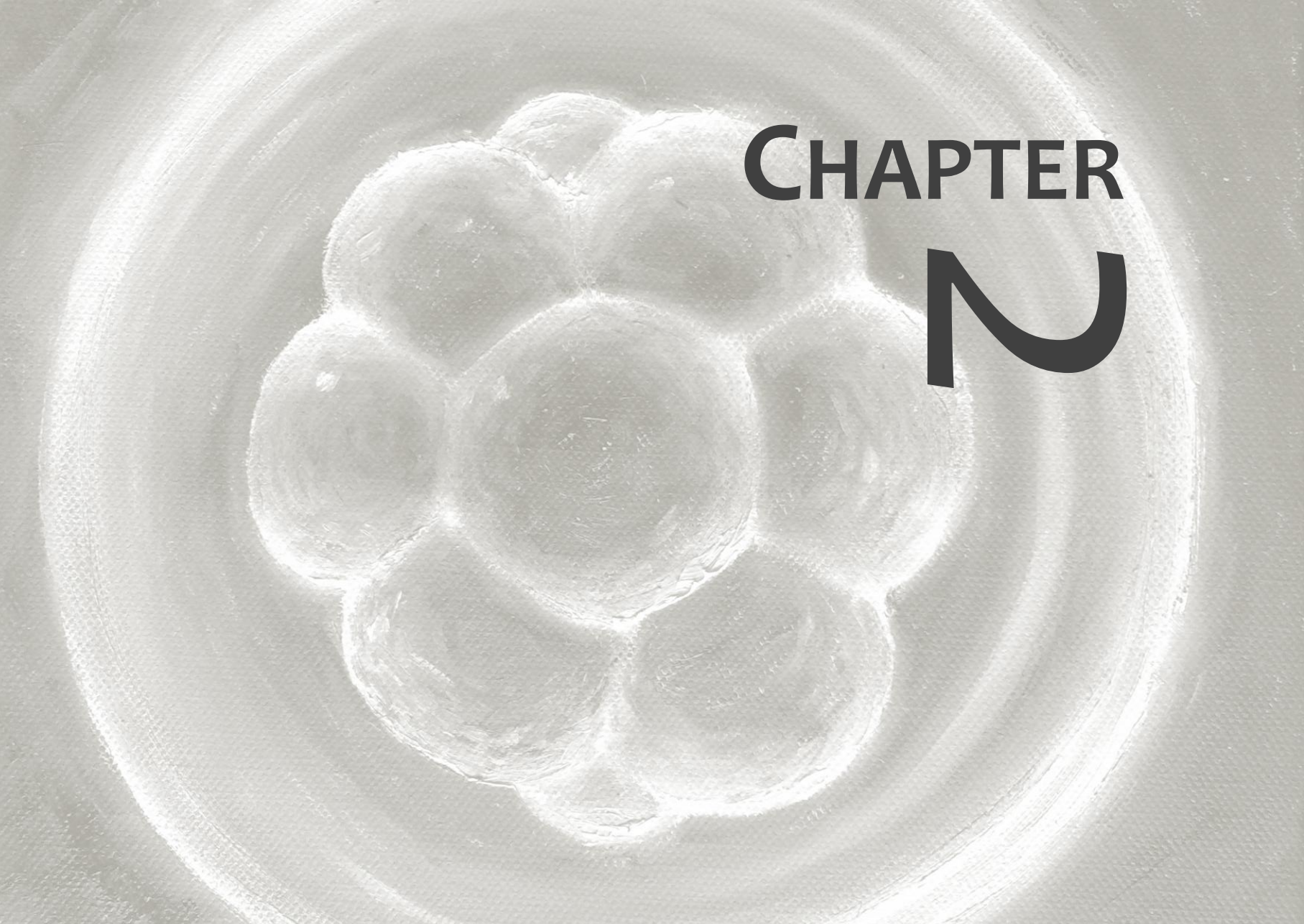

Chapter 2

METABOLOMIC PROFILING BY NEAR-INFRARED SPECTROSCOPY AS A TOOL TO ASSESS EMBRYO VIABILITY: A NOVEL, NON-INVASIVE METHOD FOR EMBRYO SELECTION

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ABSTRACT

Background: The morphology of an embryo has a limited predictive value for assessing viability and ongoing pregnancy, therefore new selection tools are needed to maintain success rates with single embryo transfer (SET). In this study, we investigated if metabolomic profiling of biomarkers of embryo culture medium by near-infrared (NIR) spectroscopy has a correlation with ongoing pregnancy in SET.

Methods: A total of 333 patients scheduled for in vitro fertilization (IVF) with SET were included in the study. Embryos were selected for transfer by morphological criteria on days 2 and 3 of in vitro culture, and left over culture media samples were analysed by NIR spectroscopy.

Results: The NIR spectral analysis produced unique metabolomic profiles that correlated to an embryo's reproductive potential. Resulting relative embryo viability scores between positive and negative pregnancy outcomes were statistically significant ($P<0.03$). A logistic regression of factors correlated to pregnancy outcomes showed that maternal age, percent fragmentation and relative embryo viability scores all demonstrated a relationship. The extent of the correlation was determined by accuracy computation, where the accuracy of assessing viable embryos on day 3 by metabolomic profiling was 53.6% and the accuracy of the morphological selection was 38.5%. In addition, the positive predictive value of metabolomic profiling was 0.365 and the negative predictive value was 0.830.

Conclusions: NIR metabolomic profiling of spent embryo culture media was able to distinguish viable embryos from non-viable embryos for reproduction.

INTRODUCTION

One of the most important complications of in vitro fertilization (IVF) treatment is a high multiple pregnancy rate. Multiple gestations lead to a higher incidence of medical (both perinatal and neonatal) and socioeconomic complications[1-4]. Single embryo transfer (SET) is an effective way to minimize the risks of multiple pregnancies post-IVF treatment[5-7]. Given that a single embryo is transferred, the embryo selection process is crucial. However, general acceptance of SET is limited as there lacks an accurate method in assessing embryo reproductive viability[8]. Currently, non-invasive embryo selection is primarily based on morphological criteria using light microscope analysis. Many studies have shown that an embryo's morphological appearance and developmental potential are biomarkers of its viability. Early cleavage[9-11] and embryos that develop at a certain cleavage rate[12-14] were shown to be good prognostic factors. Pronuclear morphology[15-17], the appearance of much fragmentation[12,13,18,19], multi nucleation of blastomeres[20] and unequally sized blastomeres[21-24] have also shown to be important. Although all of these factors are known to contribute to pregnancy results, they have a limited predictive value for ongoing pregnancy. Because of the limited predictive value, new selection tools are needed[8,25]. Additional methods which claim to be increasingly accurate, such as preimplantation genetic screening (PGS), are at a disadvantage in that one or two blastomere(s) is/are aspirated from an embryo. As an invasive measurement, this method provides little additional reproductive assessment value and shows no improvement in pregnancy rates[26]. One other study even reported lower pregnancy and live birth rates[27].

Recent studies have presented non-invasive metabolomic profiling methods of assessing an embryo's reproductive potential. It is thought that the physiology and viability of the preimplantation embryo is affected by the environment to which the embryo is exposed in vitro[28]. Suboptimal culture conditions induce cellular stress and lead to metabolic transformation. As metabolic transformation is associated with a change in viability, embryo metabolism assessment may be used to determine embryo viability before transfer[29]. The intrinsic quality of the embryo can also lead to differences in metabolism.

In previous studies, viability differences were found in embryo metabolism of pyruvate[25,30], glucose[25] and amino acid turnover[8,31,32]. Although these studies have solidified the increasing potential of metabolomic profiling, they are of limited use in routine IVF laboratories. The techniques can be very expensive and often require complex instrumentation and specific skilled staff. In addition, receiving results of these tests are not within the time frame required to make an embryo selection. So the

need for a rapid, accurate and non-invasive tool to predict embryo viability in SET in IVF remains.

In a recent publication, a method has been introduced that evaluates, by means of near-infrared (NIR) spectroscopy, the metabolomic profiles of spent culture media of IVF/ICSI embryos showing its ability to assess an embryo's reproductive potential[33]. However, this study was conducted using medium samples of multiple transferred embryos, whereas this method may be of particular value in the selection process with SET. Techniques such as proteomics and metabolomics are able to provide important clinical information concerning the modification of an embryo's in vitro environment. In relation to metabolomics by NIR spectroscopy, this information is measured biologically by ascertaining the concentrations of key functional groups such as ROH, -SH, C=C, -CH, -OH, and -NH groups. The possible constituents of the culture media that could represent these changes include albumin, lactate, pyruvate, glutamate and glucose. In this pilot study, we retrospectively investigated if metabolomic profiling of spent culture media by NIR spectroscopy, correlates with ongoing pregnancy when the transferred embryos were selected by conventional morphological selection criteria.

MATERIALS AND METHODS

PATIENT POPULATION

The ethic committee of the VU University medical center gave approval for this study. From July 2006 to April 2007, embryos from patients of 333 IVF or ICSI cycles (304, day 3; 29, day 2) with SET were included. Pregnancy outcomes were recorded for each patient at 12 weeks of gestational age. Positive pregnancy was defined as fetal cardiac activity (FCA) at this time.

STIMULATION PROTOCOL

Stimulation protocols were performed as previously described[34,35]. Briefly, patients under the age of 38 years or with previous good response in a IVF or ICSI treatment underwent controlled ovarian hyperstimulation with a 'long' protocol with GnRH-agonist (Decapeptyl [Ferring, Copenhagen, Denmark]) and gonadotropins (Gonal F [Serono, Geneva, Switzerland], Puregon [Organon, Oss, the Netherlands] or Menopur [Ferring, Copenhagen, Denmark]). In women older than 38 years or with a previous poor response a short GnRH-agonist protocol was applied.

Ovarian response was monitored by vaginal ultrasonography and serum estradiol determinations. Human chorionic gonadotropin (HCG; Pregnyl [Organon, Oss, the

Netherlands]) 10,000 IU s.c. was administered, when there was at least 1 follicle ≥18 mm and three or more follicles ≥16 mm. Ultrasonographic directed oocyte retrieval was performed 36 h later.

LABORATORY PROCEDURE

Oocyte insemination was initiated ~40 h after HCG injection using standard IVF and/or ICSI procedures. At 16-18 h after insemination, the fertilization was scored. Embryos were cultured individually in 25-µl pre-equilibrated medium drops (human tubal fluid [HTF], Lonza, Belgium with 10% protein solution, Sanquin, the Netherlands) and alongside, embryo-free media drops were incubated as controls. Morphological assessment was assigned for each embryo by combining the number of blastomeres and the percentage fragmentation of an embryo. Embryos were selected by their morphological appearance and developmental potential prior to transfer. The embryo with the highest number of blastomeres and the least fragmentation was transferred. If only one embryo was available, the transfer was performed on day 2 after oocyte retrieval, irrespective of morphology. After transfer, the spent media drop, in which the transferred embryo was cultured, and a control media drop were immediately frozen (-196°C). Samples were shipped to Molecular Biometrics (Montreal, Canada) and stored at -80°C until analysed by NIR spectroscopy.

ANALYSIS OF CULTURE MEDIA SAMPLES BY NIR SPECTROSCOPY

Spectral regions (ROH, -SH, -C=C, -CH, -OH, and -NH groups) of metabolomic profiles that discriminated between embryos with positive and negative pregnancy outcomes (as determined by FCA) were determined by a genetic algorithm (GA) search method. These spectral regions were quantified using inverse least-squares regression and leave-one-out cross validation, resulting in a relative 'embryo viability score' as a measure of an embryo's reproductive potential. For further details see Seli et al. (2007)[33].

IVF culture media samples were thawed at room temperature for 30 minutes, after which they were vortexed for 10 seconds and centrifuged for 10 minutes at 13000 RPM. Samples were analysed by NIR spectroscopy using an indium gallium arsenide (InGaAs) array based 512 element NIR spectrometer with an operating wavelength of 920-1675nm. The sample chamber was stabilized at 21 °C so spectra were recorded under controlled temperature. NIR compatible 3 mm path length sample cells were filled with 7-µl of sample media for the spectral measurements. Sample analysis time was approximately 1 minute. The measurement was repeated with the control media to account for any variation in culturing conditions.

Validation of the spectrometer was done by measuring the reproducibility of NIR spectra from the same batch of 6 different IVF culture media over a period of 10 consecutive days. The coefficient of variation was 0.4%. Instrument noise and drift were also monitored and validated using standard solutions of water-ethanol and fixed wavelength filters.

SPECTRAL MODEL DEVELOPMENT

NIR metabolomic profiles were computed by dividing each specimen's transmittance spectrum by its associated control media spectrum and were then converted to an absorbance scale. Resulting absorbance spectra were mean-centered and were used for all subsequent calculations.

Spectral regions within the NIR metabolomic profiles that discriminate most between positive and negative FCA pregnancy outcome embryos consisted of determining a parsimonious combination of spectral regions that estimate pregnancy outcomes by inverse least-squares regression and genetic algorithm (GA) optimization. This method is based on that given by Gributs and Burns[36] and by Seli et al.[33]. Briefly, models investigated were described by the formula:

$$Y = b_0 + b_1 X + b_2 X_2 + ... + b_n X_n \qquad (1)$$

where Y is the pregnancy outcome of each sample (1 = positive FCA, 0 = negative FCA), X1, X2, …, Xn are the spectral regions, and b0, b1, …, bn are their associated weighting coefficients. A GA determines the combination of X-values that best estimate Y, by employing principles of Darwinian natural selection and biological inspired operations: reproduction, crossover, and mutation.

The method first objectively selects spectral regions by a pre-processing method based on Haar wavelets. A wavelet transformation concentrates spectral information in a small number of variables, similar to jpeg compression of images. Combinations of integrated spectral regions defined by the wavelets (X-values) are used as trial solutions. Generally, a GA produces an initial population containing a number of trial solutions. These solutions are evaluated (to yield a fitness) and a new generation is created from the better of them. The process is continued through a number of generations with the aim that the population should evolve to contain an acceptable solution. The weighting coefficients (b0, b1, …, bn) of the optimal wavelets selected by the GA were then calculated by inverse least-squares regression, using known FCA pregnancy outcomes (Y). All analyses were written in and evaluated using Matlab (The MathWorks Inc., MA).

EMBRYO VIABILITY DETERMINATION

Final relative embryo viability scores (Y) were determined using the model developed (equation 1), with known spectral regions, and weighting coefficients. In theory, Y-values are discrete values of either 0, negative FCA, or 1, positive FCA. However, the model produced can estimate a continuous scale of reproductive potential. The greater the relative viability score, the higher the embryo's potential of resulting in pregnancy.

STATISTICAL ANALYSIS

Subsequent to the relative viability score estimation, Student's t-test results were used to separately determine trends within the pregnancy outcome groups. Alpha error of <0.05 was considered significant for all comparisons. Values of accuracy, positive and negative predictive values (PPV and NPV) were also computed using,

$$Accuracy = \frac{TP + TN}{TP + TN + FP + FN} \times 100 \quad (2)$$

$$PPV = \frac{TP}{TP + FP} \quad (3)$$

$$NPV = \frac{TN}{TN + FN} \quad (4)$$

where TP is the number of true positives, TN, the number of true negatives, FP, the number of false positive, and FN, the number of false negatives. Values of TP, TN, FP, and FN were determined by selecting the optimal threshold as determined by a receiver operator characteristic curve.

A logistic regression analysis was done to model the probability of pregnancy given maternal age, percent fragmentation and number of blastomeres of the embryo, type of infertility treatment (IVF or ICSI), endometrium thickness, duration and indication of infertility, and the relative embryo viability scores.

A Pearson correlation coefficient was also calculated to establish the association between the relative viability score and the morphology of an embryo.

RESULTS

The overall ongoing pregnancy rate after SET on day 2 was 17.2% (5/29) and 29.3% (89/304) after day 3 SET. There were no multiple pregnancies. NIR spectral analysis of discarded culture media samples collected from 333 SET cycles produced unique metabolomic profiles. The mathematical model (equation 1) produced relative embryo viability scores that correlated to an embryo's reproductive potential as determined by pregnancy outcomes. The mean relative viability scores of negative and positive FCA groupings after day 2 SET were 0.2295 (± 0.0985) and 0.2814 (± 0.0224), respectively ($P<0.03$). The mean relative viability scores of negative FCA and positive FCA groupings after day 3 SET were 0.2767 (± 0.1115) and 0.3076 (± 0.0974), respectively ($P<0.02$).
Table 1 shows values of accuracy, positive predictive values and negative predictive values of metabolomic profiling and morphology assessment after day 2 and day 3 SET. The accuracies of metabolomic profiling and morphology in assessing embryo reproductive potential after day 3 SET were 53.6 and 38.5%, respectively. The positive predictive values of metabolomic profiling and morphology after day 3 SET were 0.365 and 0.313, respectively. The negative predictive values of metabolomic profiling and morphology after day 3 SET were 0.830 and 0.833 respectively. The accuracies of metabolomic profiling and morphology after day 2 SET were 69.0 and 27.6%, respectively. The positive predictive values of metabolomic profiling and morphology after day 2 SET were 0.333 and 0.100, respectively. The negative predictive values of metabolomic profiling and morphology after day 2 SET were 0.941 and 0.667, respectively.

Table 1. The accuracy, the positive and negative predictive value of the metabolomic profiling and the morphology after day 2 and day 3 SET.

	Variable	Accuracy (%)	Positive Predictive Value	Negative Predictive Value
Day 2	Morphology	27.6	0.100	0.667
	Metabolomic profiling	69.0	0.333	0.941
Day 3	Morphology	38.5	0.313	0.833
	Metabolomic profiling	53.6	0.365	0.830

Logistic regression analysis (Table 2) of factors contributing to FCA revealed that after day 3 SET, maternal age, percent fragmentation of the embryo and the relative embryo viability score were positively associated with ongoing pregnancy. Number of

blastomeres, ICSI or IVF treatment, endometrium thickness, duration and indication of infertility were not associated with ongoing pregnancy and were excluded from the model. Logistic regression analysis of factors contributing to FCA for day 2 SET was denied because of the small sample size.

Table 2. Multivariate regression analysis of factors contributing to FCA after day 3 SET with the variables age, fragmentation, cell number, endometrium thickness, ICSI or IVF treatment, infertility duration, infertility indication and the relative viability score.

Day 3 SET	Variable	Coefficient	Std. Error	P-value
	Age	-0.084	0.0308	0.006
	Fragmentation embryo	-0.6703	0.2519	0.008
	Relative viability score	26.839	12.861	0.037

Figure 1 shows the relation between the relative viability score and number of blastomeres with a Pearson correlation coefficient of –0.013. The null hypothesis of no association is accepted (P=0.826). Figure 2 shows the relation between the relative viability score and percent fragmentation with a Pearson correlation coefficient of 0.024. The null hypothesis of no association is also accepted (P=0.682).

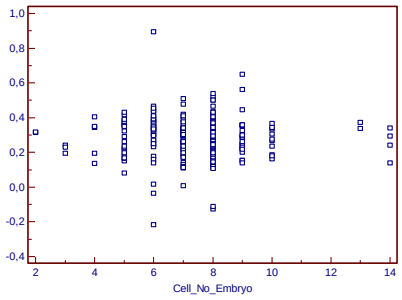

Figure 1. The absence of relation between the relative viability score and number of blastomeres (r=-0.013).

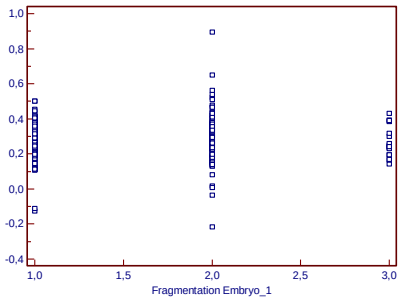

Figure 2. The absence of relation between the relative viability score and fragmentation (r=0.024).

DISCUSSION

Through this pilot study, we have demonstrated that metabolomic profiling of spent culture media by NIR spectroscopy is capable of accurately assessing the reproductive potential of a cleavage stage embryo, by correlating a relative viability score to ongoing pregnancy. Furthermore, it was suggested that the modification of culture media by oxidative modification may be an indicator of reproductive potential, and is independent of morphological selection criteria.

NIR spectroscopy is advantageous in the application of metabolomic profiling as it only requires small sample volumes (7-10-µl), is non-invasive and non-toxic to the embryo. It can be easily incorporated into a clinical setting as it is a compact, bench-top instrument that is rapid (analysis time per sample is <1 min) and simple to use. Even further, it offers objective selection criteria in comparison to morphological selection methods, where the morphology of an embryo is judged by a variation of embryologists and technicians with subjective opinions.

Our results indicate that embryos that implant and result in pregnancy affect their in vitro environment differently than embryos who fail to implant. This is in agreement with previous studies in which differences were found in embryo metabolism of glucose[25], pyruvate[25,30] and amino acid turnover[8,31,32]. In contrast, NIR spectroscopy analyses the changes in several organic functional groups (ROH, -SH, C=C, -CH, -OH, and -NH groups) associated with these individual metabolites, and is therefore an indirect assay of culture media biomarkers. These organic functional groups are sensitive to reactive oxygen species, and the variation between viable and non-viable embryos may be a result of oxidative modification.

Metabolomic profiling by NIR spectroscopy might give a new dimension to embryo selection, in addition to morphological criteria. It is well known that selection based on morphology has its limitations, although most pregnancies are from high grade embryos[8,25,30]. In the current study, although >85% of the embryos were of good quality, the ongoing pregnancy rate after day 3 SET was 29.3%. Even though there are other factors that influence pregnancy outcomes, apparently not all morphological good quality embryos will implant. In comparing the selection accuracy of both metabolomic profiling and morphology, metabolomic profiling by NIR spectroscopy is 15% higher than the accuracy of morphology after day 3 SET and even ~40% higher in the day 2 SET group, when transfer was performed irrespective of morphology. In practice, these data suggest that metabolomic profiling might be used in conjunction with morphological criteria to enhance the embryo selection procedure. While these outcomes are quite encouraging, it is important to note that the day 2 SETs were all

transferred because only one embryo was available, whereas only the morphologically best embryo was transferred on day 3.

Our data confirm the recent results by Seli et al.[33] in a clinical IVF setting, but with a larger number of cases and using single cultured and transferred embryos. Seli et al's data are very promising but with the SET data we can measure differences in metabolism between individual embryos and make one on one comparisons between pregnancy, metabolomic profiles and morphology data. In addition, we used much lower volumes (i.e. 4-5 fold) and a replaceable sample cell providing a closer representation of use in a clinical IVF setting. Most importantly, from a spectroscopy standpoint, this is the first time long wavelength NIR has been used. The long wavelength NIR allows the volume decrease.

The logistic regression of factors contributing to FCA with the day 3 SET samples showed that maternal age, fragmentation of the embryo and the relative viability score were all factors contributing to FCA. Maternal age[34,35,37] and fragmentation[12,13,18,19] are known to contribute to FCA, but the relative viability score derived from metabolomic profiling by NIR spectroscopy provides a new parameter of embryo quality. This further indicates the usefulness of the relative viability score when selecting between embryos of equal morphological quality.

The Pearson correlation coefficients between the relative viability score and number of blastomeres; and between the relative viability score and fragmentation, are both close to 0, indicating no linear correlation between these variables[38]. Considering that >85% of the transferred embryos were of good quality (<20% fragmentation, 4 blastomeres on day 2 or 6-9 blastomeres on day 3), this is a strong indication that we are looking at new aspects of an embryo's intrinsic quality. This is in agreement with an earlier study in which was stated that morphology alone cannot discriminate between sibling embryos[25].

The resulting data showed a large inter-patient or inter-embryo variability of the relative viability score. So far it is unknown what the cause of this variability is and if comparable variability will occur when we analyse individual embryos of the cohort of one patient. However, in general, a low relative viability score was highly predictive of poor pregnancy outcomes, so the data suggest that metabolomic profiling can be used in conjunction with morphology for embryo selection to distinguish between morphologically equal embryos.

Additional studies are required to provide further insight on the clinical value of metabolomic profiling by NIR spectroscopy, and the improvements in IVF embryo selection when morphology and metabolomic profiling are combined as an adjunct technique of assessing reproductive potential. Currently, prospective randomized trials

applying the relative viability score on top of the morphology to select an embryo for SET are underway.

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