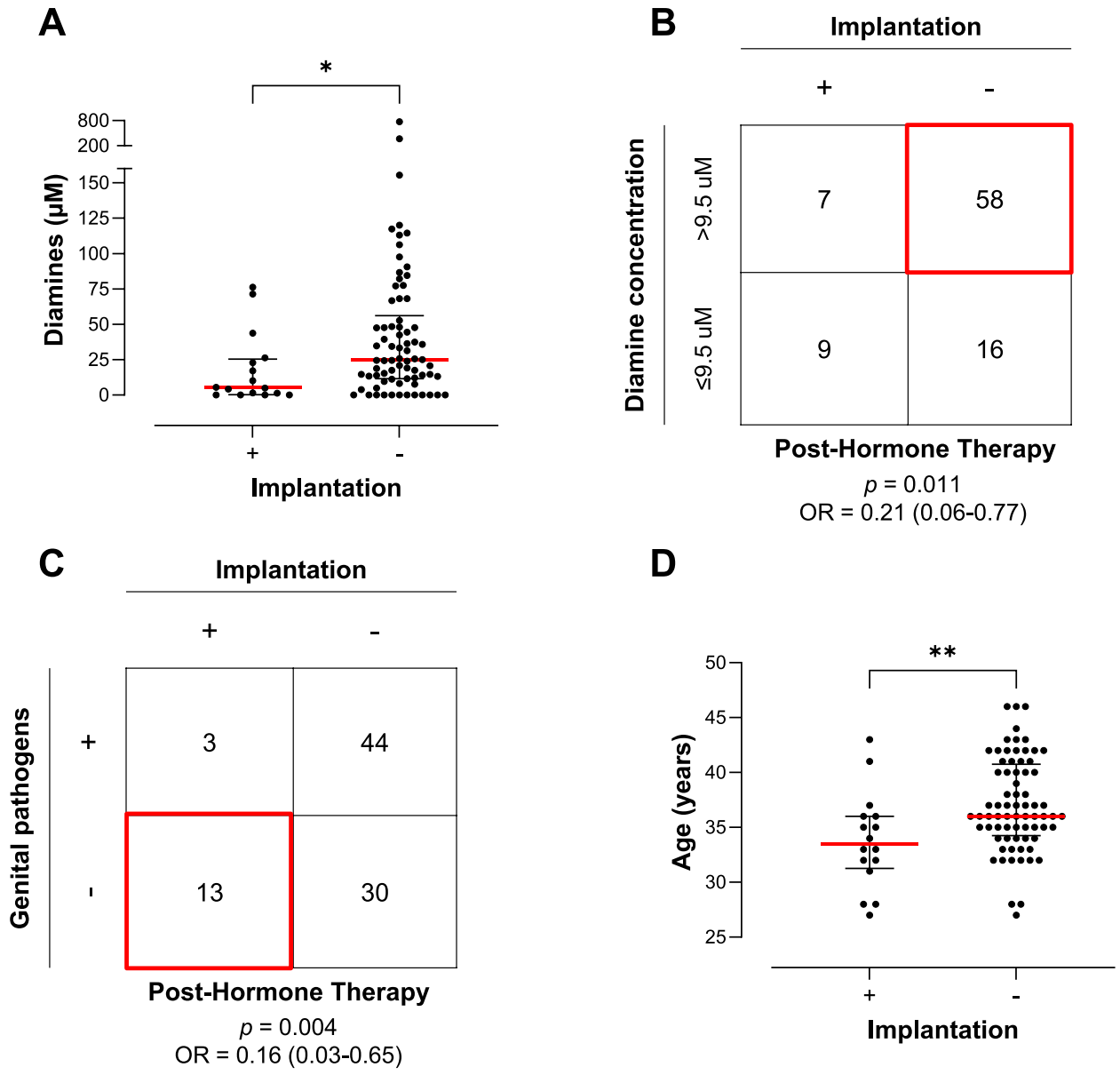

Fig. 3 Impact of diamine concentrations, presence of genital pathogens and maternal age on embryo implantation outcome. Analyses were performed on a total of 90 patients who completed the IVF-ET cycle, of which 16 had a successful implantation. **A** Median (and IQR) diamine concentrations after COH in patients with positive and negative IVF-ET outcome. Each dot represents a single patient, and red lines show median values. Asterisk indicates statistically significant differences (*, $p = 0.015$). **B** Contingency table reports the number of patients with low (≤ 9.5 μM) and high (> 9.5 μM) post-HT diamine concentrations in relation to the outcome of embryo implantation. **C** Number of patients who tested positive and negative for genital pathogens post-HT with respect to the IVF-ET results. **D** Median patient age with IQR in association with IVF-ET outcome. Each dot represents a single patient, and red lines show median values. Asterisks indicate statistically significant differences (**, $p = 0.007$). Continuous variables were compared using the Mann–Whitney test, and categorical variables were analyzed using the Fisher's exact test

treatment used for COH favored the outgrowth of genital pathogens and the increase of vaginal diamine levels, both of which were significantly associated with IVF-ET adverse outcomes. Accordingly, a diamine concentration of 9.5 μM was identified as the threshold for a dysbiotic microbiota and a measurable biomarker potentially predictive of implantation failure.

High-throughput 16S rRNA sequencing studies on the composition of the vaginal microbiota in reproductive-age women evidenced the existence of at least five 'community state types' (CSTs) of microbiota, four of which (CST I, II, III and V) are dominated by *Lactobacillus* species [4]. The most abundant species of CST-III is *Lactobacillus iners*, which is considered as an atypical lactobacillus with limited protective capabilities against

invading pathogens and is often associated with vaginal dysbiosis [41, 42]. In contrast, CST-IV is a *Lactobacillus*-depleted CST [4, 43]. Compared to the other CTSs, CST-IV is characterized by the highest production of the diamines cadaverine and putrescine [29] and is microbiologically similar to BV [10]. On the other hand, AV has been described as the 'aerobic' equivalent of BV [20, 21]. Despite BV and AV differ in various aspects [8], they are both classified as vaginal dysbiosis, characterized by a reduction in lactobacilli and an increase in microbial pathogens. An increasing body of evidence supports the association between vaginal dysbiosis and higher susceptibility to genital tract infections, strengthening the protective role of *Lactobacillus* spp. against invading pathogens [5, 6, 19]. Our data showed that, following COH, the vaginal microbiota of patients shifted toward a microbial population composed of streptococci, enterococci, enterobacteria, staphylococci and yeasts, which are commonly detected in women with AV [21]. The most abundant bacterial genus post-HT was *Streptococcus* spp. (fivefold increase) followed by *Enterococcus faecalis* (formerly *Streptococcus faecalis*) (1.6-fold increase). The involvement of streptococci in vaginal dysbiosis associated with infertility was described by other authors [44–46], who observed a significant decrease in live birth rates when streptococcal species accidentally contaminated the catheter used for ET or were retrieved from the cervical canal on the ET day [47]. Consistent with the present finding, we have previously reported that the presence of *E. faecalis* in post-HT vaginal swabs was significantly associated with reduced lactobacillary levels, and the simultaneous isolation of *E. faecalis*, *Mycoplasma hominis* and *Ureaplasma urealyticum* from genital samples of IVF-ET couples was predictive of implantation failure [48], further highlighting the role of *E. faecalis* as an emerging pathogen potentially associated with couple infertility. Along with increased AV-associated pathogens, patients also presented with high concentrations of vaginal diamines in post-HT vaginal samples, which are typical catabolites of BV-associated microbes [27, 35]. In contrast, the observation of a modest reduction of lactobacilli together with their detection in approximately 70% of patients positive for pathogens or with high diamine levels after COH, was unexpected. Several data suggest that *L. iners* is a transitional colonizer of an altered vaginal environment and may even contribute to vaginal dysbiosis [42]. However, as species identification was not performed and *L. iners* grows poorly on Rogosa agar, its prevalence and potential role in vaginal dysbiosis in our patient cohort remain unknown.

Traditionally, the diagnosis of BV and AV was based on microscopy. The conventional methods for diagnosing BV include the clinical criteria proposed by Amsel in 1983 [27] which can also be managed as a point-of-care (POC) test [49], and the laboratory criteria described by Nugent et al. in 1991, who instead used Gram-stained vaginal smears to score bacterial morphotypes [50]. The gold standard for BV remains the Nugent scoring system, and novel diagnostic approaches generally use the Nugent's criteria as the reference test [49, 51, 52]. For AV diagnosis, although Donders et al. initially suggested fresh wet-mounted vaginal smears in phase-contrast microscopy [20], alternative criteria that combine Gram staining and clinical features have recently been proposed to diagnose both AV and BV [53]. Currently, there are several assays available for BV, ranging from a few POC tests to different molecular assays [49, 52]. Among the POC, the FemExam test card 1 (Cooper Surgical) measures pH and the presence of trimethylamine in vaginal secretions [31]. Despite being included in the latest sexually transmitted infections treatment guidelines from CDC [54], FemExam card 1 is a merely qualitative and relatively costly enzymatic test with 71.4% sensitivity and 72.8% specificity versus the Nugent test [31, 55]. In this study, we assessed the presence of BV-associated microbes by using another enzymatic test, VADA [37]. Although not designed as a POC, VADA proved to be a simple and inexpensive laboratory test that quantifies the diamines cadaverine and putrescine in clinical samples with a turnaround time of few hours [37]. Compared to the Nugent score, VADA showed 65.2% sensitivity and 92.8% specificity (Mendonca et al., unpublished data). By using this approach, the value of 9.5 μM was regarded as the diamine threshold to separate eubiotic from dysbiotic vaginal microbiota. After COH, 71.3% of treated women presented with a dysbiotic vaginal microbiota (diamines > 9.5 μM), and embryo implantation failed in 89.2% of patients with vaginal dysbiosis. Interestingly, a very recent metagenomic functional analysis of vaginal samples from IVF patients showed that women with implantation failures displayed an enrichment of the biosynthesis pathways of L-lysine [56], which is the precursor amino acid of cadaverine [28]. In addition to high diamine levels, the observation that pathogen overgrowth after COH was also significantly associated with implantation failure in 93.6% of cases, reinforces the role of vaginal dysbiosis in reproductive outcomes, identifying diamines as a quantifiable biomarker of such dysbiosis and as a potential indicator of IVF-ET outcome.

During puberty and pregnancy, the establishment and maintenance of a eubiotic vaginal microenvironment, dominated by *Lactobacillus* species, depend on high estrogen levels [6]. However, COH inevitably results in estradiol concentrations exceeding physiological levels by more than tenfold, and the effects of such estradiol excess on IVF-ET outcomes remain controversial [57, 58]. Although some authors claim that high serum estradiol levels on the day of ovulation induction with hCG have

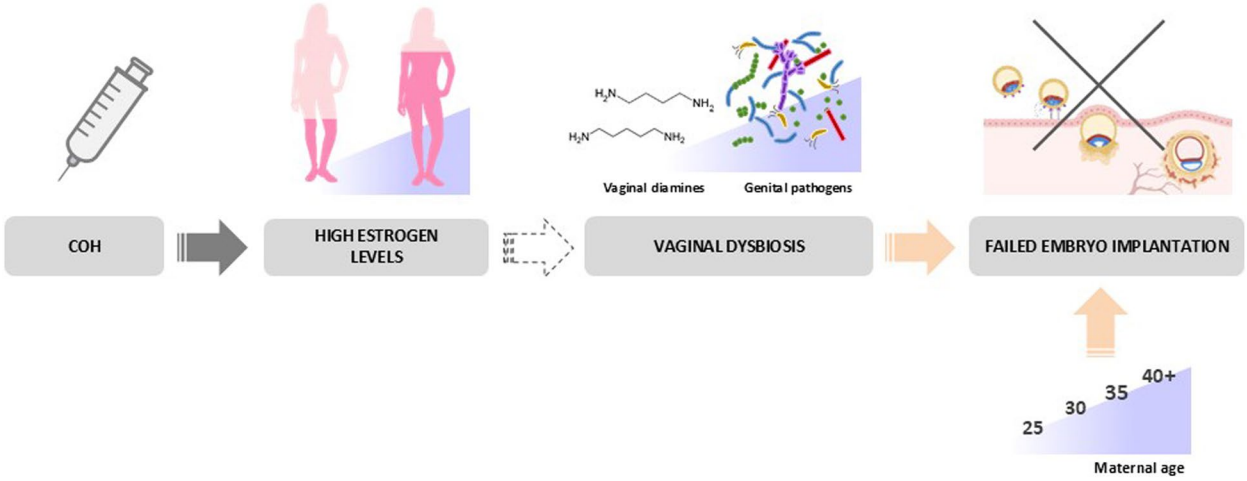

Fig. 4 Hypothetical impact of COH on vaginal microbiota and IVF-ET outcome. The drawing is a proposed hypothesis based on both the results of this study and data from literature. Treatment with exogenous gonadotropins during COH induces a supraphysiological hyper-estrogenic condition in patients undergoing IVF-ET [57, 58], which may in turn affect the vaginal microbiota. Enrichment of genital pathogens and increased levels of vaginal diamines may lead to vaginal dysbiosis. Vaginal diamines are considered as a marker of vaginal dysbiosis [30–33], which is associated with poor reproductive outcomes [13, 15, 18, 22, 73, 75, 77]. In this study, pathogen overgrowth, diamine levels above 9.5 µM, and older maternal age were significantly associated with IVF-ET failure both by univariate and multivariate analyses. The grey arrow describes an acknowledged side-effect of COH, while the grey dashed arrow represents a possible effect of hyper-estrogenism on genital microbiota, which is gaining increasing interest in the literature [79, 81, 82] and warrants further investigation. The orange arrows show results presented in this study

no negative impact on pregnancy rate, birth rate and birth weight [58–62], other investigators have reported that supraphysiologic concentrations of estradiol may instead be associated with detrimental effects on oocyte fertilization, embryo implantation, placenta formation, pregnancy and live birth rates [63–69]. In recent years, the importance of the vaginal and endometrial microbiota for ART outcomes has become increasingly recognized, and the vaginal microbiota is now being considered as a tool to predict implantation outcomes in IVF patients [70–77]. Zhao et al. reported that the vaginal microbiota of IVF-ET patients is not sensitive to hormonal changes due to COH [78], whereas more recent studies are beginning to show that the vaginal microbiota undergoes significant alterations during COH, which may subsequently negatively affect embryo implantation [79, 80]. Carosso and colleagues reported that COH treatment of infertile women significantly augments the microbial biodiversity (*i.e.* lower levels of lactobacilli and higher number of pathogenic species) both in the vagina and endometrium [81]. The authors suggested that the post-COH microbial instability may negatively affect endometrial receptivity and embryo implantation, but they did not relate the microbiota composition to the IVF outcome [81]. In contrast, our study has linked alterations of the vaginal microbiota during COH to IVF–ET outcomes and, for the first time, has proposed vaginal diamines above 9.5 µM as a biomarker of vaginal dysbiosis and potential predictor of implantation failure. Very recently, Chen and coworkers demonstrated that supraphysiological estradiol levels during IVF-ET cycles altered the vaginal microbiota resulting in the overgrowth of streptococci (particularly *Streptococcus anginosus*) and anaerobes, which were associated with adverse pregnancy outcomes [82]. Interestingly, *S. anginosus* positively correlated with peak estradiol levels in patients who did not achieve pregnancy [82]. In our cohort, the fivefold streptococcal enrichment observed after COH primarily involved species of the *S. anginosus* group, and notably, none of the patients positive for any streptococcal species achieved successful implantation. Interestingly, the authors also found that lactobacilli under hyper-estrogenic conditions did not improve reproductive outcomes [82], which aligns with our findings of implantation failure in IVF patients despite the persistence of lactobacilli following COH. Therefore, as supraphysiologic estradiol levels were likely present in our patient cohort, we hypothesize that the hyper-estrogenic condition after COH may have altered the vaginal milieu, leading to vaginal dysbiosis and potentially contributing to embryo implantation failure (Fig. 4). It is interesting to note that some authors reported higher implantation rates in frozen ET cycles compared to fresh cycles where embryos are transferred directly after COH [83–85]. In the so-called 'freeze-all' strategy, frozen embryos are instead transferred in subsequent cycles, thus avoiding the supraphysiologic hormonal levels observed during COH. This approach, originally dedicated to women at risk of ovarian hyperstimulation syndrome or with polycystic ovarian syndrome and those in need of preimplantation genetic testing, has been extended to other clinical conditions to improve endometrial receptivity and implantation rates

[86]. Dysbiosis of the vaginal and endometrial microbiota following COH may support consideration of frozen rather than fresh ET [79].

The present study has some limitations. The first is the small number of embryo implantations (16/90 = 17.8%), which limits the robustness of multivariate analysis. The second shortcoming is the absence of molecular diagnostic approaches that prevented a comprehensive characterization of the vaginal microbiota of the IVF-ET patients. In fact, culture-based methods can identify only a subset of the microbial species present in a biological sample, underestimating obligate anaerobes and nutritionally fastidious microorganisms (*i.e.*, *Ureaplasma* spp., *Mycoplasma* spp., *Fannyhessea vaginae* [formerly *Atopobium vaginae*], *Prevotella* spp., *Sneathia* spp., *Megasphaera* spp., *Mobiluncus* spp. and others). This drawback also precluded CST assignment and species-level identification of lactobacilli, making a detailed analysis of the vaginal microbiota of the cohort impossible.

Conclusions

There is an urgent need for biomarkers to predict pregnancy outcomes in IVF-EF treatments. As the female genital microbiota is increasingly being considered amongst the possible predictors of embryo implantation, it is important to identify a dysbiotic environment not only prior to ART initiation, but particularly after COH therapy and before ET. Our study has identified vaginal diamines as a readily quantifiable biomarker of vaginal dysbiosis and a potential predictor of embryo implantation outcome, which may be used to monitor the vaginal health of women undergoing ART. Although the diamine threshold of 9.5 μM requires validation in larger patient cohorts, VADA could serve as a rapid and practical tool to quantify diamines at oocyte retrieval, thereby supporting clinical decision-making by identifying patients who may need time to restore a healthier vaginal microbiota before embryo transfer.

Abbreviations

ART	Assisted reproduction technology
AUC	Area under the curve
AV	Aerobic vaginitis
BV	Bacterial vaginosis
CFU	Colony forming units
CI	Confidence interval
COH	Controlled ovarian hyperstimulation
DAO	Diamine oxidase
CST	Community state type
ET	Embryo transfer
IQR	Interquartile range
GnRH	Gonadotropin-releasing hormone
IVF	in vitro fertilization
hCG	Human chorionic gonadotropin
HT	Hormone therapy
OR	Odds ratio
Pre-HT	Before hormonal therapy
Post-HT	After hormonal therapy
POC	Point of care
ROC	Receiver operating characteristic
VADA	Vaginal diamine assay

Supplementary Information

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Supplementary Material 1: Relationship between vaginal lactobacilli before and after COH and implantation outcomes.

Supplementary Material 2.

Supplementary Material 3.

Supplementary Material 4.

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Authors' contributions

EL carried out the experiments and wrote the manuscript draft; SL performed the statistical analysis and participating in manuscript revision; KM developed and performed the VADA and collected the data; SDG carried out the statistical analysis of the manuscript draft; AL and RP participated in revising the manuscript; GM provided the clinical data; PP contributed to the study design and manuscript revision; FS interpreted the data and revised the manuscript; GP planned the study design, analysed the data and revised the manuscript; SR participated in the study design, interpreted the data and wrote the manuscript. All authors read and approved the final manuscript.

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Data availability

The datasets analysed during the current study have been uploaded as Supplementary Table S3.

Declarations

Ethics approval and consent to participate

The study protocol was approved by the ethic regional commission (CEAVSE, code 20170619). All participants signed a written informed consent.

Consent for publication

An informed written consent was signed by the participant.

Competing interests

The authors declare no competing interests.

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