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ORIGINAL ARTICLE

Improving human embryos selection in IVF: non-invasive metabolomic and chemometric approach

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Abstract We present here a new metabolomic methodology to predict embryo implantation ability in in vitro fertilization (IVF). In the present study we have included a total of 23 patients scheduled for IVF. Embryos were selected to be transferred by using morphological criteria on day 3 of in vitro culture. The relative amino acid concentrations in the embryo culture media were analyzed by HPLC–MS and HPLC–MS/MS. ^{1}H NMR metabolomic profiles were also obtained for the embryo culture media. Chemometric models were performed with SIMCA (soft independent modeling of class analogy) for samples from both, non-pregnancy and pregnancy cycles. The metabolic differences between the embryos, with pregnancy and non-pregnancy outcome, can be correlated with the relative amino acid concentrations and with ^{1}H NMR profiles. We used interval partial least square (iPLS) in order to identify the higher correlation between regions in the ^{1}H NMR spectra and the embryo implantation capability. The ^{1}H NMR regions with higher correlation are between 1.2 and 0.5 ppm, that included the signals for cholesterol backbone $–C(18)H_3$, $–CH_3$ and CH_2 groups of triglycerides, cholesterol compounds and phospholipids. Our results can allow building a quick, non invasive, useful and feasible chemometric models in order to identify embryos with a high pregnancy rate and embryos unable to achieve successful pregnancies.

Keywords Chemometric tools · HPLC–MS/MS · NMR · Embryo viability · Pregnancy potential · Culture media · IVF

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1 Introduction

Even though three million children around the world have been born by means of in vitro fertilization (IVF) (Xella et al. 2010), and approximately 250,000 ART cycles are carried out per year around the world, the pregnancy rate is still far from being considered good (Seli et al. 2007). One of the reasons is the lack of knowledge of embryo biology. Consequently, we are still forced to compensate for the low success rates by transferring more than one embryo, which result in either multiple order pregnancies (RE 2001) putting both patients and embryos at high risk (Mugford and Henderson 1995; Lieberman 1998; Bergh et al. 1999; Templeton 2000), or unsuccessful cycles.

With the growing trend in IVF clinics to transfer fewer embryos (ESHRE 2001; Templeton 2000; Hamberger and Hazekamp 2002), there is an increasing reliance on the precision of IVF laboratory techniques to maximize embryo implantation rates. The criteria for selecting embryos to be transferred are based largely on cell number

and their morphological appearance, which are relatively poor predictors of a successful implantation. More recently, the timing of embryo cleavage has also been included on the selection criteria (Racowsky et al. 2000). As a consequence, grading systems based on the embryo cleavage ratio and morphologic features have been developed (Cummins et al. 1986; Hill et al. 1989; Steer et al. 1992; Veeck 1999; Sakkas et al. 2001), leading to significant improvements in implantation and pregnancy rates, and fewer high order pregnancies (Toner 2002). Unfortunately, the accuracy of these techniques is still insufficient.

The limitations of evaluating embryos morphologically have led many researchers to look for additional technologies to assess the reproductive potential of a given embryo. However, we are coping with several important issues: the evaluation of embryo capability should be performed quickly in order to avoid the need for cryopreservation, should be non invasive in order to increase implantation rate (IR) while decreasing multiple births, and should be accessible for all the IVF laboratories, avoiding overly sophisticated methodologies.

Several metabolic parameters of developing embryos have been measured, using a number of non-invasive techniques (Gardner and Leese 2000; Sakkas and Gardner 2005; Seli et al. 2008; Vergouw et al. 2008). In recent years, various studies have shown the relationship between metabolites in the culture media, clinical pregnancy and live birth (Hardy et al. 1989; Gott et al. 1990; Conaghan et al. 1993; Jones et al. 2001; Gardner et al. 2001; Houghton et al. 2002; Brison et al. 2004; Marhuenda-Egea et al. 2010).

In this context, we sought to develop a new technique that would help embryologists in their labor. After 3 days, the embryos were transferred to the patient, and it is reasonable to think that changes in the culture medium could be related to the implantation potential for human embryos. Taking into account that amino acids are needed to synthesize new proteins, the variation in the amount of amino acids in the culture medium could be linked to embryo implantation capability (Houghton et al. 2002; Brison et al. 2004; Marhuenda-Egea et al. 2010). In our previous research we have reported that non-invasive amino acid analysis of embryo culture media correlates with successful pregnancy in women undergoing IVF (Marhuenda-Egea et al. 2010). Techniques such as high-performance liquid chromatography with mass spectroscopy (HPLC–MS) and a chemometric classification tool (soft independent modeling of class analogy, SIMCA) were used (Marhuenda-Egea et al. 2010). In the current study, we have analyzed the amino acid concentration and the metabolomic profile of embryo culture media in order to identify the embryo with the highest reproductive potential. We have applied high-performance liquid chromatography with mass spectroscopy (HPLC–MS), high-performance liquid chromatography with mass/mass spectroscopy (HPLC–MS/MS), proton nuclear magnetic resonance (^{1}H NMR) and a chemometric tools such as SIMCA (soft independent modeling of class analogy) (Esbensen 2006), interval partial least square regression (iPLS) (Norgaard et al. 2000; Winning et al. 2007), and genetic algorithm (GA) methods (Leardi and Lupiáñez-González 1998). Working with methods that select variables or intervals of variables in the original variable space can result more useful, i.e., selecting an interval of the spectrum. This selection in the original spectrum can be interpreted as chemical species. The spectroscopic information is optimally preserved with iPLS and GA methods (Leardi and Lupiáñez-González 1998; Leardi and Norgaard 2004). The iPLS method is a new graphically oriented approach for local regression modelling of spectral data, and GA is a very powerful tool for the variable selection for a PLS model (GA-PLS) (Leardi and Norgaard 2004). With a good selection of the spectral regions and good models it is possible to improve the predictive ability and the possibility for the development of new instruments for on-line monitoring of the process, such as determining the capability of the human embryo to produce a pregnancy in ART. Our objective was to develop a new, accessible, non time-consuming and biologically sensible tool that would help embryologists in their labour of selecting human embryos in IVF.

2 Materials and methods

2.1 Patient selection, treatment, and sample collection

All patients were treated in a long agonist protocol, starting with a dose of 3.72 mg of (Decapeptyl Depot, IPSEN, Slough, UK), administrated in day 18 of the previous cycle. Once the ovarian acquiescence was confirmed by means of an ultrasound scan performed after the menses, a combination of recombinant human follicle stimulating hormone (Gonal F®; Serono, London, UK) and human menopausal gonadotrophin (HMG-Lepori®; Farma-Lepori, Barcelona, Spain), was given according the patient's response. Ovarian stimulation was monitored by transvaginal ultrasound and measurement of oestradiol plasmatic levels. Ovulation was induced with 250 μg of recombinant human chorionic gonadotrophin (hCG) (Ovitrelle, Merck Serono, Barcelona). Oocytes were retrieved 36 h after hCG administration by a gentle transvaginal ultrasound-guided needle aspiration under sedation. On day one, fertilized embryos showing two pronuclei were placed into individual 50 μl drops of G1.3 culture medium (Vitrolife, Goteborg, Sweden), under standard incubation conditions to the cleavage stage and then transferred to patients on day three.

The present study had 23 participants; we evaluated 46 samples of the culture medium from human embryos obtained in IVF cycles carried out at the Bernabeu Institute of Fertility and Gynecology in Alicante, Spain, during the first semester of 2009, with known outcome (0 or 100% ongoing pregnancy rates). The transfers were Double Embryo Transfer (DET), and three DET were implanted with 50% ongoing pregnancy rates (Tables 1, 2, 3). After the 8th week, an ongoing pregnancy was confirmed by ultrasound scan (Heart activity). To make the analysis, the samples were divided in three sample sets (see Sect. 3) (Tables 1, 2, 3).

2.2 HPLC–MS and HPLC–MS/MS spectroscopy

After the embryos were transferred, the culture medium was obtained from the embryologist and used in analysis: 20 µl of culture medium, 20 µl of Met 0.1 mM as internal standard, and 60 µl of solvent A (30 mM ammonium acetate-5% acetic acid, 12.5:87.5, pH 2.5) were mixed in a tube and microinjected in the HPLC–MS system. LC–ESI–MS–MS analysis was performed as described (Thiele et al. 2008), with different modifications as explained below. LC–ESI–MS–MS analysis was performed with an Agilent (Santa Clara, CA, USA) 1100 series HPLC instrument. The LC system was coupled with Agilent 1100 Series LC/MSD Trap SL, with the possibility of carrying out MS/MS analysis. The mass spectrometer was operated in positive ESI mode, and the ion-spray voltage was set at 4 kV. Nitrogen was used as the sheath gas (30 psi), and the ion transfer capillary was heated to 350°C. Injections were performed using an HTC Pal autosampler (CTC Analytics, Zwingen, Switzerland) equipped with a 20-µl sample loop. The MS–MS parameters of the amino acids were determined by analyzing the flow injection of amino acid standard solutions using an inbuilt syringe pump. The culture medium solutions were infused in the flow of the HPLC system via a tee piece, under the following conditions: flow rate of the syringe pump, 10 µl min^{-1}; flow rate of the HPLC system, 0.3 ml min^{-1} (30 mM ammonium acetate-5% acetic acid, 12.5:87.5, pH 2.5, solvent A). LC separations were carried out with Phenomenex (Torrance, CA, USA) Luna 5 μ SCX 100 Å column, 150 mm × 2.0 mm internal diameter, at 25°C. For the elution of the

Table 1 Relative amino acid concentrations for first sample set measured by the integration of the peak areas in the HPLC–MS chromatograms and normalize with the peak area of the internal standard (Leu)

Droplet identification	Asp	Glu	Ser	Asn	Gln	Gly	Ala	Pro
Pregnancy								
1	0.404	0.941	0.181	0.348	1.030	0.025	0.110	1.620
2	0.395	0.805	0.138	0.407	0.712	0.019	0.113	1.557
3	0.443	1.019	0.212	0.474	1.160	0.032	0.110	1.661
4	0.420	0.999	0.183	0.536	0.836	0.028	0.119	1.919
5	0.354	0.804	0.185	0.386	0.849	0.023	0.089	1.428
6	0.440	0.892	0.149	0.409	0.935	0.020	0.081	1.591
7	0.329	0.742	0.130	0.337	0.804	0.000	0.099	1.374
Non pregnancy								
8	0.364	0.940	0.164	0.447	1.115	0.023	0.123	1.671
9	0.325	0.872	0.162	0.361	0.956	0.013	0.086	1.688
10	0.422	1.085	0.190	0.427	1.035	0.033	0.094	1.736
11	0.503	1.169	0.205	0.464	1.157	0.027	0.180	2.148
12	0.390	0.961	0.188	0.414	1.007	0.024	0.112	1.956
13	0.373	0.826	0.126	0.324	1.274	0.013	0.122	1.482
14	0.431	1.010	0.149	0.505	1.208	0.046	0.102	1.921
15	0.378	0.995	0.182	0.429	1.188	0.023	0.113	1.681
16	0.210	0.538	0.108	0.258	0.644	0.017	0.081	0.660
17	0.304	0.730	0.155	0.349	0.818	0.022	0.100	1.491
Pregnancy/non pregnancy								
18[a]	0.336	0.734	0.148	0.331	0.643	0.021	0.102	1.500
19[a]	0.386	0.893	0.195	0.443	0.887	0.032	0.118	1.660
20[a]	0.398	0.974	0.233	0.495	0.939	0.021	0.147	1.724
21[b]	0.416	1.019	0.151	0.415	1.165	0.018	0.115	1.690
22[b]	0.346	0.932	0.179	0.430	0.781	0.027	0.116	1.672
23[b]	0.212	0.630	0.109	0.319	0.608	0.018	0.071	0.663

Pregnancy was referred to the success of the implantation of a single embryo determined by the Positive Fetal Heart after 8 weeks

[a] Samples classified as pregnancy in the SIMCA model

[b] Samples classified as non-pregnancy in the SIMCA model

Table 2 Relative amino acid concentrations for second sample set measure by the integration of the peak areas in the HPLC–MS/MS chromatograms and normalize with the peak area of the internal standard (Leu)

Droplet identification	Asp	Glu	Ser	Asn	Gly	Ala
Pregnancy						
1	6.23	10.01	14.61	5.50	2.93	26.65
2	6.07	16.30	6.26	8.90	4.76	36.93
3	9.57	8.51	2.92	0.01	3.82	35.48
4	2.19	3.39	1.11	1.85	1.10	7.30
5	2.42	2.82	1.21	2.31	0.97	15.66
6	17.34	14.83	10.44	15.49	3.71	62.01
7	17.31	18.90	0.00	11.69	6.56	73.03
8	8.52	22.56	5.97	10.39	9.60	72.42
9	4.33	5.30	1.63	6.35	3.94	45.13
10	21.81	17.37	14.29	14.95	7.74	94.40
11	10.02	11.46	5.98	14.28	22.50	88.28
12	3.47	5.07	1.94	4.78	2.49	27.67
13	2.35	4.45	0.88	1.96	1.42	16.48
Non pregnancy						
7	11.17	12.26	0.00	18.39	6.42	94.98
8	10.46	15.39	2.67	11.18	3.78	74.11
9	10.78	11.17	3.41	13.69	6.67	90.86
10	9.22	13.29	1.93	13.61	5.82	79.88
11	9.38	17.98	0.00	12.16	5.55	75.52

Pregnancy was referred to the success of the implantation of a single embryo determined by the Positive Fetal Heart after 8 weeks

amino acids, an isocratic step was programmed with solvent A for 15 min. The overall flow rate was adjusted to 0.3 ml min^{-1}. Prior to use, the new SCX column was flushed with 150 mM ammonium acetate solution overnight. We determined the retention times for individual amino acids using a standard solution at the concentration of G1.3 culture medium (Vitrolife, Goteborg, Sweden) by HPLC–MS/MS, so as to avoid ambiguity (Thiele et al. 2008; Marhuenda-Egea et al. 2010). The areas of the HPLC–MS peaks with the same retention times as the calibration standard were integrated and subsequently used for calculating the concentrations of each individual amino acid. To calculate the relative amino acid concentration, the peak area was divided by the peak area of the internal standard.

2.3 ^{1}H NMR spectroscopy

We used the culture medium of the second sample set for the ^{1}H MNR experiment. 40 µl of the culture medium was put in a capillary tube and the capillary tube with the sample was introduced in a 5 mm nmr tube with 450 µl of ultrapure water and 50 µl of D_2O with 0.75% 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP). The spectra were referenced to TSP at 0.00 ppm.

All NMR experiments were performed on a Bruker Avance 400 MHz equipped with a 5 mm ^{1}H-BB-^{13}C TBI probe with an actively shielded Z-gradient. 1D solution state ^{1}H NMR experiments were acquired with a recycle delay of 2 s, 32,768 time domain points and with 2.556 s of acquisition time. The number of scans was 2253. Spectra were apodized by multiplication with an exponential decay producing a 5 Hz line broadening in the transformed spectrum. Direct ^{1}H NMR was performed using SPR-W5-WATERGATE (Lam and Simpson 2008). 12 ppm and −2 ppm and were outside the spectral window.

The ^{1}H NMR spectra were reduced to ASCII files using custom-written *ProMetab* software (version 2.1) (Viant

Table 3 Relative amino acid concentrations for the third sample set measured by the integration of the peak areas in the HPLC–MS/MS chromatograms and normalize with the peak area of the internal standard (Leu)

Droplet identification	Asp	Glu	Ser	Asn	Gln	Gly	Ala	Pro
Pregnancy								
1	15.25	25.55	3.38	18.35	11.44	5.57	112.75	38.81
2	11.67	16.53	4.18	14.83	6.38	4.96	95.26	45.60
3	14.35	11.39	5.10	15.66	6.31	5.22	108.44	38.11
4	15.27	18.80	6.02	17.92	10.01	6.95	115.36	49.85
5	5.62	9.07	0.00	5.75	3.10	2.19	46.70	19.04
6	9.81	15.47	3.06	12.19	7.76	2.83	63.72	33.90
Non pregnancy								
7	0.96	1.17	0.00	2.28	0.00	0.00	0.00	3.92
8	16.54	20.91	4.35	17.95	7.29	4.73	78.28	30.42
9	13.14	17.28	3.90	10.38	7.56	4.57	66.58	38.27
10	14.39	22.45	3.10	13.16	7.10	2.48	51.43	42.09
11	18.17	22.25	4.61	14.83	8.47	6.11	128.57	46.50
12	4.27	6.41	0.39	4.22	2.41	0.00	22.14	16.77

Pregnancy was referred to the success of the implantation of a single embryo determined by the Positive Fetal Heart after 8 weeks

2003) and aligned using *i*coshift (version 1.0; available at www.models.kvl.dk) (Savorani et al. 2010). All ^{1}H NMR spectra processing have been performed in MATLAB (The MathWorks, Natick, MA) using a AMD Turion X2, 2.20 GHz processor with 4 GB of RAM.

2.4 Statistical analysis

SIMCA is a classification algorithm based on producing a PCA model for each class of samples and then comparing their distance to the class confidence limits (Esbensen 2006). A personal computer with The Unscrambler 9.8 (Camo, Sweden) was used for this purpose.

The iPLS algorithm and GA-PLS, especially useful to wavelength selection, have been used. These algorithms have already been described (Leardi and Lupiáñez-González 1998; Norgaard et al. 2000; Leardi and Norgaard 2004; Winning et al. 2007) and the reader is referred to these papers for more details. MATLAB version 6.5 from MathWorks is used for the calculations, and the iPLS algorithm (included in iToolbox) GA-PLS Toolbox are available from http://www.models.kvl.dk.

3 Results and discussion

3.1 HPLC–MS and HPLC–MS/MS analysis

It has been reported that non-invasive analysis of amino acid turnover has the potential to improve significantly the prospective selection of the most viable embryos in an IVF cycle (Houghton et al. 2002; Brison et al. 2004; Marhuenda-Egea et al. 2010). We used HPLC–MS to determine the relative amino acid concentrations with the first sample set (Fig. 1). The sample set was divided in two groups (pregnancy and non-pregnancy). The SIMCA model constructed with all the relative amino acid concentrations had a distance model of 5.20. Using the relative amino acid concentrations with a higher discrimination power (Asp, Gln, Glu, Ser and Pro), the distance model increased until 19.59. The Coomans plot showed the separation between the two groups of embryos (Fig. 2): the viable and non-viable.

There are other cases, where two embryos are transferred, and only one gives pregnancy. We have also

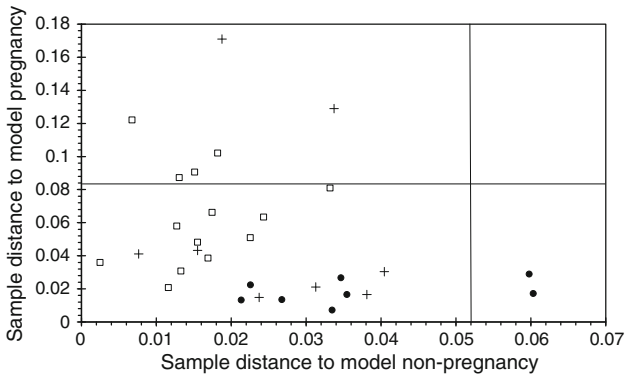

Fig. 2 Coomans plot for discrimination between pregnancy and non-pregnancy embryos. SIMCA model was made with the relative amino acid concentrations determined by HPLC–MS from the third sample set. *Empty square* non-pregnancy and *filled circle* pregnancy samples. Pregnancy was referred to the success of the implantation of a single embryo determined by the Positive Fetal Heart after 8 weeks. The *horizontal and vertical gray lines* are class membership limits calculated at a 25% confidence limits. (+) Cases in which two embryo were implanted and only one Positive Fetal Heart

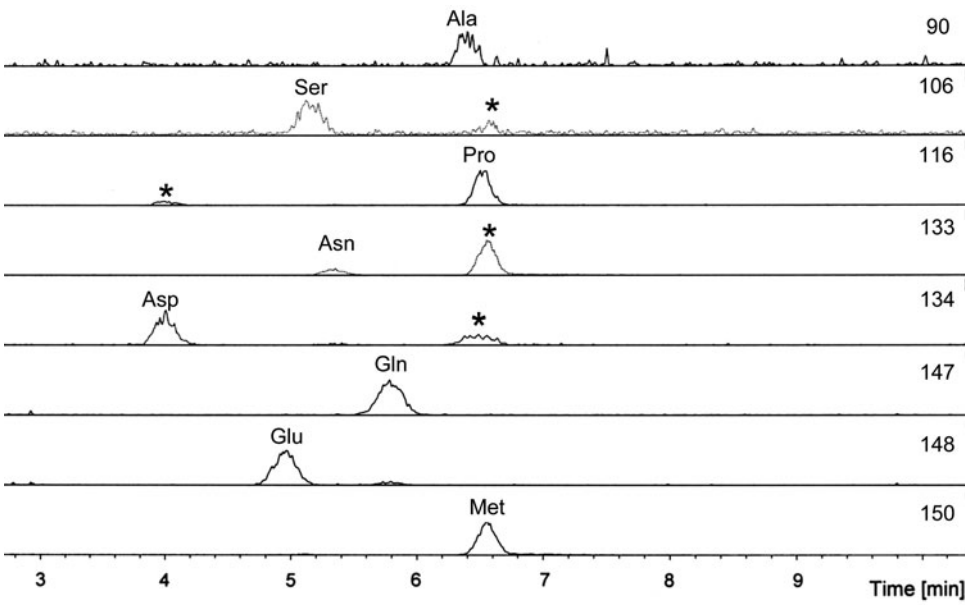

Fig. 1 Selected ion chromatograms of seven amino acids present in G1.3 culture medium and Met as internal standard (20 μM) obtained in HPLC–MS. The numbers on the right are m/z protonated precursor ion and the peaks marked with "*" correspond to contribution of another product ion from another amino acid

included four IVF cycles in our model with two embryos per cycle. For this situation, four embryos are classified as pregnancy and four embryos are classified as non-pregnancy, using HPLC–MS analysis and SIMCA model (Fig. 2). Evidently, with this analysis it is not possible to determine which embryo was pregnancy and which one was not pregnancy in IVF cycle. However, this is an important issue in IVF outcome: why, when two embryos are transferred, only one gives pregnancy? In the last sample set we have included samples of DET with only one pregnancy. The SIMCA model built with these samples previous analyzed with HPLC–MS, could discriminate between the two embryos (Fig. 2). SIMCA method, improved with the analysis of more samples, opens a real way to know the embryo with higher implantation potential. In other words, from a clinical point of view: fertility clinics could dramatically improve patient safety reducing the number of transferred embryos, decreasing the related risk of multiple order pregnancies and lastly, but not less important, increasing the number of embryos suitable to be successfully frozen. Moreover, the study of the amino acid consumption opens new ways to improve our knowledge of early human embryos physiology.

In the second sample set, we measured the relative amino acid concentrations using HPLC–MS/MS spectroscopy. We determined the most abundant cation species for the individual amino acid in order to improve the *m/z* signal (see Sect. 2). This second sample set was divided in two groups (pregnancy and non-pregnancy), as in the first sample set, and the relative amino acid concentrations determined by HPLC–MS/MS were analyzed by PCA and classified with SIMCA. The SIMCA model had a distance of 7.85. The Coomans plot displayed the separation between the relative amino acid concentrations from culture medium of embryos with pregnancy and non-pregnancy outcome (Fig. 3).

Also HPLC–MS/MS analysis was used with the third sample set. This sample set was also divided in two groups (pregnancy and non-pregnancy). The constructed SIMCA model had a distance of 33.28. The relative amino acid concentration for Ser and Gly had low modeling power and they were removed to a new SIMCA model. Coomans plot for this new model shows the separation between the culture media from embryos with pregnancy and non pregnancy, with a distance of 59.77 (Fig. 4).

With different sample sets analyzed by HPLC–MS and HPLC–MS/MS, it was possible to obtain good SIMCA models that allow us to classify the embryos in pregnancy and non pregnancy. An important feature in our analysis is that the amino acids can be detected without any modification or derivatization. The derivatization is the usual method used to analyze amino acids (Houghton et al. 2002; Brison et al. 2004). Here we use a method without any

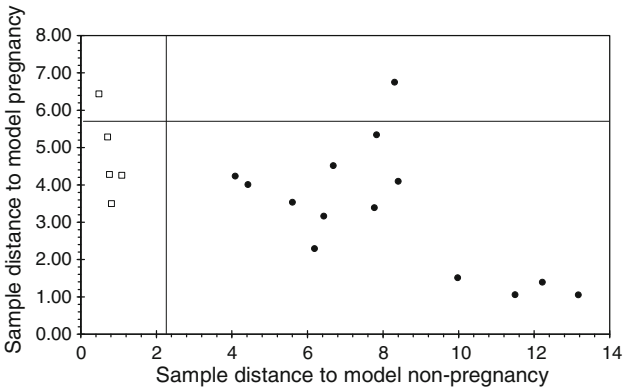

Fig. 3 Coomans plot for discrimination between pregnancy and non-pregnancy embryos. SIMCA model was made with the relative amino acid concentrations determined by HPLC–MS/MS from the first sample set. *Empty square* non-pregnancy and *filled circle* pregnancy samples. Pregnancy was referred to the success of the implantation of a single embryo determined by the Positive Fetal Heart after 8 weeks. The *horizontal and vertical gray lines* are class membership limits calculated at a 25% confidence limits

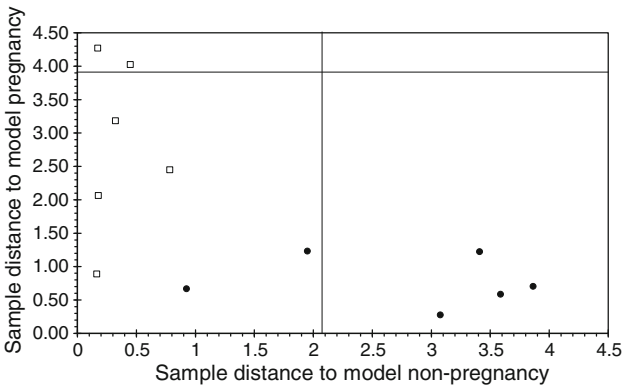

Fig. 4 Coomans plot for discrimination between pregnancy and non-pregnancy embryos. SIMCA model was made with the relative amino acid concentrations determined by HPLC–MS/MS from the second sample set. *Empty square* Non-pregnancy and *filled circle* pregnancy samples. Pregnancy was referred to the success of the implantation of a single embryo determined by the Positive Fetal Heart after 8 weeks. The *horizontal and vertical gray lines* are class membership limits calculated at a 25% confidence limits

derivatization (Thiele et al. 2008)), since derivatization could bring an external error source in the analysis by sample work out. Our objective was to analyze the sample with the minimum operator participation (see Sect. 2). By HPLC–MS and HPLC–MS/MS it was possible to achieve our goal and to analyze the relative amino acid concentration with the minimum operator participation.

Clearly, it is difficult or impossible to view the data from HPLC–MS or HPLC–MS/MS grouping in seven dimensions (one dimension for each amino acid). A multidimensional region method is needed for where a sample point is expected to be located. Principal component analysis (PCA) is the perfect tool for this task (Esbensen

2006). Since each class has its own distribution, the distance of each sample from each class must be calculated. This should not be surprising, as these samples were used as the training set. The most significant result is that no sample from any other case in the set is classified incorrectly in another class (Figs. 2, 3, 4). In other words, the embryos with a high implantation rate (pregnancy) are in one group and, as a more important practical application, the embryos with low implantation rate (non-pregnancy) are in another group (Figs. 2, 3, 4). The PCA models are based on the samples from a known class. This means that samples that had the same relative amino acid concentrations would still be classified within those with the same case. In addition, using SIMCA, a sample can be a member of two or more classes, or a member of none of the classes modeled (Figs. 2, 3, 4). With all these considerations in mind, the near-zero error classification is impressive, and lends credibility to the postulate that there are real relative amino acid concentration differences between these samples that can be attributed to non-pregnancy and pregnancy cases.

The SIMCA models can be improved if the data for amino acids with low modeling power are removed, i.e., for the third sample set, the model distance increased from 33.28 to 59.77, since some amino acid concentrations were removed from consideration. This feature could make us think about the possibility of selecting some amino acids as biomarkers of embryo viability. However, the modeling power of the relative amino acid concentrations in the SIMCA model is determined by the analytical technique, HPLC–MS or HPLC–MS/MS. HPLC–MS shows a low sensitivity for the amino acids with low molecular mass, such as glycine (76 g/mol) and alanine (90 g/mol). HPLC–MS/MS is founded on the fragmentation of the ions and in the detection of the majority fragment. Therefore, the sensitivity in HPLC–MS/MS for an amino acid is determined by the abundance of the majority fragment.

3.2 ^{1}H NMR spectroscopy

^{1}H NMR spectroscopy is a non-destructive technique and it is possible to use the same sample in HPLC–MS/MS. We took out the NMR tube sample and it was prepared for the HPLC–MS/MS analysis presented above. The evaluation of the third sample set by means of ^{1}H NMR spectroscopy and multivariate analysis, using PCA and SIMCA, also showed differences between the culture medium from embryos with pregnancy and non-pregnancy outcomes. NMR is a holistic technique, in a ^{1}H NMR spectrum it is possible to see all the metabolites present in the culture medium. The major problem, with these samples from G1.3 culture medium, is the difference between the metabolite concentrations, i.e., the lactate concentration is

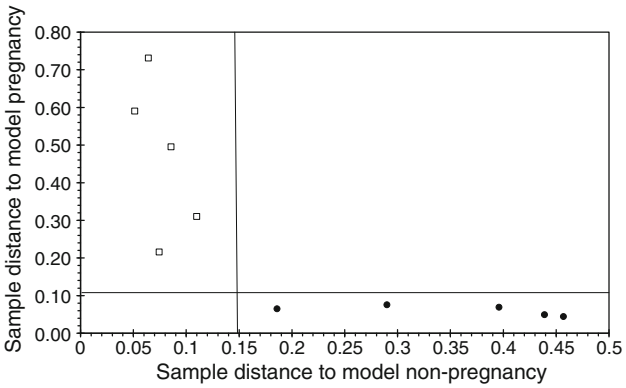

Fig. 5 Coomans plot for discrimination between pregnancy and non-pregnancy embryos. SIMCA model was made with the ^{1}H NMR spectral region between 1.2 and 0.5 ppm from the second sample set. *Empty square* Non-pregnancy and *filled circle* pregnancy samples. Pregnancy was referred to the success of the implantation of a single embryo determined by the Positive Fetal Heart after 8 weeks. The *horizontal and vertical gray lines* are class membership limits calculated at a 25% confidence limits

10.5 mM and the amino acid concentrations are 0.1 mM (except Gln with 1 mM). Another evident problem is the volume of the sample: we work with around 40 µl.

The SIMCA model constructed had a model distance of 5.29, working with the spectral region between 4.5 and 0.5 ppm. The spectral region with a particular high discrimination power was selected in order to improve the model. We selected the spectral region between 1.2 and 0.50 ppm. It was possible to obtain a very good SIMCA model with a model distance of 57.66. The Coomans plot displayed the separation between the spectra from culture medium of embryos with pregnancy and non-pregnancy (Fig. 5).

With another chemometric approach, using iPLS and GA-PLS algorithms (Norgaard et al. 2000; Winning et al. 2007), is possible to go more in depth into the spectroscopy data from embryo culture medium. It was possible to model the embryo pregnancy rate by sequential application of iPLS and GA-PLS algorithms. Interval PLS removes the non-informative intervals and genetic algorithm can work with a significantly lower number of variables. A better correlation was also found in the spectral region between 1.2 and 0.5 ppm, using iPLS regression. Working with this region, we should select the region between 0.83 and 0.79 ppm, with a root mean square error of cross validation (RMSECV) of 3.15% with two components (Fig. 6). Applying genetic algorithms (GA-PLS) in the interval selected by iPLS, RMSECV was 2.21% (selecting two spectral regions: around 0.82239 ppm and around 0.79941 ppm) (Fig. 7). The region between 1.2 and 0.5 ppm showed signals of the cholesterol backbone $-C(18)H_3$, $-CH_3$ and CH_2 groups of triglycerides, cholesterol compounds and phospholipids (Kristensen et al.

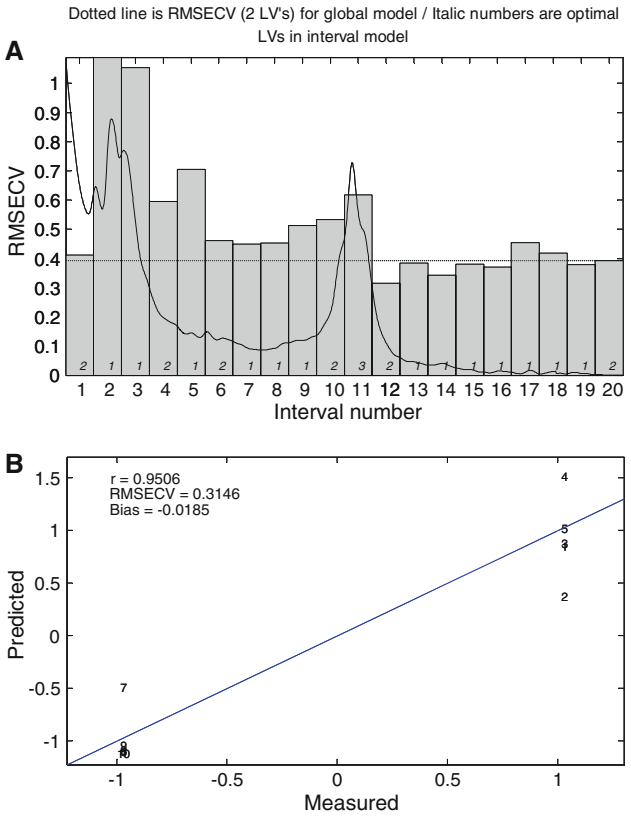

Fig. 6 **a** Cross-validated prediction performance (RMSECV) for 20 interval models (*bars*) and for the ^{1}H NMR spectral region between 1.2 and 0.5 ppm (*line*) plotted together with the normalized mean spectrum. The italic numbers on the bars indicate the optimal number of PLS components used in each interval model. **b** Predicted versus measured plot for the ^{1}H NMR spectral region for the best interval 12 (0.83–0.79 ppm) from a iPLS model with two components

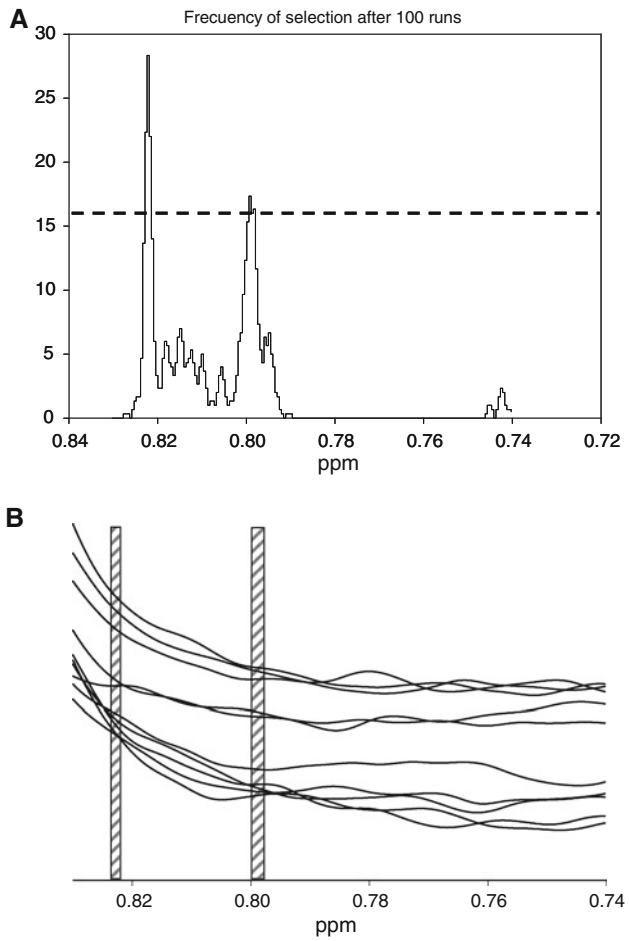

Fig. 7 **a** Frequency of selection of original ppm after GAPLS (100 runs) in ^{1}H NMR spectral region selected by iPLS (0.83–0.79 ppm). **b** Location of ^{1}H NMR spectral region selected by GAPLS

2010). With the chemometric approach it is possible to go more in depth into the prediction of embryo pregnancy capability, since a good ppm selection can open the door to discover the molecule key in the embryo pregnancy capability. If our results and approach presented here go in a positive direction, we can design new strategies in order to significantly improve human embryo selection in ART.

^{1}H NMR spectroscopy is a very useful technique in the metabolomic approach to the biological process, and it was used for analyzing the metabolic profile of the embryo culture medium and to find molecules that correlate with the embryo implantation rate (Seli et al. 2008). The Seli group (Seli et al. 2008), using ^{1}H NMR spectral integration found that glutamate levels were higher in spent culture media samples of embryos that result in pregnancy compared with those that fail to implant. They proposed that the elevated levels of glutamate in culture media of embryos that result in pregnancy may be related to lowering the levels of potentially detrimental ammonium in culture media better than nonviable embryos (Seli et al. 2008). Seli et al. (2008) also found a trend toward decreased alanine levels in the culture media of embryos with higher reproductive potential (Seli et al. 2008). In the ^{1}H NMR spectra of the culture media, we did not quantify the amino acid concentrations such as Seli et al. (2008). Our approach was very different. We attempted to find out the ^{1}H NMR spectral regions of samples of embryo culture media with a reproductive potential correlation using chemometric tools, such as SIMCA, iPLS and GAPLS. The next step should be to find the molecule or molecules that generated the signal selected by the chemometric tools.

4 Concluding remarks

The chemometric models constructed with data from HPLC–MS, HPLC–MS/MS or ^{1}H NMR show that there are metabolic differences between the embryos with pregnancy and the embryos with non-pregnancy. These metabolic differences can be detected and applied as a classification tool to select the embryos with the highest

implantation rate. Consequently, we can hypothesize that all the amino acids present in the embryo culture medium (G1.3) play a crucial role in the metabolism of the embryos during the culture, and the efforts to find one or two amino acid as embryo viability biomarkers in the culture medium could be reconsidered. On the other hand, the chemometric analysis of ^{1}H NMR profiles showed that the better correlation with embryo pregnancy capability was in the main lipid spectral region (1.2–0.5 ppm). Our hypothesis in future research will be study the role of lipid molecules present in the embryo culture medium.

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