For the present study we asked whether the endometrial fluid lipidomic may be a useful approach to predict endometrial receptivity in freeze-all cycles. For this case-control study, endometrial fluid samples were collected from 41 patients undergoing freeze-all cycles. Samples were split depending on the pregnancy outcome: positive group ($n = 24$) and negative group ($n = 17$). Data were acquired by the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were applied. A list of potential biomarker ion ratios was obtained and the values were used to build a receiver operating characteristic (ROC) curve to predict pregnancy success. The lipid categories were attributed by LIPID MAPS database. Ion ratios were established according to their correlations and used for the analysis. The PCA showed a tendency of separation between the studied groups, whereas the PLS-DA was able to clearly distinguish them. Fifteen ratios (13 hyper-represented in the negative and two hyper-represented in the positive group) were selected according to their importance for model prediction. These ratios were used to build the ROC curve, which presented an area under curve of 84.0% (95%CI: 69.2–97.4%; $p = 0.009$). These findings suggest that lipidomic profiling of endometrial fluid may be a valuable tool for identifying the time interval comprising the window of implantation.

KEYWORDS
endometrium, lipidomics, mass spectrometry, window of implantation, WOI

1 INTRODUCTION

Since the first reported pregnancy following in vitro fertilization (IVF; Steptoe & Edwards, 1978), assisted reproductive technologies (ART) have rapidly evolved. It is estimated that 1–3% of children born in developed countries are conceived through ART. In the most recent world report by the International Committee for Monitoring ART, over 237,000 infants were born through ART worldwide in a single year (Sullivan et al., 2013). Nevertheless, its efficiency in terms of live birth is still low. In a recently published report, comprising 584,835 stimulated IVF cycles, the overall birth rate were 22.5% and it was estimated that 4.45 cycles are needed, across all age groups, to achieve one live birth event following IVF and fresh embryo transfer (Sunkara, LaMarca, Polyzos, Seed, & Khalaf, 2016).

Embryo implantation is an essential event for the human reproduction, but it is a critical and limiting stage in the process of human reproduction (Su & Fazleabas, 2015). It depends on three critical events: (a) the presence of a viable embryo, (b) the acquisition
of a receptive endometrium, and (c) proper dialog between them (Domínguez, Pellicer, & Simon, 2003).

The endometrium is a highly dynamic tissue that undergoes cyclic cellular proliferation, differentiation, and immune cell trafficking in response to changing circulating ovarian-derived steroids (Valdes, Schutt, & Simon, 2017). Synchronized production of progesterone and estrogen mediates structural and functional changes in the uterus that enable the blastocyst to attach (Zhang et al., 2013). The rising estrogen level during the first part of the menstrual cycle increases endometrial cell proliferation (proliferative phase). Following ovulation, progesterone secreted by the luteinized follicles leads to the differentiation of these cells (secretory phase). At this point, the endometrium is mature and ready for embryo implantation (Achache & Revel, 2006).

The embryo is unable to adhere to it through most of the menstrual cycle in humans, except during a short, self-limited period, in which the endometrial tissue acquires a functional and transient status that permits blastocyst adhesion. This limited period of time is referred to as the “window of implantation” (WOI), and in humans, this “receptive phase” lasts for only approximately 2 days, occurring about 8–10 days after ovulation (Direito, Bailly, Mariani, & Ecochard, 2013; Valdes et al., 2017).

The fertilization takes place at the same time that secretory transformation begins in the endometrium. If both are normal, then development will be synchronous and implantation is possible. However, if the endometrium is not optimally receptive when the embryo is ready to implant, asynchrony takes place (Franasiak, Ruiz-Alonso, Scott, & Simon, 2016).

Accumulated evidence has suggested that there is an alteration of endometrial receptivity in patients with recurrent implantation failure (RIF; i.e., failure of three IVF cycles in which one or two high-grade quality embryos were transferred to the patient in each cycle). In the pre-IVF era, classical histologic analysis showed that women with repeated donor insemination failures have altered endometrial progression in relation to their menstrual cycle (Li et al., 1993). Moreover, hysteroscopy studies have shown that 18–50% of RIF patients have abnormalities of the uterine cavity (Demirol & Gurgan, 2004; Margalioth, Ben-Chetrit, Gal, & Eldar-Geva, 2006), whereas temporal displacement of the WOI has been described in one out of four RIF patients (Ruiz-Alonso et al., 2013).

Uterine pathologies, when diagnosed, may be corrected; however, the obvious fact that implantation requires synchrony between the endometrium and the embryo needs more attention. Therefore, determining the ideal timing for embryo transfer is crucial. Predictors of the uterine receptive state are needed to enable a better understanding of the causes of endometrial-based infertility and help women with RIF due to a possible asynchrony between embryo and endometrium, to achieve pregnancy.

The "omics" revolution has stimulated the concept of molecular profiling in biological systems. Despite having already been an intensive area of research in the 1960s (Han & Gross, 2005; Oresic, Hanninen, & Vidal-Puig, 2008), lipid research has recently gained prominence with the emergence of lipidomics (Postle, 2012).

Lipidomics can be defined as the large-scale study of lipid species and their related networks, and metabolic pathways that exist in cells or any other biological system (Gross, 2017). Lipids have several functions that go beyond cell membrane structure and energy storage. Lipid mediators participate in signal transduction pathways, proliferation, apoptosis, and membrane trafficking in the cell (Loizides-Mangold, 2012). There is evidence that lipid homeostasis is essential for the maintenance of health, and that both metabolism and lipid homeostasis are involved in the pathogenesis of important diseases. However, there is limited understanding of the role of lipids in organisms when compared to other fields such as gene or proteomic expression (Oresic et al., 2008).

Modern approaches to lipid analysis are preferably performed by mass spectrometry (MS; Want, Cravatt, & Siuzdak, 2005), which allows the study of intact lipid molecule species from samples with very low volumes (Schwudke et al., 2006). Thus, the study of signaling molecules, such as lipids, in the endometrial fluid is fundamental in creating new diagnostic tools and therapeutic targets for future treatment of RIF.

In light of this, the goal of the present study was to make use of the analytical power of mass spectrometry to identify lipid biomarkers capable of differentiating receptive from nonreceptive endometrium.

2 | RESULTS

2.1 | Patient and cycle characteristics

Patient and cycle characteristics did not differ among the groups (Table 1).

2.2 | Lipid analysis

The raw data were processed and 265 ions were used for statistical analysis. Ion ratios were established according to their correlations and those variables were used for the analysis.

The fold-change analysis detected 13 ratios with a two-fold increased representation in the positive group and 84 ratios with a two-fold increased representation in the negative group (Figure 1a). According to t test, 16 ratios were differentially represented among the groups (p < 0.05; Figure 1b), and the volcano plot analysis detected five ratios two-fold differentially represented among the groups with statistical significance (Figure 2).

The PCA analysis showed a tendency of separation between the studied groups (Figure 3a), while the PLS-DA was able to clearly distinguish the positive from the negative group (Figure 3b).

Fifteen ratios (13 hyperrepresented in the negative group and two hyper-represented in the positive group) were selected based on their importance for the model prediction (Figure 4). These ratios were used to build the receiver operating characteristic (ROC) curve, which presented an area under the curve of 84.0% (95%CI: 69.2–97.4%; p = 0.009; Figure 5), and were considered possible biomarkers of endometrium receptivity.
The ions identified by the LIPID MAPS database were: phosphoethanolamine, phosphatidic acid, diacylglycerol, triacylglycerol, glycosyl diacylglycerol, phosphatidylcholine, neutral sphingolipid, and lysophosphatidylglycerol.

Functional enrichment analysis revealed that an increase in the ratio of triacylglycerol and phospholipids leads to remodeling of low-density lipoproteins, which may be associated with changed steroid syntheses and release, and therefore displacement in the WOI.

3 | DISCUSSION

Implantation involves a complex sequence of events that are crucial to pregnancy success. However, the implantation process efficiency is relatively low (Gnoth, Godehardt, Godehardt, Frank-Herrmann, & Freundl, 2003; Racowsky, 2002). Indeed, despite the marked improvements in IVF success rates over the last few decades, there is still a wide range of patients, in whom high-quality embryos are transferred, that experiences RIF, which may be caused by factors related to the endometrium (Timeva, Shterev, & Kyurkchiev, 2014).

Extensive research has been carried out in a search for markers that can be used clinically to define the exact time of the WOI. This would determine the right time for embryo transfer. For the present study, the use of a fast and simple method of mass spectrometry, to identify possible lipid biomarkers of the receptive endometrium, was tested. Fifteen ion ratios, mostly correlated with implantation failure, were identified in the endometrial fluid.

The development of microarray technology allows the investigation of the transcriptomics of human endometrium in the different phases of the menstrual cycle, including within the receptivity phase (Horcajadas, Pellicer, & Simon, 2007). Accumulated knowledge about transcriptomic profiles has suggested potential transcriptomic predictors of endometrial receptivity, enabling identification of the WOI (Díaz-Gimeno, Ruiz-Alonso, Blesa, & Simon, 2014; Ruiz-Alonso et al., 2013; Ruiz-Alonso, Galindo, Pellicer, & Simon, 2014).

However, the overlap between the studies is relatively small. In addition, changes in gene expression are not necessarily reflected in changes in translated proteins, nor does gene analysis consider posttranscriptional, translational, or posttranslational changes that relate to cyclical transitions. Of the hundreds of gene expression changes typically identified by microarray, relatively few are common to more than two studies (Haooui, Dechaud, Assou, De Vos, & Hamamah, 2012; Horcajadas et al., 2006). Although the metastature of endometrial receptivity has recently been described (Altmay et al., 2017), comparisons of proteomic data with published gene expression data in similar cohorts of women (Burney et al., 2007; Chen et al., 2009; Haooui et al., 2012) have also revealed an overall lack of correlation between the two, suggesting that posttranscriptional or translational regulation is an important feature of endometrial remodeling.

### TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>Positive group (n = 24)</th>
<th>Negative group (n = 17)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age</td>
<td>32.5 ± 3.8</td>
<td>33.3 ± 4.3</td>
<td>0.521</td>
</tr>
<tr>
<td>Paternal age</td>
<td>39.1 ± 6.8</td>
<td>38.2 ± 6.5</td>
<td>0.667</td>
</tr>
<tr>
<td>BMI</td>
<td>24.1 ± 3.6</td>
<td>24.2 ± 3.4</td>
<td>0.564</td>
</tr>
<tr>
<td>FSH dose</td>
<td>2060.3 ± 538.2</td>
<td>2220.3 ± 613.1</td>
<td>0.381</td>
</tr>
<tr>
<td>Aspirated follicles</td>
<td>214 ± 12.4</td>
<td>210 ± 10.0</td>
<td>0.931</td>
</tr>
<tr>
<td>Retrieved oocytes</td>
<td>18.1 ± 10.9</td>
<td>16.4 ± 8.1</td>
<td>0.597</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>88.1 ± 9.1</td>
<td>85.9 ± 13.0</td>
<td>0.535</td>
</tr>
<tr>
<td>Obtained embryos</td>
<td>12.2 ± 7.5</td>
<td>11.6 ± 5.2</td>
<td>0.806</td>
</tr>
<tr>
<td>High-quality embryo rate (%)</td>
<td>46.4 ± 16.8</td>
<td>45.8 ± 20.7</td>
<td>0.930</td>
</tr>
<tr>
<td>Thaw embryo survival rate (%)</td>
<td>95.7 ± 1.5</td>
<td>94.3 ± 3.1</td>
<td>0.547</td>
</tr>
<tr>
<td>Transferred embryos</td>
<td>1.6 ± 0.48</td>
<td>1.7 ± 0.49</td>
<td>0.875</td>
</tr>
<tr>
<td>Endometrial thickness (mm)</td>
<td>9.4 ± 1.3</td>
<td>10.0 ± 1.0</td>
<td>0.153</td>
</tr>
</tbody>
</table>

Abbreviation: BMI: Body mass index.

The ions identified by the LIPID MAPS database were: phosphoethanolamine, phosphatidic acid, diacylglycerol, triacylglycerol, glycosyl diacylglycerol, phosphatidylcholine, neutral sphingolipid, and lysophosphatidylglycerol.

Functional enrichment analysis revealed that an increase in the ratio of triacylglycerol and phospholipids leads to remodeling of low-density lipoproteins, which may be associated with changed steroid syntheses and release, and therefore displacement in the WOI.

![Figure 1](wileyonlinelibrary.com)
Besides the studies with transcriptome, other studies evaluated the application of proteomics in the identification of receptive endometrium (Brosens et al., 2010; Chen et al., 2009; DeSouza et al., 2005).

Although analysis of tissue biopsies has some merits, it requires the direct biopsy of endometrial tissue before the embryo transfer cycle. To avoid the biopsy previous studies have focused on the proteomic analysis of uterine secretions, either as a direct aspirate or through flushing (Casado-Vela et al., 2009; Hannan et al., 2010; Parmar et al., 2008; Salamonsen et al., 2013; Scotchie, Fritz, Mocanu, Lessey, & Young, 2009).

In fact, uterine secretion analysis offers a means to avoid these difficulties, because it has already been demonstrated that aspiration of endometrial fluid does not affect pregnancy rates in the same cycle (C. Boomsma, Kavelaars, Eijkemans, Lentjes et al., 2009; van der Gaast, Beier-Hellwig, Fauser, Beier, & Macklon, 2003). Moreover, the uterine fluid, and in particular the glandular secretions that provide many of its components, is critical for implantation (Salamonsen et al., 2013).

Proteomic analysis may be considered a promising technique for understanding the functional changes experienced by the endometrium. However, its low sensitivity, which limits the number of proteins that can be evaluated, challenges its application (C. Boomsma, Kavelaars, Eijkemans, Amarouchi et al., 2009). Plasma-derived proteins, including albumin and gamma globulins, along with hemoglobin, if there is blood contamination during sample collection, represent most of the total protein in the samples, thereby masking the less abundant proteins and making their detection and analysis difficult (Hannan, Stoikos, Stephens, & Salamonsen, 2009).

Therefore, other “omics” approaches, such as metabolomics, may be more useful in guiding the identification of the receptive endometrium. Of the four kinds of biological molecules that comprise the human body, that is, nucleic acids, amino acids, carbohydrates, and lipids, lipids stand out among the various cellular metabolites for their sheer number of distinct molecular species. Lipids are extremely abundant in biological systems, constituting 50% of the mass of cell membranes of most animals (Oresic et al., 2008), as well as
presenting an important degree of specialization in specific cellular compartments (Sprong, van der Sluijs, & van Meer, 2001).

However, compared with genomic and proteomic analysis, lipidomic analysis has far been the least used method among all the research on the endometrium (Wenk, 2005). On the other hand, we know that the endometrium has a very important lipid component for reproduction. Among those lipids, prostaglandins (PGs) were probably, the most studied agents. Accumulated evidence has demonstrated a key role for PGs in embryo implantation in animals (Arosh, Banu, & McCracken, 2016; Waclawik, Kaczmarek, Blitek, Kaczynski, & Ziecik, 2017) and humans (Vilella, Ramirez, Berlanga et al., 2013)

Sordelli et al. (2012) demonstrated the participation of PGs in the process of implantation, by preparing the uterine milieu for embryo invasion during the WOI, in animal models. PGE2 and PGF2α levels were significantly increased during the WOI (Vilella, Ramirez, & Simon, 2013) and defective endometrial PG synthesis had been correlated with RIF (Achache, Tsafrrir, Prus, Reich, & Revel, 2010).

Vilella et al. (2013) investigated the lipidomic profile of endometrial fluid in natural cycles, hormonal replacement therapy cycles, and controlled ovarian stimulation (COS) cycles, and a significant increase in PGE2 and PGF2 was noted during the WOI. Interestingly, after inserting an intrauterine device in the hormonal replacement therapy patients, the secretion of both PGs significantly decreased throughout the cycle. Other used the MS based lipidomics for the rapid assessment of endometriotic tissues (Adamyan et al., 2018) and to study subtypes of endometrial cancer (Cummings et al., 2018).

In our study we aimed to evaluate the lipid signature of the endometrial fluid and to identify which lipids were differentially represented in the positive- and negative-implantation groups; however any specific group of lipids was measured. Prostaglandins were not among the lipids that contributed most to the model, here established. On the other side, the functional enrichment analysis of the identified ions revealed that an increase in the triacylglycerol and phospholipid ratio leads to the remodeling of low-density lipoproteins, which may be associated with changes in steroid synthesis and release.

Whether the increase in triacylglycerol and phospholipid ratio in endometrial fluid represents systemic changes is to be elucidated, however, it is well known that the acquisition of the receptive endometrium process is regulated by an interplay of the maternal steroid hormones, estrogen and progesterone (Zhang et al., 2013). Estrogen initiates hypertrophy and hyperplasia of endometrial epithelia. Progesterone transforms this prepared endometrium into a secretory tissue and creates an environment within the uterine milieu that is conducive to embryo attachment (Plant & Zeleznik, 2015).

Steroid hormones act through their intracellular receptors, which are ligand-inducible gene regulatory factors. It is therefore likely that steroids trigger the expression of a unique set of genes during the peri-implantation period and that these eventually lead to synthesis of new proteins that prepare the uterus to accept the invading blastocyst (Carrascosa, Horcajadas, & Moreno-Moya, 2018).

Several studies correlated implantation failure with the increase in progesterone level on the day of human chorionic gonadotropin (hCG) trigger in COS cycles (Andersen, Devroey, & Arce, 2006; Check, Choe, Katsoff, Summers-Chase, & Wilson, 1999; Haouzi et al., 2009; Haouzi et al., 2014; E. Kolibianakis et al., 2002; E.M. Kolibianakis, Venetis, Bontis, & Tarlatzis, 2012; Shapiro et al., 2011; Venetis, Kolibianakis, Bosdou, & Tarlatzis, 2013; Xu et al., 2012). The exact mechanism by which this happens is not yet fully elucidated, but some authors suggest that under high levels of

**FIGURE 4** Variable influence on projection score: Ion ratios with higher contribution for difference among the groups [Color figure can be viewed at wileyonlinelibrary.com]

**FIGURE 5** ROC curve: Based on the VIP scores. ROC: receiver operating characteristic; VIP: variable influence on projection [Color figure can be viewed at wileyonlinelibrary.com]
progesterone, endometrial maturation is abnormally accelerated, which would anticipate the WOI (Van Vaerenbergh et al., 2009; Van Vaerenbergh et al., 2011), leading to asynchrony between embryonic development and endometrial receptivity (Labarta et al., 2011; Melo et al., 2006; Papanikolaou, Bourgain, Kolibianakis, Tournaye, & Devroey, 2005).

To decrease the bias concerning the association between embryo quality and implantation success, we selected exclusively young patients, into whom one or two top-quality blastocysts were transferred. Moreover, to diminish the ovarian stimulation influence in the endometrium, for the present study, samples were collected from freeze-all cycle patients undergoing endometrium preparation.

There is growing evidence in the literature suggesting that supraphysiologic doses of hormones, used during COS, may decrease endometrial receptivity. (Check et al., 1999; Haouzi et al., 2009; E. Kolibianakis et al., 2002; Shapiro et al., 2011). This phenomenon can be explained by a possible asynchrony between the embryo and the endometrium, a fact that was not detected in egg-donor cycles or embryo thaw cycles (Richter, Shipley, McVeary, Tucker, & Widra, 2006; Shapiro, Daneshmand, Garner, Aguirre, & Ross, 2008). Such evidence has led to the practice of cryopreservation of all oocytes or embryos of the cohort and transfer in a subsequent cycle, free of the action of gonadotrophins, the so-called freeze-all policy (Chang et al., 2017; Roque, 2015; Roque, Valle, Guimaraes, Sampaio, & Geber, 2015), with the possibility of leading to superior results (Braga et al., 2017; Chang et al., 2017; Magdi et al., 2017; Roque, 2015; Roque et al., 2015; Roque, Valle, Guimaraes, Sampaio, & Geber, 2017; Shapiro, Daneshmand, Garner, Aguirre, & Hudson, 2014; Zhu et al., 2018). In addition, the freeze-all policy allows the personalized embryo transfer, at the defined moment of the WOI and the stage of development of the embryo, after access to the stage of endometrial development.

The volume of endometrial fluid collected in our experiment was extremely low, which could have hindered lipid extraction and analysis. However, mass spectrometry technology enables a small-sample-size and quick-result analyses (Bou Khalil et al., 2010; Ferreira et al., 2010), which can provide a sensitive and accurate assessment of endometrial receptivity through easy samples collection from the uterus via a minimally invasive approach.

Despite the high sensitivity, the model did not allow exact identification of the lipids, which may limit the clinical relevance of the study. In fact, lipid identification analysis, by itself, represents a constant challenge due to the great structural diversity of these molecules. It is estimated that there are around 200,000 different lipid isoforms (Oresic et al., 2008). This issue could be potentially overcome by the MS-MS technology. However, the present study focused on untargeted lipidomics, aiming to suggest lipid biomarkers for embryo implantation, which is the first step towards the long process of biomarkers discovery, according to the metabolomics workflow. In fact, further studies for molecular elucidation and biomarkers measurements should be performed, including others analytical methods and platforms, which would also elucidate molecular composition, measurement, and validation.

It is also important to highlight that the small sample volume makes it impossible to apply all steps in the discovery of a biomarker in a single sample. Although, this is a preliminary study to evaluate whether it is possible to, noninvasively, access biomarkers of endometrial receptivity by endometrial fluid lipidomic. Still concerning the small sample volume, it could also be argued that small differences in sample volume would possibly impact the results. It could be true if a quantitative analysis was performed, however the for the present study qualitative chemometrics analysis was performed, and small differences in samples volume was minimized.

Another limitation for the present study would be the timing of sampling. It would be argued that samples collection immediately before embryo transfer would limit the time for any intervention. However, this was a pilot test, and collecting samples by the embryo transfer moment avoid additional distresses for the patients. Previous research works have shown that fluid aspiration performed before embryo transfer can be performed with no adverse effect on pregnancy rates in IVF cycles (C.M. Boomsma, Kavelaars, Eijkemans, Lentjes et al., 2009; van der Gaast et al., 2003), besides, it is known that WOI is stable over the cycles.

In conclusion, the findings presented here suggest that endometrial fluid lipidomics may be a powerful approach to defining the exact time of the WOI. This would be extremely important for determining the right time for embryo transfer and, consequently, would diminish the incidence of RIF, a substation challenge in assisted reproduction.

4 | MATERIALS AND METHODS

4.1 | Experimental design, inclusion, and exclusion criteria

For this case-control study, endometrial fluid samples from 41 patients undergoing embryo transfers from January 2016 to December 2016, in a university-affiliated assisted reproduction center, were analyzed. Written informed consent, in which patients agreed to share the outcomes of their cycles for research purposes, was obtained, and the study was approved by the local institutional review board (#1.281.695).

Samples were split into two groups depending on the pregnancy outcome: positive group, 100% implantation (n = 24) and negative group, 0% implantation (n = 17).

To exclude any possible influence of COS on endometrial receptivity, the study included exclusively freeze-all cycles. Moreover, since the goal of the present study was to evaluate the endometrial receptibility potential and not the embryo viability, the study included samples from cycles in which one or two high-quality blastocysts were transferred. In addition, only cycles of females aged <37 were included. All cases with severe spermatogenic alterations, including frozen and surgically retrieved sperm, were also excluded from the study. Finally, all cycles for the positive pregnancy group presented a 100% implantation rate.

Clinical pregnancy was defined as the presence of a gestational sac that could be visualized using ultrasound 4–6 weeks after embryo transfer, and miscarriage was defined as pregnancy with a total loss of gestational sacs before 20 weeks’ gestation.
Written informed consent, in which patients agreed to share the outcomes of their cycles for research purposes, was obtained, and the study was approved by the local institutional review board.

4.2 | COS and laboratory procedures

COS was achieved by pituitary blockage using a GnRH antagonist (GnRH – Cetrotide®; Merck KGaA, Darmstadt, Germany); ovarian stimulation was performed using recombinant FSH (Gonal-F; Merck KGaA, Darmstadt, Germany).

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) was administered to trigger the final follicular maturation. The oocytes were collected 35 hr after hCG (Ovidrel®; Merck KGaA) administration through transvaginal ultrasound ovum pickup.

The recovered oocytes were assessed to determine their nuclear status, and those in metaphase II were submitted to intracytoplasmic sperm injection following routine procedures (Palermo, Colombero, & Rosenwaks, 1997). On the third day of development all obtained embryos were vitrified.

4.3 | Embryo vitrification and warming

Both vitrification and the warming procedures were performed using the Cryotop method (Kuwayama, Vajta, Kato, & Leibo, 2005). Briefly, vitrification was achieved by exposure of embryos initially to the equilibration solution, followed by a 30-s exposure to the vitrification solution. Individual embryos were then picked up in an extremely small volume (<0.1 ml) of vitrification solution, to facilitate rapid cooling, and placed on top of a very fine polypropylene strip attached to a hard-plastic handle. As soon as the embryo was placed onto the thin polypropylene strip of the Cryotop, it was immediately submersed vertically into liquid nitrogen. Then the thin strip was covered with a hard-plastic cover on top of the Cryotop sheet.

For warming, the protective cover was removed from the Cryotop whereas it was still submerged in liquid nitrogen, and the polypropylene strip of the Cryotop was immersed directly into the thawing solution at 37°C for 1 min. Embryos were retrieved and transferred into dilution solution for 3 min and then washed twice in the washing solution for 5 min each. The tools and solutions required for the vitrification and warming processes were obtained from Kitazato® (Tokyo, Japan).

4.4 | Embryo transfer and sample collection

After warming, intact embryos were cultured until the blastocyst stage when embryo transfer was performed. Immediately before embryo transfer, endometrial fluid was aspirated using an embryo transfer catheter (Wallace®, CooperSurgical, Trumbull, CT), attached to a 5 ml syringe. The external catheter was inserted through the cervix with the inner catheter tip shielded within it, to avoid contamination with cervical mucus. Once within the uterine cavity, the inner catheter was advanced, and a slight suction was applied for 30 s before it was again withdrawn within the external catheter, which was withdrawn from the uterus.

After collection, the tip of the catheter containing the endometrial fluid was cut and stored at −20°C, until lipid extraction and analyses.

4.5 | Endometrial preparation

After menses, patients received 200 µg of transdermal 17β oestradiol every 3 days (Estradot®, Noven Pharmaceuticals, Miami, FL) and endometrial thickness and morphology were evaluated by ultrasound examination every other day. When the endometrium showed proliferative morphology and thickness of at least 7.5 mm, 800 mg of progesterone (Utrogestan®, Farmoquímica, Rio de Janeiro, Brazil) was vaginally administered per day and embryo transfer was performed after 5 days.

Both 17β oestradiol and progesterone were administered concomitantly for 10 days after embryo transfer and were suspended in the case of a negative βhCG test. In the case of a positive βhCG test, the 17β oestradiol and progesterone treatments were maintained until weeks 6 and 12 of gestation, respectively.

4.6 | Sample preparation, lipid extraction, and mass spectrometry

Lipids were extracted using the method reported by Bligh and Dyer (1959) with modifications, as reported elsewhere (Cataldi et al., 2013). Briefly, the catheter tip was placed in a microtube, into which 150 ml of ultrapure water was added, followed by the addition of 190 µl of chloroform and 375 µl of methanol. The mixture was stirred in a vortex for 2 min at 2,000 rpm before the addition of another 150 ml of water and 190 ml of chloroform. After 10 min of centrifugation at 2,000 rpm the mixture was separated into two phases; the lower chloroform phase was collected, concentrated with a Speed-Vac apparatus, and reconstituted into 100 µl of an acetonitrile/water (80:20; v/v) solution.

A volume of 1 µl of the extract was spotted onto the well of the mass spectrometer plate. After drying at room temperature, the extract was overlaid with 1 µl spot of 2.5-dihydroxybenzoic acid matrix solution (Sigma-Aldrich, St Louis, MO) at a concentration of 10 mg/ml−1 in 50% acetonitrile containing 2.5% trifluoroacetic acid.

Spectra were acquired in the positive mode using an Autoflex III matrix-assisted laser desorption/ionization time-of-flight mass spectrometer equipped with Smartbeam® Laser Technology (Bruker Daltonics, Bremen, Germany), using a range of 600–1,000 m/z.

4.7 | Data processing and statistical analysis

The results were obtained using FlexControl 3.0 software (Bruker Daltonics) and data analyses were performed using MetaboAnalyst 3.0 software (http://www.metaboanalyst.ca).

The intensity values were normalized by log2. The Pearson correlation analyses were performed and ions presenting an
increased correlation coefficient were used to obtain ion ratios, which were used for further analysis.

Univariate analyses were performed to obtain an overview of the data. For that, fold-change analysis, t tests, and volcano plots were performed. The t test determined whether the group distinctions were statistically significant and the volcano plots were used to compare the size of the fold change to the statistical significance level.

Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were applied to the data set. The PCA, an unsupervised method, was used to detect intrinsic clusters based on the lipid profile, and the PLS-DA, a supervised method, was used to maximize the group discrimination. From these analyses, a list of the most important ions responsible for group discrimination was obtained.

Variable influence on projection values were used to build a ROC curve and to evaluate the model’s potential to predict pregnancy success or failure.

Lipid attribution was performed using the LIPID MAPS database (http://www.lipidmaps.org/), considering their main subclass, with a maximum mass tolerance of 0.1 Da, whereas the maximum mass error was 50 ppm. For the attribution, only molecules containing hydrogen (M+H+), sodium (M+Na+), and potassium (M+K+) as adducts were considered. Functional enrichment analysis was performed using Cytoscape software (www.cytoscape.org/).

Patient and cycle characteristics, along with clinical and laboratorial 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 patented using z score. Maternal and paternal age, total FSH dose used for COS, number of aspirated follicles, retrieved oocytes, obtained embryos, fertilization rate, and endometrial thickness were compared between the groups using the Student t test. Variables were described as mean ± standard deviation and the considered significance level α was 5%.

5 | CONCLUSION

Endometrial fluid lipidomics may, in the future, assist in the identification of the receptive endometrium and be a valuable approach to determine the exact moment for embryo transfer and diminish the incidence of repeated implantation failure, one of the main challenges of assisted reproduction technology.

ACKNOWLEDGMENTS

The authors thank Erika Ono, PhD, for the key help in collecting the samples.

ORCID

Daniela Paes Almeida Ferreira Braga http://orcid.org/0000-0003-1333-6593
Daniela Antunes Montani http://orcid.org/0000-0002-0637-6848

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How to cite this article: Braga DPAF, Borges E Jr., Godoy AT, et al. Lipidomic profile as a noninvasive tool to predict endometrial receptivity. Mol Reprod Dev. 2018;1–11. https://doi.org/10.1002/mrd.23088