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RESEARCH ARTICLE

Metabolomic profile as a noninvasive adjunct tool for the diagnosis of Grades III and IV endometriosis-related infertility

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Abstract

The aim of the present case-control study was to develop a noninvasive adjuvant tool for the diagnosis of endometriosis. Serum samples from 100 patients undergoing intracytoplasmic sperm injection were split into two groups according to the cause of infertility: an endometriosis group ($n = 50$), consisting of samples derived from patients with Grade III and IV endometriosis, and a control group ($n = 50$), comprising samples derived from patients with isolated male factor infertility. The metabolomic profile of each sample was obtained, through mass spectrometry. Partial least squares discriminant analysis was able to clearly classify the endometriosis and control groups. Ten potential biomarkers were selected based on their importance for model prediction. These ions were used to build the receiver-operating characteristic curve, which presented an area under the curve of 0.904 (95% confidence interval: 0.796–0.985). To validate the model, 30 other samples from infertile women without any evidence of endometriosis were tested. Considering these ions as possible biomarkers, the model was able to correctly classify 84% of the patients. Finally, a similar prediction potential was observed in the model validated set, when samples from the disease-free group were tested. Serum metabolomics may be useful as a noninvasive adjunct tool for the selection of patients who must undergo laparoscopy for definitive endometriosis diagnosis.

KEYWORDS

biomarker, endometriosis, mass spectrometry, metabolomics

1 | INTRODUCTION

Although the first studies of endometriosis were conducted more than 150 years ago, many aspects of the disease, including its pathogenesis, are still unknown. It is generally accepted that endometriosis is a heterogeneous disease defined as the presence of endometrial-type mucosa outside the uterine cavity (Vercellini, Vigano, Somigliana, & Fedele, 2013). Despite the fact that endometriosis significantly reduces quality of life, endometriosis-induced pain can often be confused with menstrual cramps, and because a complete diagnosis requires laparoscopic surgery (Ahn, Singh, & Tayade, 2017; Dunselman et al., 2014), instead of establishing the diagnosis of endometriosis by invasive approaches, empirical medical treatment for pain symptoms may be prescribed, and the diagnosis is usually postponed (Hudelist et al., 2012; Nnoaham et al., 2011). It was reported that adolescent girls who suffer from the symptoms of endometriosis delay seeking medical attention by 4.6 years, and by the time they do, it takes another 4.7 years to obtain a diagnosis (Greene, Stratton, Cleary, Ballweg, & Sinaii, 2009).

Even though laparoscopy has been considered the gold standard diagnostic technique for pelvic pain, a visual diagnosis of endometriosis during laparoscopy has been demonstrated to be unreliable. While endometriosis may be present in an apparently

normal peritoneum (Khan et al., 2014), a positive finding through the use of laparoscopy is of limited value without histological confirmation (Wykes, Clark, & Khan, 2004). It has been reported that laparoscopy positive for endometriosis will not be confirmed by histology in 50% of cases (Walter, Hentz, Magtibay, Cornella, & Magrina, 2001). Moreover, laparoscopy has been pointed out as an invasive, expensive, and potentially risky procedure (Surrey, Soliman, Yang, Du, & Su, 2017).

Approximately 5–10% of reproductive-age women are affected by endometriosis, and at least one-third of these are infertile (de Ziegler, Borghese, & Chapron, 2010). Hormone treatments can alleviate the symptoms of the disease, but it is not a choice for infertile patients trying to conceive (Practice Committee of the American Society for Reproductive, 2012). Therefore, the remaining possibilities for these patients are laparoscopy or assisted reproductive technology (ART; de Ziegler et al., 2010). However, concerns remain about elevated oestradiol levels caused by controlled ovarian stimulation (COS), which might negatively affect estrogen-dependent endometriosis (Giudice & Kao, 2004).

To clarify this issue, Santulli et al. (2016) evaluated 264 matched pairs of endometriosis patients and disease-free women undergoing in vitro fertilization. The authors concluded that ART does not aggravate the symptoms of endometriosis or negatively impact the quality of life in endometriosis-related infertility patients.

In light of this, what would be the rationale for subjecting patients with endometriosis-related infertility to laparoscopy to confirm the diagnosis of endometriosis, as ART may be performed, and pregnancy may be achieved? Nonsurgical methods for the diagnosis of endometriosis could potentially improve the quality of life of these patients (Surrey et al., 2017).

Imaging examinations such as ultrasound and magnetic resonance may suggest endometriosis and effectively guide surgical intervention for patients with suspected endometriosis; however, alone, these techniques have limited utility in the diagnosis of endometriosis, as it lacks adequate resolution to identify adhesions or superficial peritoneal implants (Hsu, Khachikyan, & Stratton, 2010).

Several studies have demonstrated the utility of CA-125 for the diagnosis of endometriosis and its correlation with disease severity (Mabrouk et al., 2012; Socolov et al., 2011). However, CA-125 is not specific for endometriosis, being an ovarian cancer marker. In addition, the specificity and sensitivity to detect all endometriosis stages are low (Hirsch et al., 2016; Mol et al., 1998).

In the postgenomic era, several studies have put forward efforts to stimulate the concept of molecular profiling in biological systems. Metabolomics enables the characterization of endogenous small molecules, referred to as metabolites, that are the products of biochemical reactions, revealing connections among different pathways that operate within a living cell (Wang et al., 2011). Thus, metabolomics emerges as a powerful tool to identify biomarkers for given conditions in different types of biological samples.

In the last decade, studies have focused on the identification of potential metabolic biomarkers of endometriosis in follicular fluid (Cordeiro et al., 2015; Karaer, Tuncay, Mumcu, & Dogan, 2018; Marianna et al., 2017), blood (Dutta et al., 2012; Letsiou et al., 2017; Vicente-Munoz et al., 2016; Vouk et al., 2012; Zheng, Pan, & Liu, 2011), urine (Vicente-Munoz et al., 2015), and even endometrial tissues (Li et al., 2018); however, these potential biomarkers have not been properly validated. Therefore, the goal of the present study was to make use of the analytical power of mass spectrometry (MS) to develop an adjuvant tool for the diagnosis of Grades III and IV endometriosis in infertile patients.

2 | RESULTS

Except for the pregnancy rate, the patient and cycle characteristics did not differ between groups (Table 1).

A total of 429 and 484 ions for the positive and negative ionization modes were analyzed, respectively. Considering components one, two and three, the PLS-DA was able to clearly distinguish the Endometriosis Group from the Control Group for both positive (Figure 1a) and negative (Figure 1b) ionization modes.

Ten potential biomarkers were selected based on their importance for model prediction, five in the positive and five in the negative ionization mode. Two metabolites were identified by the database. Triacylglycerols and α-amino acids were overs abundant in the serum of positive endometriosis patients, whereas the other ions were not identified by the currently available database. The relative abundance of individual biomarkers, the average abundance of the compounds in each group and metabolite identification are described in Table 2.

These ions were used to build the ROC curve, which presented an area under the curve (AUC) of 0.904 (95% confidence interval [CI]: 0.796–0.985, Figure 2a), indicating the accuracy of the biomarkers for sample classification in the Control or Endometriosis groups. Considering these ions as possible biomarkers, the model was able to correctly classify 84% of the patients (Figure 2b). A similar prediction potential was observed in the statistical validation set, when 30 more samples from infertile women without evidence of endometriosis, the disease-free group, were tested (Table 3).

When individual ROC curves were constructed, all 10 potential biomarkers presented AUCs between 0.70 and 0.88.

3 | DISCUSSION

The "omics" revolution led to the introduction of metabolomics, an emerging field that speeds up the understanding of global metabolic characteristics, elucidation of metabolic mechanisms, and identification of metabolic biomarkers (Peng, Li, & Peng, 2015). For the present study, the analytical application of MS was successfully tested as an adjuvant tool to diagnose Grades III and IV endometriosis in infertile patients. The data revealed 10 potential biomarkers of endometriosis, with a prediction potential of 90.4%.

Diagnosing endometriosis is challenging because symptomatology may vary widely and the disease course is unpredictable.

TABLE 1 Patient and cycle characteristics for the endometriosis and control groups

Variables	Endometriosis ($n = 50$)	Control ($n = 50$)	p value
Maternal age (years)	33.6 ± 3.3	34.4 ± 2.5	.176
Paternal age (years)	39.0 ± 8.2	37.2 ± 5.3	.198
Maternal BMI	24.5 ± 4.4	24.604 ± 3.1	.904
Total dose of FSH	2554.9 ± 634.7	2580.9 ± 669.5	.845
Oestradiol level	3279.2 ± 2341.5	2475.7 ± 2058.5	.183
Follicles	16.1 ± 8.2	14.7 ± 10.3	.178
Oocytes	11.9 ± 7.8	10.0 ± 7.7	.140
Oocyte yield (%)	75.2 ± 21.6	73.1 ± 23.6	.160
Mature oocytes	8.3 ± 6.4	7.7 ± 5.7	.138
Mature oocytes rate (%)	73.3 ± 21.6	78.5 ± 18.5	.144
Fertilization rate (%)	81.4 ± 20.3	85.2 ± 15.8	.300
High-quality embryos on Day 3 (%)	57.2 ± 24.1	54.0 ± 24.7	.747
Blastocyst formation rate (%)	40.7 ± 34.7	43.2 ± 28.3	.787
Transferred embryos	2.1 ± 0.6	2.0 ± 0.6	.385
Clinical pregnancy rate (%)	16/50 (32.0)	36/50 (72.0)	.007
Miscarriage rate (%)	2/16 (12.5)	5/36 (13.8)	.347
Implantation rate (%)	30.7 ± 39.8	32.8 ± 27.9	.121

Note: Values are means ± standard deviation unless otherwise noted.
Abbreviations: BMI, body mass index; FSH, follicle-stimulating hormone.

A thorough history and careful physical examination are imperative. According to the European Society of Human Reproduction and Embryology (ESHRE) guidelines for the management of women with endometriosis, symptoms that are predictive of the diagnosis of endometriosis are dysmenorrhea, noncyclical pelvic pain, deep dyspareunia, infertility, and fatigue (in the presence of any of the above), as well as other nongynaecological cyclical symptoms such as dyschezia, dysuria, hematuria, rectal bleeding, and shoulder pain (Dunselman et al., 2014). These symptoms may be a clue to the presence of the disease and may help to identify which patients should undergo video laparoscopy.

Laparoscopy has been considered the gold standard method for endometriosis diagnosis; however, in a recently published study including 24,915 women who underwent a hysterectomy and 37,308 who underwent a laparoscopy, it was concluded that both of these treatments are associated with a substantial burden of negative outcomes, including significant health care resource use. It was described that endometriosis patients are at considerable risk of surgical complications, subsequent surgeries and hospital admissions, both during and after their initial therapeutic laparoscopy or hysterectomy (Surrey et al., 2017).

This, associated with the fact that laparoscopy is far from 100% accurate (Khan et al., 2014; Walter et al., 2001; Wykes et al., 2004), raises the question of whether laparoscopy should be indicated in all suspected cases of endometriosis. Laparoscopy obviously has its merit in both diagnosis and treatment of endometriosis (Schipper & Nezhat, 2012); however, it could be argued that better screening of patients to be submitted to laparoscopy, not only for the presence of specific symptoms and by physical examination but also for the presence of serum biomarkers, may benefit patients.

Recently, metabolomics technology has been applied aiming to identify endometriosis biomarkers. Although many studies have shown remarkable changes in the metabolite profile (Dutta et al., 2012; Dutta et al., 2018; Li et al., 2018) of endometriosis patients' serum, the identification of possible biomarkers is still sparse, with insufficient sensitivity and specificity. Moreover, potential biomarkers were not properly validated.

The model presented here revealed that triacylglycerol (TG) and α-amino acids are metabolites overs abundant in patients presenting with endometriosis. Intriguingly, Domínguez et al. (2017), found reduced levels of saturated diacylglycerols and saturated TGs in the endometrial fluid of patients with endometriosis when compared with controls. Excessive accumulation of TGs, however, is associated with human diseases such as obesity, diabetes mellitus, and steatohepatitis. Although TGs are essential for normal physiology, excessive accumulation of TG in human adipose tissue results in obesity and in nonadipose tissues is associated with organ dysfunction (Yen, Stone, Koliwad, Harris, & Farese, 2008).

As for α-amino acid, previous reports have detected changes in its profile in patients with ovarian cancers (Miyagi et al., 2017), suggesting that alterations in amino acid metabolism may be related to the inflammation cascade leading to ovarian cancers and probably to endometriosis. In fact, several studies have indicated an increased risk of ovarian cancer among women with endometriosis (Aris, 2010; Brinton et al., 2005; Heidemann, Hartwell, Heidemann, & Jochumsen, 2014; Vercellini et al., 2000). Furthermore, histopathology has

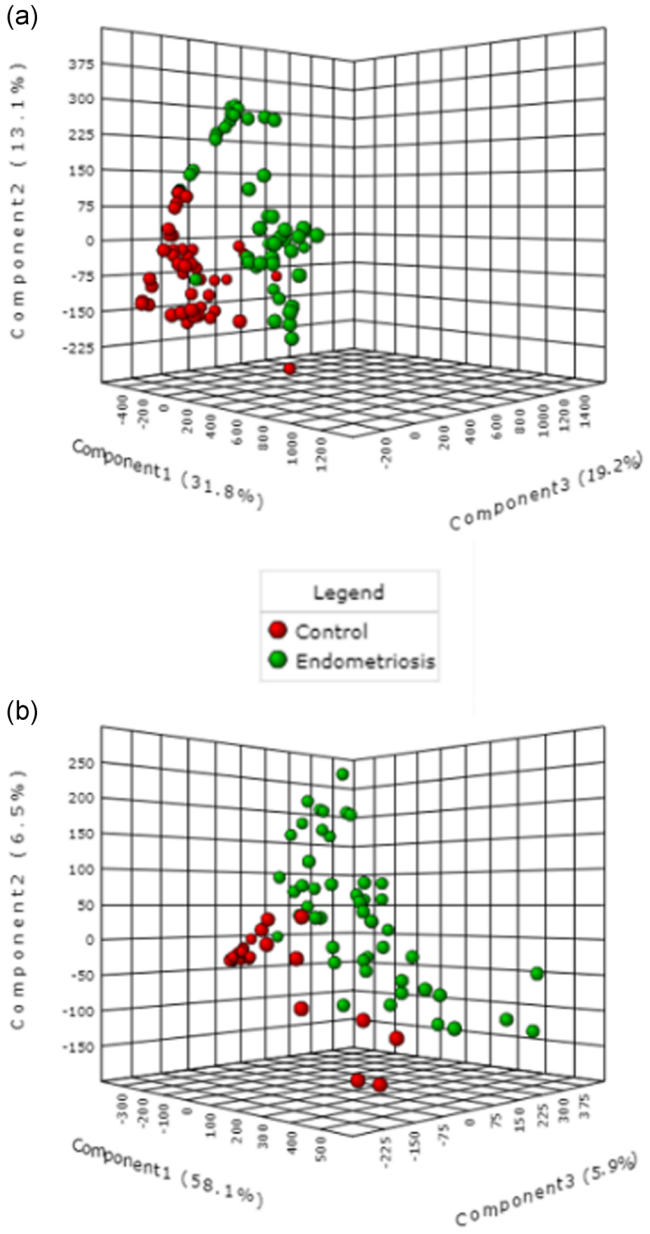

FIGURE 1 Partial least square discriminant analysis (PLS-DA) score plot: variance among groups (Endometriosis in red and Control in green) according to PLS-DA considering the components 1, 2, and 3, for the positive (a) and negative (b) ionization modes

suggested atypical endometriosis as a transition between benign endometriosis and malignancy (Wiegand et al., 2010).

The value of the model presented here was proven when samples from infertile women without evidence of endometriosis were tested. The achieved prediction potential was close to 80%. Nevertheless, the study has some pitfalls. Even though the model has been tested in another population of disease-free patients, the biomarkers should be validated in independent sets for both endometriosis and control groups.

Since endometrial tissue communicates with the blood directly and through extracellular fluids, several metabolites may be secreted into the bloodstream. Therefore, a serum marker is expected to be reliable to diagnose endometriosis; however, for that, ion identification is an essential step in defining the importance of the proposed biomarker and its role in pathogenesis. There are several databases for metabolite identification; however, these databases are limited due to the different instruments used to generate the spectrum; therefore, most compounds are useful but unidentified.

For the present study, 10 ions were determined to contribute the most to the prediction model; however, only two of these were identified. Even so, the metabolomic profiles of histologically confirmed endometrioses patients were clearly distinct from those of disease-free patients. The ions selected by the model presented a high predictive power and were able to correctly classify 84% of the samples.

These lines of evidence suggest that serum metabolomics may be a valuable approach to the diagnosis of endometriosis and may be used as an adjunct tool for the selection of patients who must undergo laparoscopy to obtain a definitive diagnosis. Nevertheless, these data must be validated in a larger patient population and in independent sets of subjects.

4 | MATERIALS AND METHODS

4.1 | Experimental design

For this case-control study, serum samples from 100 patients undergoing intracytoplasmic sperm injection (ICSI), from January 2017 to December 2017, in a private, university-affiliated in vitro fertilization center, were collected.

Samples were collected from female patients aged below 38 years in whom embryo transfers were performed on Day 5. Samples were split into two groups according to the cause of infertility: the endometriosis group ($n = 50$), consisting of samples derived from patients with Grade III and IV endometriosis, classified according with the American Society for Reproductive Medicine (ASRM; Practice Committee of the American Society for Reproductive, 2012), and the control group ($n = 50$), comprising samples derived from patients with isolated male factor infertility. Clinical diagnosis and classification of subjects in the endometriosis group were performed through laparoscopic surgery followed by histology to confirm the presence of endometriotic lesions. The metabolomic profile of each sample was obtained and analyzed. To validate the model, 30 other samples from infertile women without any evidence of endometriosis (the disease-free group) were tested.

Written informed consent, in which patients agreed to share the outcomes of their ART cycles for research purposes, was obtained, and the study was approved by the local institutional review board.

4.2 | Controlled ovarian stimulation and laboratory procedures

Controlled ovarian stimulation was achieved by pituitary blockage using a GnRH antagonist (GnRH; Cetrotide®; Merck KGaA,

TABLE 2 Relative abundance of individual biomarkers, average abundance of the compounds in each group, and metabolite identification based on their respective m/z

m/z	VIP score	AUC	Identification	Adduct	Formula	Error (ppm)	Group average (intensity)	
Positive ionization							Control	Endometriosis
758.7234	6.58	0.70	–	–	–	–	5523.76	23410.78
786.7585	5.44	0.71	–	–	–	–	2460.38	13588.32
758.7155	4.43	0.70					15377.56	4519.02
782.7239	4.25	0.70	Triacylglycerol	$(M + NH4)^+$	C48H92O6	0.00	3443.86	11624.00
369.4541	3.92	0.72	–	–	–	–	5054.12	11531.98
Negative ionization								
279.3316	6.65	0.87	–	–	–	–	3755.58	15441.52
215.1182	5.18	0.82	α-amino acid	$(M - H)^-$	C8H16N4O3	15.00	3049.46	9079.76
255.3261	5.15	0.88	–	–	–	–	2075.80	8039.44
281.3487	5.08	0.74	–	–	–	–	4868.14	14340.04
283.36375	3.74	0.73	–	–	–	–	2206.54	6372.40

Abbreviations: AUC, area under the curve; ROC, receiver-operating characteristic; VIP, variable influence on projection.

Darmstadt, Germany); ovarian stimulation was performed using recombinant follicle-stimulating hormone (FSH; Gonal-F®; Serono).

Follicular growth was monitored using transvaginal ultrasound examination starting on Day 4 of gonadotropin administration. When adequate follicular growth and serum E2 levels were observed, recombinant hCG (Gonal-F®; Merck KGaA, Darmstadt, Germany) was administered to trigger final follicular maturation. Oocytes were collected 35 hr after hCG (Ovidrel®; Merck KGaA, Darmstadt, Germany) administration through transvaginal ultrasound ovum pickup.

The recovered oocytes were assessed to determine their nuclear status, and those in metaphase II were submitted to ICSI following routine procedures (Palermo, Colombero, & Rosenwaks, 1997).

4.3 | Embryo culture and morphology evaluation

Approximately 16 hr after ICSI, fertilization was confirmed by the presence of two pronuclei and the extrusion of the second polar body. The embryos were maintained in a 50 µl drop of culture medium (Global®; LifeGlobal, CT) supplemented with 10% protein supplement and covered with paraffin oil in a humidified atmosphere under 6% CO_2 at 37°C for 3 days.

The embryo morphology was assessed 16–18 hr post ICSI and on the mornings of Days 2, 3, and 5 of embryo development using an inverted Nikon Diaphot microscope (Eclipse TE 300; Nikon, Tokyo, Japan) with a Hoffmann modulation contrast system under ×400 magnification.

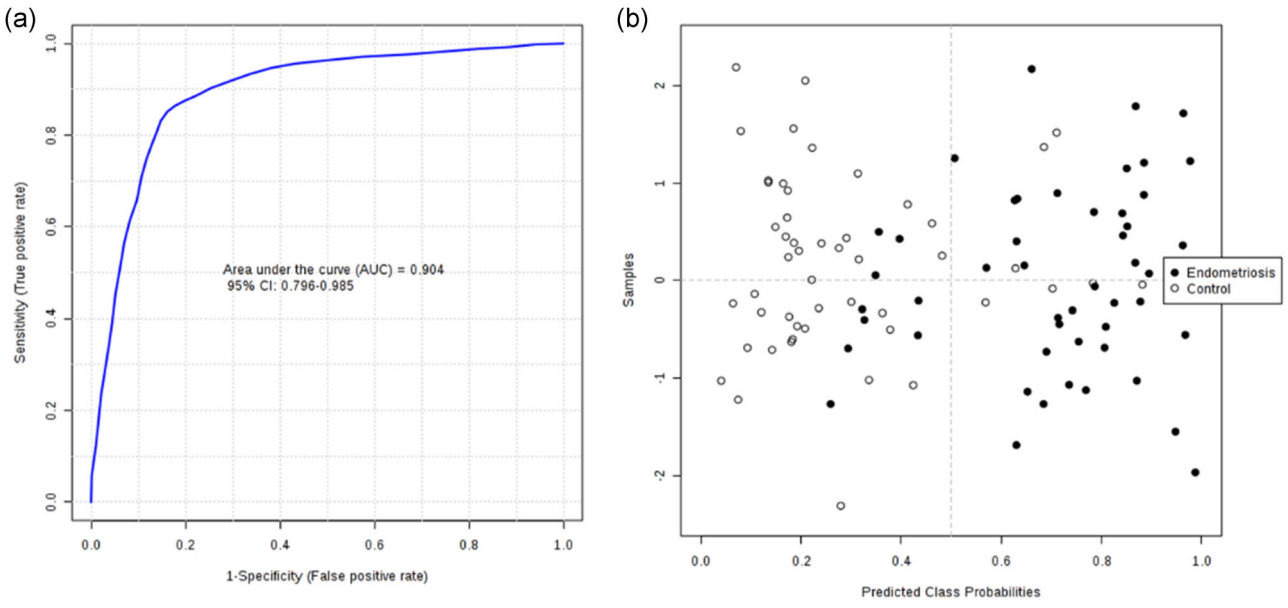

FIGURE 2 (a) ROC curve considering the ions selected by PLS-DA. (b) Sample classification based on the ROC curve analysis, in which 84% of samples were correctly classified. AUC, area under the curve; CI, confidence interval; ROC, receiver-operating characteristic

TABLE 3 Validation set classification—Disease-free group sample classification according to the predictive model

Sample	Probability	Classification	Sample	Probability	Classification
1	.89	Control	16	.91	Endometriosis
2	.89	Control	17	.89	Control
3	.89	Control	18	.89	Control
4	.89	Control	19	.89	Control
5	.89	Control	20	.89	Control
6	.89	Control	21	.89	Control
7	.78	Control	22	.89	Control
8	.89	Control	23	.89	Control
9	.89	Control	24	.60	Endometriosis
10	.88	Control	25	.96	Endometriosis
11	.89	Control	26	.89	Control
12	.89	Control	27	.89	Control
13	.89	Control	28	.89	Control
14	.89	Control	29	.78	Endometriosis
15	.89	Control	30	.89	Control

To evaluate the cleavage-stage morphology, the following parameters were recorded: the number of blastomeres, the percentage of fragmentation, the variation in blastomere symmetry, the presence of multinucleation, and the defects in the zona pellucida and cytoplasm. The high-quality cleavage-stage embryos were defined as those with all of the following characteristics: four cells on Days 2 or 8–10 cells on Day 3, less than 15% fragmentation, symmetric blastomeres, the absence of multinucleation, colorless cytoplasm with moderate granulation and no inclusions, the absence of perivitelline space granularity and the absence of zona pellucida dysmorphism. Embryos lacking any of these characteristics were considered to be of low quality.

To evaluate the blastocyst morphology, embryos were given a numerical score from one to six based on their degree of expansion and hatching status as follows: (a) an early blastocyst with a blastocoel that was less than half of the volume of the embryo; (b) a blastocyst with a blastocoel that was greater than half of the volume of the embryo; (c) a full blastocyst with a blastocoel that completely filled the embryo; (d) an expanded blastocyst; (e) a hatching blastocyst; and (f) a hatched blastocyst. Full, expanded, hatching, and hatched blastocysts were classified as complete blastocysts.

Full blastocysts onwards, presenting morphologically normal inner cell mass (ICM) and trophectoderm (TE) were defined as high-quality blastocysts. A tightly packed ICM presenting many cells was defined as a high-quality ICM. Similarly, the TE was classified as high quality by the presence of many cells forming a cohesive epithelium.

4.4 | Embryo transfer and clinical follow-up

Embryo transfers were performed on Day 5 of embryo development. Up to three embryos were transferred per patient, according to the ASRM and Society for Assisted Reproductive Technology guidelines for the number of embryos to transfer (Practice Committee of the American Society for Reproductive Medicine. Electronic address & Practice Committee of the Society for Assisted Reproductive, 2017). A pregnancy test was performed 10 days after embryo transfer. All women with a positive test had a transvaginal ultrasound scan 2 weeks after the positive test. Clinical pregnancy was diagnosed when the fetal heartbeat was detected. Pregnancy rates were calculated per transfer. The implantation rate was calculated by dividing the number of gestational sacs with fetal heartbeats by the number of transferred embryos. Miscarriage was defined as clinical pregnancy loss before 20 weeks.

4.5 | Endometrial preparation

On the day of ovum pickup, patients received 600 mg of progesterone vaginally per day divided into three doses, until embryo transfer. Progesterone was suspended in the presence of a negative β-hCG test or maintained until 6 to 12 weeks of gestation in the presence of a positive β-hCG test.

4.6 | Sample preparation and metabolite extraction

Blood samples were collected from fasted patients, on the morning of Day 3 of the menstrual cycle, using an ethylenediaminetetraacetic acid tube. Samples were centrifuged at 4°C and 800*g* for 10 min to obtain plasma, which was immediately stored at −20°C until metabolite extraction.

Metabolite extraction was performed by adding 250 µL of HPLC grade methanol/chloroform (2:1 v/v; both solvents were purchased from Merck Millipore, MA, EUA) to 50 µl of serum. The mixture was vortexed for 1 min and incubated on ice for 30 min for protein

precipitation. After incubation, samples were centrifugated at 8,000*g* for 10 min at 4°C. Finally, 200 µl of the organic layer was transferred to a 96-well plate for MS analysis. Blanks were prepared by concentrating 200 µl of the solvent solution.

4.7 | Metabolomics

Spectra were acquired in both positive and negative ionization modes using an Apollo II electrospray ion source mass spectrometer (Bruker, Billerica) coupled to a UFLC Prominence Binary Liquid Chromatograph (Shimadzu, Kyoto, Japan). The plate was stored in a SIL-30AC autosampler at 10°C before analysis. Each sample (1 µl, without column) was carried to the analyzer with solvent injection: acetonitrile/2-propanol (4:1, v/v) solution with 20 mol/L ammonium formate (20 mmol/L; Sigma-Aldrich, St Louis), at a flow rate of 200 µl/min during 2 min. High-resolution MS data were acquired with the electrospray ionization (ESI) source set as follows: nebulizer gas at 2.0 bar, dry gas at 8.0 L/min, the dry temperature at 180°C, and voltage at 4.5 kV. The mass/charge ratios were scanned (m/z 100–1,000 Da).

Sodium formate clusters in isopropyl alcohol within the m/z 50–1,200 Da range were used as a calibration standard. Acetonitrile and isopropyl alcohol (LC-MS grade) were purchased from Scharlau (Barcelona, Spain).

Data from ESI-MS were calibrated with sodium formate signals in DataAnalysis 4.1 software (Bruker Daltonics, MA). The ion list and intensities were transferred to Excel software (version 2016) for statistical analyses, and ions with intensity greater than 200 were considered exclusively.

Aiming to ensure the reproducibility of the study, a pool of samples from both groups was run alongside every 20 samples so that quality control could be guaranteed. Moreover, hypothetical samples containing the solution used for metabolite extraction (blank samples) were run to generate a reference spectrum of possible noise signals from the analysis.

4.8 | Data processing and statistical analysis

Data analyses were performed using MetaboAnalyst (version 3.0) software (http://www.metaboanalyst.ca). A mass tolerance of 0.01 Da was adopted for signal alignment, and the m/z of blank samples were removed from the analysis. Ions containing more than 50% of intensities 0,0 in both groups were also removed from the analysis.

The intensity values were standardized by Paretto for each ionization mode, and the supervised test, partial least square discriminant analysis (PLS-DA) was applied to the data set to determine the discriminatory components based upon the combination of variable influence on projection (VIP) values.

Five ions from each ionization mode were selected as potential biomarkers and were used to build a single ROC curve, considering the biomarkers as a set and individually. In addition, biomarkers were tested by statistical model validation using 30 new samples from infertile women without evidence and/or diagnosis of endometriosis (the disease-free group).

Metabolite identification was performed using the Human Metabolite Database (https://hmdb.ca), considering the maximum mass error as 20 ppm. For the identification, only molecules containing hydrogen $(M + H)^+$, sodium $(M + Na)^+$, and potassium $(M + K)^+$, and ammonium $(M + NH4)^+$ as adducts were considered. Deprotonation $(M-H)^-$ was considered for negative ionization.

Patient and cycle characteristics, along with clinical and laboratory results, were analyzed using the SPSS Statistics 21 (IBM, New York, NY) statistical program. Variables were tested for normality distribution and group homogeneity using the Shapiro--Wilk and Levene tests, respectively. When necessary, samples were standardized using the *z*-score.

Maternal and paternal age; the total FSH dose used for COS; the number of aspirated follicles, retrieved oocytes, and obtained embryos; the fertilization rate and the implantation rate were compared between groups using Student's *t* test, whereas pregnancy and miscarriage rates were compared by χ^2 analysis. Variables were described as the mean percentage ± standard deviation, and the significance level α was 5%.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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