REPRODUCTION
RESEARCH

Prediction of embryo implantation potential by mass spectrometry fingerprinting of the culture medium

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Abstract

This study has evaluated the performance of a multivariate statistical model to predict embryo implantation potential by processing data from the chemical fingerprinting of culture medium samples used for human embryo culture. The culture medium for 113 embryos from 55 patients undergoing ICSI was collected after embryo transfer. The samples were split into positive ($n=29$) and negative ($n=84$) implantation groups according their implantation outcomes (100% or 0% implantation). The samples were individually diluted and analyzed by electrospray ionization mass spectrometry (ESI-MS). The *m/z* ratios and relative abundances of the major ions in each spectrum were considered for partial least square discriminant analysis. Data were divided into two subsets (calibration and validation), and the models were evaluated and applied to the validation set. A total of 5987 ions were observed in the groups. The multivariate statistical model described more than 82% of the data variability. Samples of the positive group were correctly identified with 100% probability and negative samples with 70%. The culture media used for embryos that were positive or negative for successful implantation showed specific biochemical signatures that could be detected in a fast, simple, and noninvasive way by ESI-MS. To our knowledge, this is the first report that uses MS fingerprinting to predict human embryo implantation potential. This biochemical profile could help the selection of the most viable embryo, improving single-embryo transfer and thus eliminating the risk and undesirable outcomes of multiple pregnancies.

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Introduction

The number of children born through assisted reproductive technologies (ART) has been increasing worldwide. However, ~70–80% of *in vitro*-produced embryos fail to implant and 66% of IVF cycles fail to result in pregnancy (Patrizio & Sakkas 2009, Seli *et al*. 2010, Assou *et al*. 2011). This low efficiency contributes to the practice of multiple embryo transfer, which seeks to overcome implantation failure and maximize pregnancy rates. Unfortunately, this procedure frequently leads to the inconvenient outcome of multiple pregnancies (Pandian *et al*. 2009, Setti & Bulletti 2011).

Embryo selection methods based on detailed morphological parameters have long been known to be associated with successful ART. Morphological assessment is the easiest way to predict viability, but most studies suggest that the morphology of embryos with high-quality morphological appearance is insufficient to predict a successful implantation (Katz-Jaffe *et al*. 2009, Assou *et al*. 2011, Mastenbroek *et al*. 2011).

Morphological assessment at the third day of development is not considered a reliable and precise tool to predict embryonic potential, as even the embryos with the best morphological features experience inconsistent implantation success and may spontaneously abort during pregnancy. Furthermore, morphologically normal embryos can be genetically abnormal, as a significant proportion of aneuploid embryos is capable of achieving the highest morphological scores (Singh & Sinclair 2007, Alfarawati *et al*. 2011, Assou *et al*. 2011).

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The limitations of strategies based on morphological embryo grading at day 3 embryos have prompted the investigation of adjunctive technologies for the non-invasive biochemical assessment of embryo viability in ART (Hamel *et al*. 2008, Katz-Jaffe *et al*. 2009, Aydiner *et al*. 2010, Assou *et al*. 2011). These approaches include the measurement of glucose, lactate, pyruvate, or amino acid levels in the embryo culture media, as well as the assessment of oxygen consumption by the embryo, and genomic and proteomic profiling (Gardner *et al*. 2001, Houghton *et al*. 2002, Brison *et al*. 2004, Lopes *et al*. 2005, 2007, Dominguez *et al*. 2008, Katz-Jaffe *et al*. 2009). Most recently, analytical examination of the embryonic metabolome (Seli *et al*. 2007, 2008, 2010, Botros *et al*. 2008, Bromer & Seli 2008, Scott *et al*. 2008, Katz-Jaffe *et al*. 2009, Aydiner *et al*. 2010, Ferreira *et al*. 2010, Ahlstrom *et al*. 2011, Cortezzi *et al*. 2011, Hardarson *et al*. 2012) has also been evaluated, particularly applying spectroscopy methods. The metabolome refers to the complete stock of small compounds, such as metabolic intermediates and signaling molecules that are found within a biological sample. The metabolome changes continuously, depending on the activation and interaction of the various metabolic pathways within the cell. Metabolomics is also the endpoint of the 'omics cascade' and most closely related to the phenotype, constituting a proper means to study functional biological systems and providing valuable information that is complementary to the data obtained from gene and protein expression studies. Whereas genomics and proteomics can supply important information regarding expected functions, metabolomics provides a unique, immediate reflection of all biological functions, increasing our understanding of the cross talk among the genome, transcripts, proteins, metabolites, and phenotypes in cells during early human development (Nagy *et al*. 2008, Urbanski *et al*. 2008).

As the number of ART cycles increases worldwide, improvement of the ability to perform fast, reliable, and noninvasive embryo viability prediction has become a crucial target for reproductive medicine (Aydiner *et al*. 2010, Assou *et al*. 2011). Mass spectrometry (MS) fingerprinting is a global screening approach that compares and classifies samples based on their metabolite patterns. This fast analytical approach allows informative, straightforward chemical characterization with the ultimate goal of identifying distinctive metabolites. MS fingerprinting normally applies direct sample infusion and requires minimal sample preparation (Villas-Boas *et al*. 2005, Dettmer *et al*. 2007). Spectra obtained in high-resolution modes provide large amounts of MS data (several *m/z* vs ion abundances) that require multivariate statistics (or chemometrics) for sample classification (Blekherman *et al*. 2011, Horgan *et al*. 2011, Theodoridis *et al*. 2011). One of these commonly used procedures is the partial least squares discriminant analysis (PLS-DA), which is an aim-directed method of classification, which is performed in order to sharpen the separation between groups of observations. In PLS-DA analysis, the peaks in the spectra that contribute to the separation of the samples receive a higher weight, improving the understanding of which variables carry the class-separating information and thereby yielding a model with better discriminatory ability compared with unsupervised principal component analysis (PCA; Dill *et al*. 2010, Mishur & Rea 2012).

This case–control study has evaluated the use of the PLS-DA multivariate method of classification and the analytical power of a straightforward and high-throughput classification tool based on MS chemical fingerprinting with minimal sample preparation and minute analysis (MS fingerprinting) to characterize the patterns of individual culture medium samples for embryo culture of patients undergoing ICSI according to implantation outcomes.

Results

General characteristics

The patients from the positive and negative implantation groups were similar with respect to female age, FSH administered, number of follicles, and number of metaphase II (MII) oocytes recovered. Male age ($P=0.03$) and high-quality embryo rate ($P=0.015$) were significantly different (Table 1).

Data analysis of mass spectra

Overall, the plotted scores of the calibration samples were able to categorize the samples of both groups according to their implantation outcomes (Fig. 1). More than 10 000 ions were observed by electrospray ionization quadrupole time-of-flight MS (ESI-Q-ToF-MS) in the negative ion mode (Fig. 2) within the *m/z* range of 100–1500. Figure 2 shows the spectra for positive and negative implantation groups, with the restricted *m/z* range of 100–600, which comprises the most abundant ions. The data were pre-processed to reduce spectral noise; specifically, ions with

Table 1 Patient characteristics for the positive and negative implantation groups.

	Positive group ($n=15$)	Negative group ($n=40$)	*P*
Female age	35.5±5.4	36.3±5.0	0.25
Male age	41.1±6.8	37.4±5.8	0.03
FSH (IU)	2169.2±964.6	2311.5±525.5	0.25
Follicles	20.7±20.8	13.8±10.9	0.06
MII oocytes	10.2±9.2	6.8±5.9	0.06
Fertilization rate (%)	72.6±26.3	75.7±24.4	0.33
High-quality embryo rate (*n*) (%)	(20) 68.9	(35) 42.7	0.015

Values are expressed as the means±s.e.m., unless otherwise noted. Student's *t*-test.

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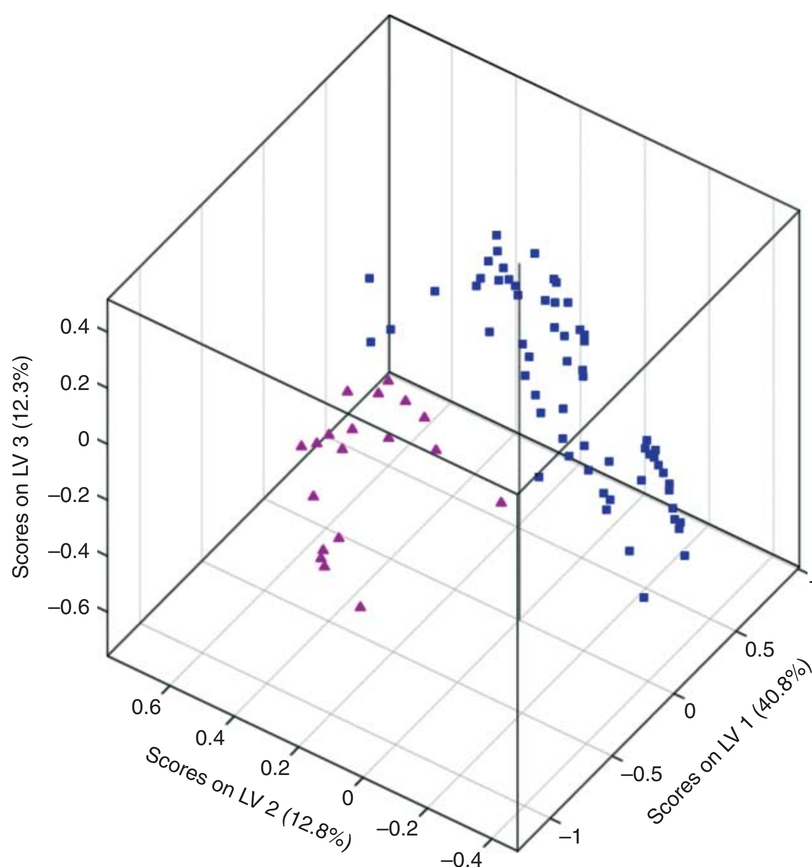

Figure 1 A plot of the scores for the calibration samples from the positive (pink triangles) and negative (blue squares) implantation outcomes.

abundances lower than 50 counts were discarded, reducing the number of ions to 5987 in the *m/z* range of 100–1285. This procedure was performed for all the spectra. The PLS-DA model was constructed using the calibration set (75 samples) and predicting the validation set (38 samples). From the calibration set of 75 calibration samples, 18 were from the positive implantation group and 57 were from the negative implantation group. The validation group comprised 38 samples, 11 from the positive and 27 from the negative implantation group. There was no overlap in patients between the calibration and validation sets, as there are nonpregnant patients, which means 0% implantation embryo samples or pregnant patients, with 100% implantation embryo samples.

The ability of the multivariate model to assess the calibration samples was optimized using cross-validation by leave-one-out, which means that *n* (equal of the number of samples) calibration models are constructed, always leaving one sample out. The final optimized model parameters are based on the mean of all models (residuals, variance, etc.). The obtained PLS-DA model described more than 82% of the data variance using six latent variables (LV) and was able to classify all the calibration samples correctly. In the validation process, new samples of the positive group were correctly identified with 100% accuracy (11/11), whereas the negative samples were correctly identified with 70% accuracy (19/27), as Fig. 3 shows. The control culture medium samples without embryos, which were subjected to the same conditions as the embryo culture drops, were statistically classified as belonging to the negative implantation group according to the statistical model. This finding raises the possibility that an embryo with a low probability of implantation does not influence or minimally influences the chemical profile of the medium. Likelihood ratios were calculated for assessing the value of performing a diagnostic test, by measuring sensitivity, specificity, predictive values, and receiver operating characteristic area under the curve of 0.94 (95% CI 0.92–0.96) (McGee 2002, Kentsis *et al*. 2012). The calibration likelihood ratios for positive and negative were zero. Sensitivity and specificity were 100%. Both positive and negative predictive values were 100% in the calibration set. The validation likelihood ratio positive was 3.33, while the likelihood ratio negative was 0. Sensitivity was 100% and specificity 70%. The positive predictive value was 58% and the negative predictive value was 100% in the validation set.

ESI-MS provides ion identification according to the *m/z* value and also the relative abundance. Through an analysis of the loading values, which correspond to the most significant *m/z* values for the separation of the two experimental groups, we were able to identify several ions with the highest potential for acting together as

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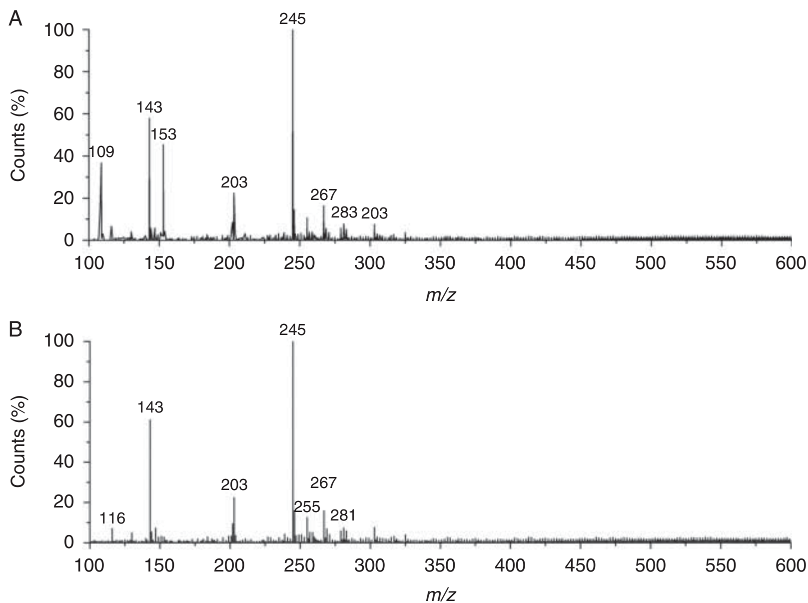

Figure 2 ESI-MS peak profiles of representative samples from culture media from the positive (A) and negative (B) implantation groups.

biomarkers, which are the ions that have contributed most to the major differences in group identity (Fig. 4 and Tables 2 and 3). Table 4 shows the identities of some of these metabolites represented by their relative masses.

Discussion

An implantation failure rate of up to 80% is known to occur primarily due to the absence of a viable embryo and/or the inability to select such an embryo (Mansour & Aboulghar 2002, McReynolds *et al*. 2011). Strategies aimed at overcoming the low rate of successful embryo implantation have, to date, primarily focused on super-physiological implantation rates, by means of obtaining more oocytes and transferring more embryos. This practice, however, has been severely criticized because it leads to a high rate of twins and a related higher risk of adverse maternal and infant morbidity and mortality, as well as increased health costs (Nagy *et al*. 2008, Roberts *et al*. 2011, Setti & Bulletti 2011). To achieve the highest possible live birth rates after IVF whilst minimizing the risk of multiple pregnancies, improvement in the current morphological selection procedure would be beneficial (Nagy *et al*. 2008, Seli *et al*. 2010, Mastenbroek *et al*. 2011).

By employing direct injection ESI-MS analysis without prior chromatographic separation, MS fingerprinting allows a sample to be processed within a few minutes (Villas-Boas *et al*. 2005, Dettmer *et al*. 2007). This rapid approach produces a large amount of chemical data that improves selectivity with no significant compromises in reproducibility or accuracy (Dettmer *et al*. 2007). In this study, we applied direct fingerprinting by ESI-Q-ToF-MS.

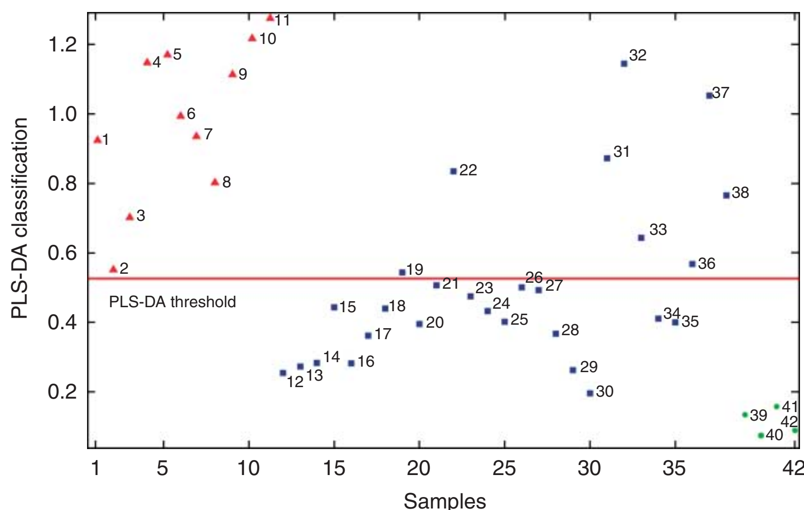

Figure 3 Scores from the validation samples, showing positive samples (red triangles), negative samples (blue squares), and incubation medium (green circles), separated into classes by the PLS-DA threshold. Eight negative implantation samples were classified as positive.

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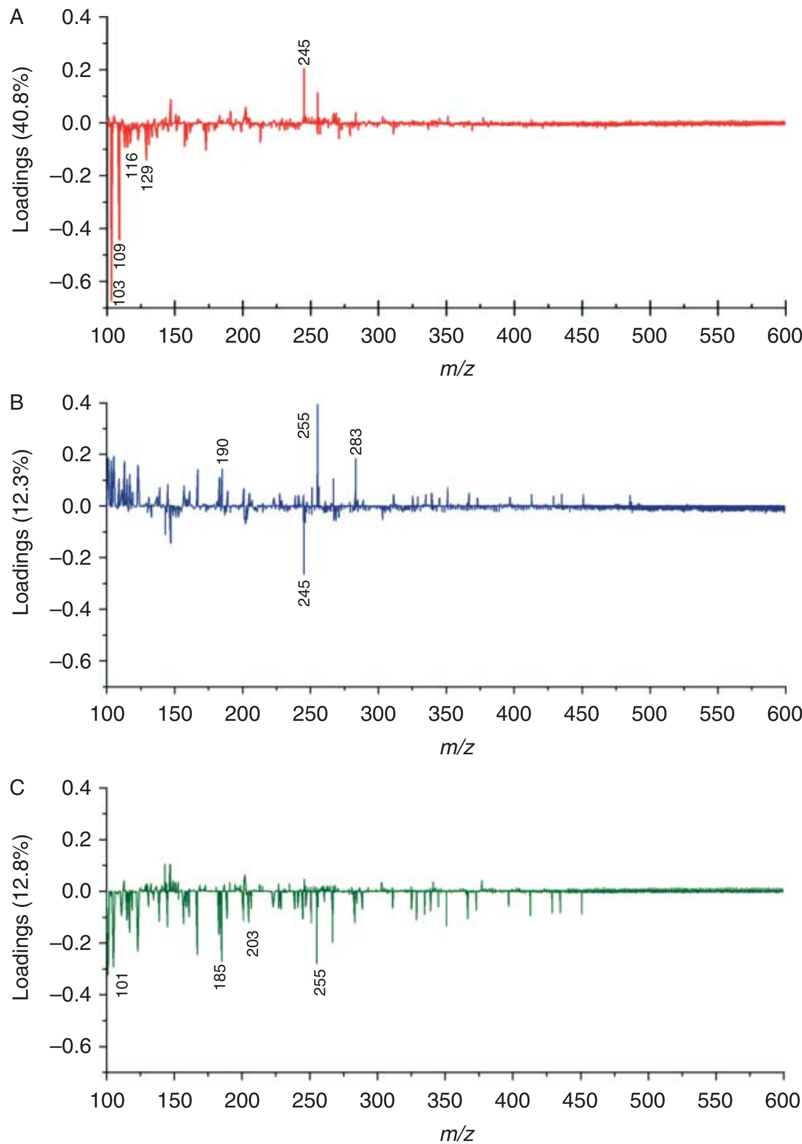

Figure 4 PLS-DA plots of the loading values, showing the most significant ions for separation in two groups based on *m/z* values at the latent variable 1 (A), LV2 (B), and LV3 (C).

MS fingerprinting analysis has been applied to a variety of biological models and even for bovine embryo culture medium quality control (Sawaya *et al*. 2004, Catharino *et al*. 2006, Marques *et al*. 2006, de Souza *et al*. 2007*a*, 2007*b*, Souza *et al*. 2008, Ferreira *et al*. 2009, Saraiva *et al*. 2009). Although diverse analytical techniques that are primarily based on spectroscopy technologies, such as near-infrared, RAMAN, and proton NMR, have been used as noninvasive tools to predict embryo viability (Seli *et al*. 2007, 2008, Nagy *et al*. 2008, Scott *et al*. 2008, Ahlstrom *et al*. 2011, Hardarson *et al*. 2012), to our knowledge, this is the first report that uses MS fingerprinting to predict human embryo implantation potential. Whereas spectroscopic techniques are based on the measurement of physical events resulting from the interaction of organic molecules with electromagnetic radiation, MS characterizes the molecular composition of a sample (Ferreira *et al*. 2010).

Table 2 The five most abundant ions in each latent variable (variables not directly observed but are inferred through a mathematical model).

Importance[a]	1°	2°	3°	4°	5°
Latent variable 1	103.1	109.0	245.1	129.0	255.2
Latent variable 2	255.2	245.1	105.0	283.3	101.0
Latent variable 3	101.0	105.0	255.2	185.0	167.0
Latent variable 4	105.0	185.0	101.0	167.0	143.1

[a]Units in *m/z*.

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Table 3 The ten most abundant ions around all latent variables (LV).

R	0.982	0.646	0.299	0.212	0.205	0.192	0.184	0.179	0.165	0.160
m/z	103.1	190.0	245.1	101.0	129.0	105.0	255.2	185.0	255.2	167.0
LV	1	1	1	3	1	3	3	3	1	3

MS has enabled, for instance, the analysis of extracellular metabolites secreted into or consumed from the culture medium by cells, providing valuable information about their physiological states (Dettmer *et al*. 2007).

MS allied with data analysis using the PLS-DA model has been successfully employed in biomarker and pathway identification (Duan *et al*. 2011, Wang *et al*. 2012*a*, 2012*b*, Yang *et al*. 2012). The statistical model developed in this work was able to describe more than 82% of the data variance using six LV and data mean-centering. LV are variables not directly observed but inferred through a mathematical model from other observed variables. In the calibration steps, the samples were successfully categorized into two clusters, based on their positive and negative implantation outcomes (Fig. 1). In the validation process, new samples of the positive group were correctly identified with 100% success, and 70% of negative samples were correctly identified. Therefore, although embryo culture media with negative and positive implantation outcomes have shown similar chemical profiles (Fig. 2) in terms of most abundant ions, some less abundant ions or their relative abundances are unique for each group and therefore PLS-DA analysis was able to separate the validation samples in both groups (Fig. 3). Of the negative implantation samples, eight samples (30%) were incorrectly classified as positive.

By the PLS-DA loading analysis, which identifies the most important ions for separating the two groups based on their *m/z* values, we were able to observe several ions with higher potential for acting as whole as biomarkers, i.e. those ions that have contributed to the major differences that distinguish the groups (Fig. 4 and Table 2). Not only the presence or absence of specific ions but also their relative abundances in the spectra allowed separation between the positive and negative implantation groups.

The model was able to predict embryo implantation potential in cases where the implantation result was positive but only with 70% accuracy in cases of negative implantation. This result is reasonable as implantation is known to involve an intricate dialog between the embryo and the endometrium, which is supposed to be receptive (Achache & Revel 2006, Teklenburg *et al*. 2010, Mastenbroek *et al*. 2011). We are aware that the ideal experimental design for the evaluation of embryo viability would involve only single-embryo transfers. Due to the costs and psychological aspects of human ART, this arrangement was unfeasible. Nonetheless, we are pursuing this ultimate goal of single-embryo transfer especially for women who present a high risk of problems in multiple gestation and who consent to single-embryo transfer. As a consequence, the maternal effect cannot be ruled out in this work, but there are no practical means of controlling this variable. Hence, we have included in the experiment primarily patients with clinically sound endometrial tissue.

In the biomarker analysis, PLS-DA could be an important tool in mining the MS data. Because each LV has a different percent variance captured by Model at Y-block, the ion with the sixth greatest abundance on the first LV may be as important as the first ion on LV2 or LV3. Therefore, to normalize the weight of each loading around all LV and facilitate the selection of the top ten ions for use as biomarkers, it was adjusted using the proposed equation 1, as follows:

$$K_i = \frac{l_{i,j}}{\sum_{i=1,j=1}^{i}\left(\sqrt{\left(l_{i,j}\right)^2}\right)} \times V_j \qquad (1)$$

In this equation, K_i is the normalized importance of the i-th loading of the j-th LV, $l_{i,j}$ is the numeric value of the i-th loading of the j-th LV, and V_j is the captured variance in the j-th LV. Following this operation, the top ten *m/z* were re-organized in sequence (Table 3).

Some ions, which are putatively assigned below, indicate a high potential to be biomarkers by PLS-DA analysis, such as the deprotonated ($[M-H]^{-1}$) amino acid L-valine (*m/z* 116), detected only at embryonic culture media from the negative implantation group. Other amino acids, such as L-tryptophan, detected as the ion of

Table 4 Identification of some ions in embryo culture media.

Compound	Molecular formula	Theoretical mass *m/z* $[M-H]^-$	Measured mass *m/z* $[M-H]^-$	Error (ppm)
L-valine	$C_5H_{11}NO_2$	116.0712	116.072	6.89
L-tryptophan (-H_2O)	$C_{11}H_{12}N_2O_2$	185.0715	185.0661	−29.2
L-histidine hydrochloride (-H_2O)	$C_6H_9N_3O_2.HCl - H_2O$	190.0383	190.0349	−17.9
L-tryptophan	$C_{11}H_{12}N_2O_2$	203.082	203.0879	29.05
Palmitic acid	$C_{16}H_{32}O_2$	255.2324	255.2287	−14.50
Oleic acid	$C_{18}H_{34}O_2$	281.248	281.2428	−18.49
Stearic acid	$C_{18}H_{36}O_2$	283.2637	283.2551	−30.36
Arachidonic acid	$C_{20}H_{32}O_2$	303.2324	303.2287	−12.20

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m/z 185 $[M-H_2O-H]^{-1}$, L-histidine hydrochloride of *m/z* 190 $[M-H_2O-H]^{-1}$, and deprotonated L-tryptophan of *m/z* 203, were detected in both groups, in different abundances. Two fatty acids were exclusively detected in the negative implantation group: deprotonated palmitic acid (*m/z* 255) and oleic acid (*m/z* 281). Furthermore, two fatty acids were detected only in the positive implantation group: deprotonated stearic acid (*m/z* 283) and arachidonic acid (*m/z* 303), as seen in Table 4.

There are controversial studies regarding the viability index of oocytes/embryos established by metabolomic tests based on spectroscopy methods as a stronger predictor of implantation potential than traditional morphological assessment (Nagy *et al*. 2008, Ahlstrom *et al*. 2011, Hardarson *et al*. 2012). However, in this MS study, the patients with positive implantation had embryos with significantly higher morphological quality, which suggests that morphology is still valuable and should be evaluated as an adjunct parameter (Seli *et al*. 2010).

Noninvasive technologies to predict embryo implantation potential, such as MS and spectroscopy approaches, represent an independent but complementary tool to morphological parameters for the assessment of embryo viability (Nagy *et al*. 2008, Seli *et al*. 2010). MS fingerprinting combined with PLS-DA of culture media provides a faster, high-throughput auxiliary tool for embryo viability prediction with higher analytical power, thus much more informative, compared with spectroscopy methods, due to its ability to detect and define the structure of metabolites at the micromolar concentration level, typical of physiological concentrations (Botros *et al*. 2008, Ferreira *et al*. 2010).

Less abundant ions detected via ESI-MS with statistical significance have contributed to the major differences between the groups. These divergences are related to the sum of the depletion and appearance (turnover) of diverse molecules in the culture media, which is probably related to embryo metabolic activity. Our findings suggest distinct chemical profiles for viable and nonviable embryos, besides the maternal receptivity effect. An improved knowledge of embryo viability should facilitate the identification of the embryos that are most competent for development. In turn, this identification will allow the more accurate selection of the best embryo, the reduction of the number of embryos transferred, and, consequently, the chance of a multiple gestation and its undesirable outcomes (Botros *et al*. 2008, Bromer & Seli 2008, Nagy *et al*. 2008, Aydiner *et al*. 2010, Seli *et al*. 2010, Assou *et al*. 2011).

Distinct chemical patterns revealed by MS fingerprinting followed by the application of multivariate statistics (PLS-DA) were observed in culture media from the positive and negative implantation groups. Less abundant but a diagnostic set of ions were found to function as a whole as biomarkers. As the aim of this study was to use a straightforward and high-throughput classification tool based on chemical fingerprinting of individual embryo culture medium, our next step will focus on the more refined identification of the possible biomarkers and understanding the biochemical pathways involved in this chemical differentiation. For these steps, the spectra will be acquired using a high-resolution and accuracy linear ion trap combined with a Fourier transform ion cyclotron resonance mass spectrometer equipped with a chip-based direct infusion nanoelectrospray ionization source for more stable ionization. This equipment provides high mass accuracy (better than 2 ppm), which allows the identification of the elemental composition of metabolites, increasing dramatically their identification.

So far, the diagnostic power of such patterns is not conclusive, as implantation results are known to also depend on the simultaneous cross talk between a competent embryo and a receptive maternal endometrium. Nevertheless, this technology seem to provide fast, reliable, and noninvasive prediction tool to help the selection of the best embryo to be transferred, probably increasing the successful implantation rates of single-embryo transfer programs. MS fingerprinting can be therefore used as an adjunct to morphological evaluation, thus minimizing the risks of undesirable outcomes of multiple pregnancies.

Materials and Methods

Patients

Culture medium samples were taken from 113 embryos obtained from 55 patients undergoing oocyte retrieval for ICSI. The samples were collected between November 2010 and March 2011. Of these ICSI cycles, five were for oocyte donation. In the 50 cycles without oocyte donation, 390 MII oocytes were retrieved. Of these oocytes, 299 (76.6%) presented normal fertilization morphology, defined as the presence of two distinct pronuclei.

The inclusion criteria were ICSI cycle using ejaculated sperm, normal serum hormonal levels (E2 $\leq$70 pg/ml and FSH $\leq$14 mU/ml), women with regular menstrual cycles, not having clinically significant pelvic abnormality and/or uterine abnormality and presence of both ovaries. Only patients without spontaneous abortion were included in this study.

The samples were classified according to the embryo implantation outcomes: 29 culture medium samples were retrieved from embryos of 15 patients with positive pregnancy results and 100% successful implantation (the positive implantation group), which means that only cycles where all the transferred embryos implanted were included. Moreover, 84 culture medium samples were retrieved from the embryos of 40 patients showing negative pregnancy outcomes (or 0% implantation, defined as the negative implantation group).

The implantation rate was defined as the number of gestational sacs observed by ultrasonography per transferred embryo 5 weeks after embryo transfer. These positive results were confirmed until 12 weeks of pregnancy.

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In the positive implantation group, the causes of infertility were defined within the following categories: male factor (40%), female factor (30%), unexplained infertility (10%), oocyte donation (10%), previous abortion (5%), and endometriosis (5%). Four out of the 15 patients (26.6%) had a male and female combined factor. Among the negative implantation group, the causes of infertility included female factor (42.6%), male factor (29.6%), endometriosis (13%), previous abortion (7.4%), oocyte donation (5.6%), and unexplained infertility (1.8%). In this group, 20% (8/40) patients had a male and female combined factor.

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

Controlled ovarian stimulation and oocyte retrieval

The patients began receiving recombinant (rec)FSH treatment (Gonal-F, Serono, Geneve, Switzerland) daily on the third day of their menstrual cycles. The first ultrasound control and the E2 plasma dosage tests were performed at the seventh day of the cycle. The dose of recFSH was adjusted depending on the response of each patient, as determined by ultrasound monitoring of the follicle size. GNRH antagonist was administered when the dominant follicle reached 14 mm in mean diameter. Oocyte retrieval through transvaginal ultrasonography was performed 35 h after the administration of recombinant human chorionic gonadotropin (rhCG, Ovidrel, Serono).

Preparation of oocytes and morphological assessment

The retrieved oocytes were maintained in culture medium (Global for Fertilization, LifeGlobal, Guilford, CT, USA) supplemented with 10% human synthetic albumin (HSA, Irvine Scientific, Santa Ana, CA, USA), which was covered with mineral oil (Ovoil, Vitrolife, Kungsbacka, Sweden) and stored at 37 °C and 6% CO_2 for 5 h. The surrounding cumulus cells were removed by exposure to a HEPES-buffered medium containing hyaluronidase (80 IU/ml, Irvine Scientific). The remaining cumulus cells were then mechanically removed by gentle pipetting with a hand-drawn Pasteur pipette (Humagen Fertility Diagnostics, Charlottesville, VA, USA). Oocyte morphology was assessed immediately before sperm injection using an inverted Nikon Diaphot microscope (Eclipse TE 300; Nikon, Tokyo, Japan) with a Hoffmann modulation contrast system under 400× magnification. Oocytes that released the first polar body were considered to be mature and were used for ICSI.

ICSI

ICSI was performed on all MII oocytes using the technique described by Palermo *et al.* (1992). The oocytes were individually placed in 4 µl droplets of buffered medium (Global w/HEPES, LifeGlobal). The sperm cells were placed in a central 4 µl droplet of polyvinylpyrrolidone solution (PVP, Irvine Scientific) in a 50×40 mm glass culture dish (WillCo-dish, Willco Wells, Vineland, NJ, USA) covered with warm mineral oil (Ovoil, Vitrolife) on a heated stage (37.0±0.5 °C) of an inverted microscope.

Assessment of fertilization, embryo quality, and embryo transfer

After the ICSI procedure, the presumptive embryos were individually maintained in a 50 µl drop of culture medium (Global, LifeGlobal) supplemented with 10% HSA and covered with mineral oil in a humidified atmosphere with 6% CO_2 at 37 °C until transfer, which occurred on the third day of development.

Approximately 18 h after ICSI, fertilization was confirmed by the presence of two pronuclei and the extrusion of the second polar body. Subsequently, embryos were transferred to new drops of culture medium and were then individually cultured for 48 h. The quality of the embryos was evaluated under an inverted microscope. High-quality embryos were defined as having the following characteristics: eight to ten cells on the third day of development, <15% fragmentation, symmetric blastomeres, and absence of multinucleation and zona pellucida dysmorphisms. Embryos lacking any of the above characteristics were considered to be of medium or low quality.

Embryo transfer was performed on the third day of development. One to three embryos from each couple were transferred to the patient. Due their low morphological quality, in one case, four embryos were transferred to a single patient, which resulted in a negative implantation outcome. Embryo selection for transfer was based on embryo morphology. Following embryo transfer, the remaining culture medium was individually collected and stored at −20 °C. Four samples of equilibrated culture medium without embryos were considered controls and were analyzed as a check test.

Sample preparation and MS analysis

Subsequent to confirmation of implantation, the culture medium samples were divided according to their implantation outcomes (100 or 0% implantation). The samples were individually diluted at a rate of 1:100 in a solution of methanol/water (1:1) with 0.1% ammonium hydroxide (v/v). After dilution, the samples were injected directly and analyzed by ESI-Q-ToF-MS (Waters, Manchester, UK) using a syringe pump at the flow rate 10 µl/min. The operating conditions of the equipment were as follows: capillary voltage, 4.5 kV; source temperature, 100 °C; desolvation temperature, 80 °C; and cone voltage, 45 V. The spectra were acquired in the negative ion mode from the pure solvent and from the samples for typically 1 min within the mass to charge ratio (*m/z*) 100–1500 range. The background was subtracted from all of the spectra, and the relative abundances of the detected ions in each spectrum were considered for further statistical analysis.

Multivariate statistical analysis

The MS datasets from the samples were organized in a matrix using the XS (Extended Statistics) module of the MarkerLynx (Waters, Milford, MA, USA) software. The data were truncated at 0.1 Da of peak separation and a threshold of 50 counts of intensity and were exported to MatLab. The analysis of the data by PLS-DA was conducted in MatLab version 7.0 using PLS_toolbox (EigenVector Co., Wenatchee, WA, USA). In each

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sample, the intensity in ion counts of the ion peak with the highest value was normalized to 1, and the samples were randomly divided into a calibration set with 75 samples and a validation set with another 38 samples and categorized in clusters in two groups, based on their positive and negative implantation outcomes. Four samples of equilibrated culture medium without embryos were used as controls and were analyzed by the previously developed multivariate method as a check test, besides the calibration or validation set. Likelihood ratios were calculated for assessing the value of performing a diagnostic test, by measuring sensitivity, specificity, and predictive values (McGee 2002, Kentsis *et al*. 2012).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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