Non-invasive embryo assessment in IVF

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Chapter ๓

NON-INVASIVE METABOLOMIC PROFILING AS AN ADJUNCT TO MORPHOLOGY FOR NON-INVASIVE EMBRYO ASSESSMENT IN WOMEN UNDERGOING SINGLE EMBRYO TRANSFER

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ABSTRACT

Objective: To determine whether metabolomic profiling of spent embryo culture media correlates with reproductive potential of human embryos.
Design: Retrospective study.
Setting: Academic and a private assisted reproductive technology (ART) programs.
Patient(s): Women undergoing single embryo transfer after IVF.
Intervention(s): Spent embryo culture media were collected after single embryo transfer on day 3 (n=304) or day 2 (n=181) and analysed by near-infrared spectroscopy. Near-infrared spectral regions were correlated to reproductive potential using a genetic algorithm optimization. Models of these spectral regions were used to calculate viability indices, and were validated by blinded analysis of a subset (n=60) of samples. Implantation rates were also compared between embryos of higher (≥0.3) and lower (<0.3) viability indices, and within each morphology grade.
Main Outcome Measure(s): Viability index and embryo viability.
Result(s): Mean viability indices of embryos that resulted in positive fetal cardiac activity were significantly higher compared to embryos that did not for both day 2 and day 3 embryos. Blinded validation of the day 2 model proved to be significant. Increasing viability index values correlated with an increase in pregnancy. Viability indices were found to be independent of morphology for both day 2 and day 3 embryos. Implantation rates were significantly higher among embryos with viability indices ≥0.3.
Conclusion(s): Metabolomic profiling of human embryo culture media using near-infrared spectroscopy is independent of morphology and correlates with reproductive potential of embryos.

INTRODUCTION

In more than 90% of IVF cycles performed in the United States, multiple embryos are simultaneously transferred to maximize the likelihood of a live birth[1]. Consequently, more than 30% of IVF pregnancies are twins or higher order multiple gestations, and more than half of all IVF neonates are the products of multiple gestations[2], a frequency 15- to 20-fold greater than with spontaneous conceptions[3].

The high multiple pregnancy rates (PR) associated with IVF have significant public health consequences[4]. In addition to health risks for the mother, including a 2- to 4-fold increase in pregnancy-induced hypertension and postpartum hemorrhage[5], the increased rate of preterm delivery in multiple infant pregnancies compromises the survival of neonates and increases their risk of lifelong disability[6,7]. Consequently, the medical and financial complications associated with multiple pregnancies has now led a number of countries to impose legal restrictions on the number of embryos transferred in IVF cycles[4]. Unfortunately, although guidelines on the number of embryos to be transferred have also been issued in the United States[8], their effectiveness has been limited, due to the patient's own financial pressures and IVF providers wishing to protect their publicly reported success rates.

Another significant problem associated with IVF is the failure of approximately eight of 10 transferred embryos to implant and two of three IVF cycles failing to result in pregnancy[4,9,10]. Our inability to determine the embryos with highest reproductive potential seems to be at least in part responsible for failed IVF cycles, as women undergoing IVF using thawed embryos after a failed fresh cycle achieve a 7%–11% implantation rate per embryo transferred, and 13%–17% ongoing PR per transfer[11,12].

To increase implantation rates, and possibly limit multiple pregnancies, an improvement in the currently used embryo assessment methodologies would be beneficial. This motivation has led many investigators to pursue adjunctive technologies to determine an individual embryo's reproductive potential. Several metabolic parameters of developing embryos and of the spent embryo culture media have been studied using a variety of non-invasive techniques[13]. For example, Gardner and colleagues[14] reported that glucose uptake was greatest in human blastocysts of highest grade, whereas Brison et al.[15] found that elevated asparagine, and decreased glycine and leucine levels in embryo culture media correlate with pregnancy. These and a significant number of additional studies suggest that embryos with positive and negative reproductive potential alter their environment differently and that this is reflected in the surrounding metabolites[4].

The complete array of small molecule metabolites that are found within a biological system constitutes the metabolome[16]. Metabolomics studies this dynamic inventory of metabolites as small molecular biomarkers representing the functional phenotype in a biological system, and attempts to determine and quantify metabolites associated with physiologic and pathologic states[17]. We have recently reported that non-invasive metabolomic profiling of embryo culture media using Raman and near-infrared (NIR) spectroscopy (vibrational analytic techniques suitable for rapid analysis of aqueous samples [reviewed in Ref. 18]) correlates with pregnancy outcome in women undergoing IVF[19]. We also performed two blinded studies with small sample sizes, and demonstrated that metabolomic models developed using NIR or Raman may predict embryo viability[19,20].

In the current study, we first determined whether a robust metabolomic model using NIR to predict reproductive potential of individual embryos can be established using a large number of samples derived from single embryo transfer (SET) on day 3 and day 2. Then, in a blinded analysis of 60 day 2 samples, we tested whether our model is predictive of pregnancy outcome. Finally, we assessed whether combining non-invasive metabolomic analysis of embryo culture media with morphological assessment may help improve determination of the reproductive potential of embryos in IVF.

MATERIALS AND METHODS

PATIENT SELECTION, TREATMENT, AND SAMPLE COLLECTION

All patients participating in the study were recruited from the VU University medical center (VUmc) in Amsterdam, The Netherlands, and Kato Ladies Clinic (KLC) in Tokyo, Japan. Institutional Review Board (IRB) approval was obtained in each center before the initiation of the study. All patients undergoing IVF with a SET from July 2006 to April 2007 at VUmc and from January to April 2007 at KLC were considered for participation in the study.

At VUmc, stimulation protocols were performed as previously described[21,22]. Briefly, patients less than age of 38 years or with previous good response in an IVF treatment underwent controlled ovarian hyperstimulation (COH) with a long protocol with GnRH agonist (GnRH-a) (Decapeptyl; Ferring, Copenhagen, Denmark) and gonadotropins (Gonal F; Serono, Geneva, Switzerland; Puregon; Organon, Oss, The Netherlands, or Menopur; Ferring). In women older than 38 years or with a previous poor response a short GnRH-a protocol was applied. Human chorionic gonadotropin (Pregnyl; Organon, Oss, The Netherlands), 10,000 IU SC, was administered when patients had two or more

follicles 18 mm or greater in mean diameter. Oocyte collection by transvaginal ultrasound (TVS)-guided needle aspiration of the follicles under deep conscious sedation was performed 36 h later.

At KLC, a minimal stimulation protocol was followed. Ovarian stimulation was accomplished using clomiphene citrate (CC, Clomid; Shionogi, Tokyo, Japan), in combination with hMG (Humegon; Organon). Clomiphene citrate was given at a dose of 50 mg/day from cycle day 3 until the day before the induction of oocyte maturation. The hMG administration was started on cycle day 8 at a dose of 75–150 IU at 2-day intervals depending on the serum E2 concentrations and ultrasound findings. The final stages of oocyte maturation were achieved by triggering an endogenous gonadotropin surge using 300 mg of GnRH analogue by inhalation (Sprecur; Aventis Pharma, Tokyo, Japan). Follicular aspiration was performed 34–35 h later. Luteal support was provided by daily oral administration of 30 mg/day dydrogesterone (Dephaston; Daiichi-sieyaku, Tokyo, Japan) for 12 days.

Retrieved oocytes were rinsed, graded, and placed in bicarbonate-buffered human tubal fluid (HTF) (Lonza, Verviers, Belgium; with 10% protein solution; Sanquin, Amsterdam, The Netherlands) at 37°C under 5% CO_2 in air at VUmc and in Sage® cleavage media, (Pasadena, CA) at 37°C under 5% CO_2, 5% O_2, 90% N_2, at KLC. Oocyte insemination was initiated approximately 40 h after hCG injection using standard IVF or intracytoplasmic sperm injection (ICSI) procedures.

At 16–18 h after insemination (day 1), each oocyte was examined for evidence of fertilization and placed into individual droplets of 25-µl of HTF media at VUmc, and 20-µl of Sage cleavage media at KLC, for culture to the cleavage stage. Embryos were cultured individually in both centers.

A standard embryo scoring system based on cleavage rate and morphology was used for the evaluation of embryo quality in both centers[13,21]. For example, a day 2 and day 3 grade A embryo was a four cell and ≥ seven cell embryo, respectively, with less than 5% fragmentation, whereas a grade B embryo was a day 2 and day 3 embryo with four cells and ≥ seven cells, respectively, with between 5% and 20% fragmentation. The single embryo with the highest number of blastomeres and the least fragmentation was transferred. Transfer was performed on day 2 at the KLC and on day 3 at the VUmc.

After removal of the embryos in preparation for transfer, the spent media were placed individually into labelled cryovials, snap frozen in liquid nitrogen, and then stored at -80°C. A control sample incubated under the same conditions without an embryo was also collected and used for normalization. Pregnancy outcomes were recorded for each patient at 12 weeks' gestational age. Positive pregnancy was defined as fetal cardiac activity (FCA) at that time.

A total of 304 spent embryo culture media samples collected at VUmc after SET on day 3 and 181 spent embryo culture media samples collected at the KLC after SET on day 2 were included in the study. An alternative analysis of the data obtained from the samples collected at VUmc has been previously reported[23].

NIR SPECTRAL ACQUISITION

All IVF culture media samples were thawed at room temperature for 30 min, after which they were vortexed for 10 seconds and centrifuged for 10 min at 13,000 rpm. The NIR transmittance spectra of media samples were measured using an indium gallium arsenide array-based 512-element NIR spectrometer with an operating wavelength of 920–1,675 nm. Spectrometer-compatible 3-mm path length sample cells were filled with 7-µl of sample culture media and placed in the sample chamber (maintained at 21.0° ± 0.1°C) for spectral measurements. Sample analysis time was approximately 1 min. The measurement was repeated with the control medium to account for any variation in culturing conditions between embryos.

SPECTRAL MODEL DEVELOPMENT

The NIR metabolomic profiles were computed by dividing each sample's transmittance spectrum by its associated control media spectrum and were then converted to an absorbance scale. Resulting absorbance spectra were mean-centered and used for all subsequent calculations. Discrimination between FCA+ and FCA- embryos consisted of determining a parsimonious combination of spectral regions from the NIR metabolomic profiles that estimated pregnancy outcomes by inverse least-squares regression and genetic algorithm (GA) optimization. This method is based on that given by Gributs and Burns[24] and by Seli et al.[19]. Briefly, models investigated were described by the formula:

$$Y = b_0 + b_1X + b_2X_2 + \ldots + b_nX_n \quad (1)$$

where Y is the pregnancy outcome of each sample (1 = FCA+, 0 = 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 using 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).

Final embryo viability indices were determined by a leave-one-out cross-validation method, using the model developed (equation 1) with known spectral regions and calculated weighting coefficients. Independent models were computed for both VUmc and KLC IVF culture media sample groups. All analyses were written in and evaluated using the Matlab (The MathWorks Inc., Natick, MA). Interestingly, in relation to the predictability between the day 2 and day 3 algorithms we noted that there was significant overlap between the two pregnancy algorithms. Both these algorithms examined areas of the spectra largely in the 1,200- to 1,500-nm spectral range.

STATISTICAL ANALYSIS

Subsequent to viability index estimation, Student's t-tests were used to separately determine trends within the pregnancy outcome groups. The Fisher's exact test was also used to determine implantation rate differences among viability index subsets. Alpha error of less than 0.05 was considered significant for all comparisons. Values of true positives (TP), true negatives (TN), false positives (FP), and false negatives (FN) were calculated by first determining an optimal cut-off value from receiver operating characteristic curves, a graphic plot of the sensitivity versus 1-specificity. The optimal cut-off was determined as that with the highest efficiency as calculated by sensitivity + specificity for each value. Percent accuracy of the test was then computed using the equation $[(TP+TN)/(TP+TN+FP+FN)] \times 100$ at the optimal cut-off. Pearson correlation coefficients were also calculated to establish the association between the viability index and the implantation rate, as well as the association between viability index and the morphology of an embryo. All statistical analyses were done using the Matlab (The MathWorks Inc.) programming package.

RESULTS

METABOLOMIC PROFILE GENERATED BY NIR SPECTROSCOPIC ANALYSIS OF SPENT CULTURE MEDIUM CORRELATES WITH PREGNANCY OUTCOME OF DAY 3 EMBRYOS

A total of 304 spent embryo culture media samples were collected at VUmc after SET on day 3 and evaluated using NIR spectroscopy. Of the 304 embryos transferred, 89 implanted and resulted in fetal cardiac activity detected by ultrasonography at the 12th gestational week (FCA+), whereas 215 did not (FCA-). All samples were analysed successfully and were included in the data analyses.

The NIR spectra of spent culture media were measured and pre-processed as previously described[19]. Mean spectra for FCA+ and FCA- were then computed after the spectra were converted to the absorbance scale and mean-centered at each wavelength. Next, using the GA as described in the Materials and Methods section, four areas in the spectroscopic range of NIR were identified as most discriminatory between the two groups. The optimal weights of these regions were also calculated.

Using the mathematical model (equation 1) that takes into account these regions and their weights, a viability index was then calculated for each sample. Mean viability index of day 3 embryos with proven reproductive potential (FCA+) was significantly higher (0.31 ± 0.10) compared to embryos that failed to implant (FCA-) (0.28 ± 0.11) ($P<0.05$) (Table 1). The metabolomic profiling of the 304 day 3 samples from VUMC have previously been reported as part of a study by Vergouw et al.[23]. These samples and their analysis were included in the current study as they were further analysed to assess whether metabolomic profiling may provide an adjunct to morphology for non-invasive embryo assessment in women undergoing SET.

METABOLOMIC PROFILE GENERATED BY NIR SPECTROSCOPIC ANALYSIS OF SPENT CULTURE MEDIUM CORRELATES WITH PREGNANCY OUTCOME OF DAY 2 EMBRYOS

Spent embryo culture media samples (n=121, 32 FCA+, 89 FCA-) from women undergoing SET on day 2 were also collected and evaluated using NIR spectroscopy. All samples were analysed successfully and were included in the data analyses.

Similar to that described previously for day 3 embryos, NIR spectra of spent culture media were measured and pre-processed. Mean spectra for FCA+ and FCA- were then computed after the spectra were converted to the absorbance scale and mean-centered at each wavelength. Next, GA optimization was performed and five wavelet areas in the spectroscopic range of NIR were identified as most discriminatory between the FCA+ and FCA- groups, and were given relative weightings. Using the mathematical model (equation 1) that takes into account these regions and their weights, a viability

index was calculated for each sample. The model used to assess day 2 samples was different from the model described previously for day 3 samples. The NIR spectroscopic analysis of spent culture media of embryos with proven reproductive potential demonstrated higher viability indices (0.34 ± 0.17) than those that failed to implant (0.24 ± 0.16) (P<0.01) (Table 1).

Table 1. The near-infrared spectroscopic analysis of spent culture media of embryos with positive and negative fetal cardiac activity (FCA) in relation to viability index scores (mean ± SD).

Center	Study design	n	Day of Transfer	Viability index FCA + (mean ± SD)	Viability index FCA – (mean ± SD)	P-value
VUMC	Model development	304	Day 3	0.31 ± 0.10	0.28 ± 0.11	<0.05
KLC	Model development	121	Day 2	0.34 ± 0.17	0.24 ± 0.16	<0.01
KLC	Blinded analysis	60	Day 2	0.33 ± 0.18	0.26 ± 0.15	<0.05

METABOLOMIC PROFILE GENERATED BY NIR SPECTROSCOPIC ANALYSIS OF CULTURE MEDIUM PREDICTS PREGNANCY OUTCOME OF EMBRYOS AFTER SINGLE EMBRYO TRANSFER ON DAY 2 IN A BLINDED ANALYSIS

Next, spent culture media of 60 embryos (16 FCA+, 44FCA-) collected at KLC, after SET on day 2,were evaluated with NIR spectroscopy by an observer blinded to pregnancy outcome, using the model established for day 2 samples, as described previously. These 60 samples were not part of the initial cohort (n=121) that was used for metabolomic model development. The NIR spectra were generated, mean centered, and viability indices were determined. Viability indices of embryos with proven reproductive potential (FCA+) were higher (0.33 ± 0.18) compared to embryos that failed to implant (FCA-) (0.26 ± 0.15) (P<0.05) (Table 1). In addition, the method identified implantation potential with an accuracy of 71.7%.

VIABILITY INDICES DETERMINED IN THE BLINDED ANALYSIS OF DAY 2 EMBRYO CULTURE MEDIA SHOW A POSITIVE CORRELATION WITH THE ABILITY OF THE EMBRYO TO RESULT IN A PREGNANCY

After demonstrating the ability of the metabolomic model to predict reproductive potential in a blinded analysis, we tested whether increasing values of viability index represent higher likelihood of the embryo to result in a pregnancy. Viability indices of the 60 embryos transferred on day 2 determined by blinded analysis, as described previously (Table 1), were separated into quartiles. We observed a positive correlation (Pearson correlation coefficient=0.5241, P<0.001) between increasing viability index values and the reproductive potential of individual embryos (Figure 1).

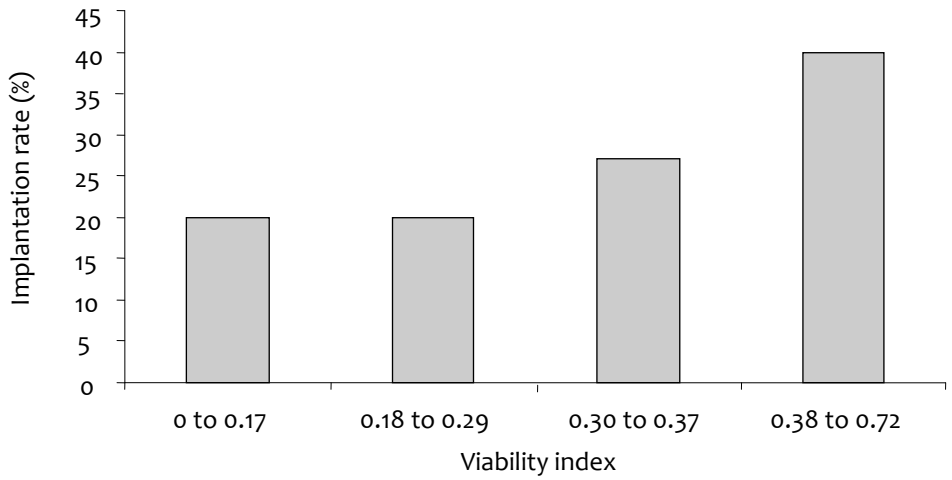


Figure 1. Viability indices determined in the blinded analysis of day 2 embryo culture media show a positive correlation with pregnancy. Viability indices determined by blinded analysis of the spent culture media of 60 embryos transferred on day 2 were analysed in quartiles. A positive correlation between increasing viability index value and the reproductive potential of individual embryos was observed (Pearson correlation coefficient=0.5241, P<0.001).

VIABILITY INDEX IS INDEPENDENT OF MORPHOLOGY FOR BOTH DAY 3 AND DAY 2 EMBRYOS

Next, we assessed whether the viability index represents a parameter independent of embryo morphology. Figure 2 shows the relation between the relative viability index and morphological grade for day 3 and day 2 embryos. The Pearson correlation

coefficients for day 3 and day 2 embryos were 0.0002 and -0.1223, respectively. The null hypothesis of no association is accepted for both day 3 (P=0.9975) and day 2 (P=0.1009) samples.

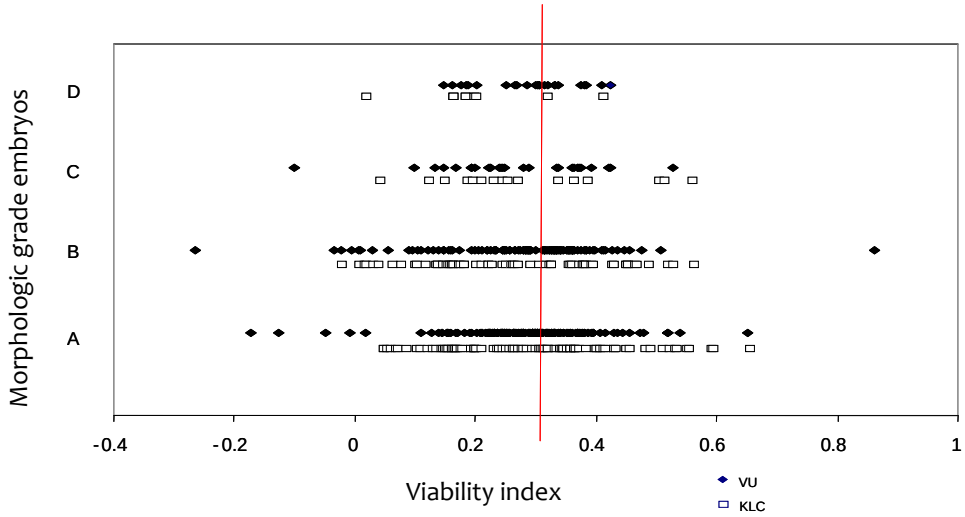


Figure 2. Relationship between the viability index and morphologic grade for day 3 and day 2 embryos. Viability indices of day 3 (n=304, diamonds) and day 2 (n=181, squares) embryos are shown for each morphologic grade. The Pearson correlation coefficients for day 3 and day 2 embryos were 0.0002 and –0.1223 respectively. The null hypothesis of no association is accepted for both day 3 (P=0.9975) and day 2 (P=0.1009) samples. The vertical line indicates the cut-off predicted by receiver operating characteristic analysis.

METABOLOMIC PROFILING MAY PROVIDE AN ADJUNCT TO MORPHOLOGY FOR NON-INVASIVE EMBRYO ASSESSMENT IN WOMEN UNDERGOING SINGLE EMBRYO TRANSFER

After establishing the predictive value of metabolomic profiling (Table 1) and demonstrating that it constitutes a parameter independent of morphology (Figure 2), we investigated whether combining metabolomic profiling with morphological assessment may improve our ability to determine an embryo's reproductive potential. We first determined whether the embryos that have a viability index value above the optimal cut-off value determined by the receiver operating characteristic curve analysis (≥0.3) showed a higher PR compared to those that have a lower viability index (<0.3). Among embryos that underwent SET on day 3 (VUmc), those with a viability index ≥0.3 demonstrated a 10% higher pregnancy compared to embryos with a viability index <0.3

(P<0.05) (Figure 3). Similarly, in embryos transferred on day 2 (KLC), a 19% higher PR was observed in embryos with a viability index ≥0.3 compared to those with a viability index <0.3 (P<0.001) (Figure 3).

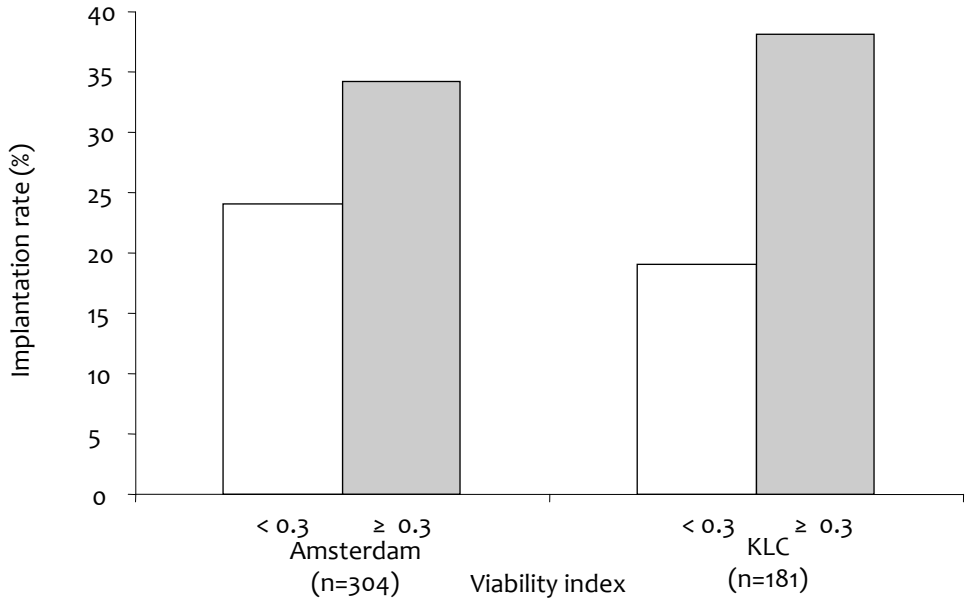
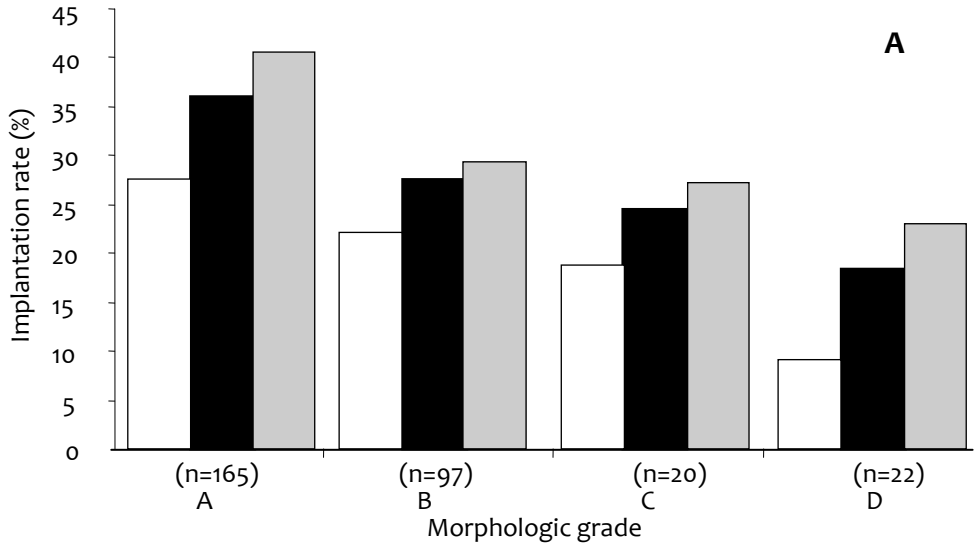


Figure 3. Implantation rates of day 2 and day 3 embryos with viability indices below and above the cut-off value (0.3) determined by receiver operating characteristic analysis. Both day 3 and day 2 embryos with a high viability index (≥0.3) demonstrated a higher implantation rate compared to those with a lower viability index (<0.3) (day 3, P<0.05; day 2, P<0.001).

Next, we assessed the outcomes of embryos with different morphological grades separately for both day 3 (Figure 4A) and day 2 (Figure 4B) embryos, and observed a similar trend. Among embryos that underwent SET on day 3 (VUmc), the difference in PR among embryos with a viability index ≥0.3 compared to embryos with a viability index <0.3 was 13%, 7%, 10%, and 14%, for grade A, B, C, and D embryos, respectively (P=not significant [NS]). In embryos transferred on day 2 (KLC) the difference in PR among embryos with a viability index ≥0.3 compared to embryos with a viability index <0.3 was 12%, and 30% for grade A, and grade B embryos, respectively (P=0.06 and P<0.01, respectively). In this group, the number of grade C and D embryos was very low and precluded statistical comparison.



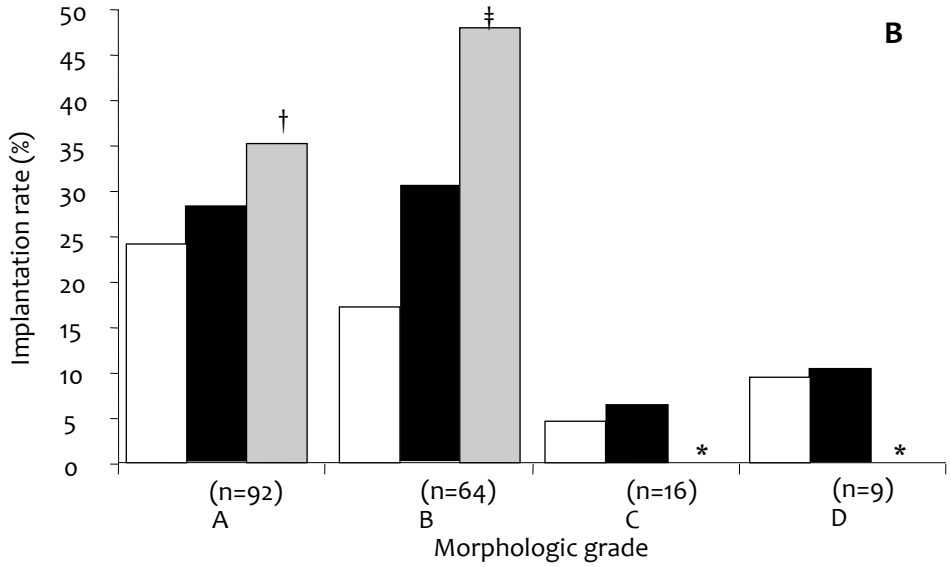


Figure 4. Implantation rates of day 3 and day 2 embryos with different morphologic grades and with viability indices below or above the optimal cut-off value (0.3) determined by receiver operating characteristic analysis. A: implantation rates of day 3 embryos comparing morphologic grades and a viability index of less than or greater than 0.3. B: implantation rates of day 2 embryos comparing morphologic grades and a viability index of less than or greater than 0.3. Black columns show implantation rates for different morphologic grades. White and grey columns show implantation rates for embryos of the same morphologic grade and a viability index <0.3 or ≥0.3 respectively. n: number of samples analysed for each morphologic grade. *low number of samples, † P=0.06, ‡ P<0.01.

DISCUSSION

In this study we analysed spent culture media of embryos with known outcome after SET on day 2 or day 3. First, we used NIR spectroscopy to establish metabolomic models that correlate with cycle outcome (Table 1). We then validated our approach by blinded analysis of a subset of embryos (Table 1). Our findings are consistent with previous reports using NIR[19] and Raman[19,20] and strongly suggest that in vitro cultured embryos with a high reproductive potential alter their environment differently compared to embryos that do not result in a pregnancy, and that the difference is detectable using spectroscopy.

We also observed a positive correlation between viability indices reflective of metabolomic profile and the ability of embryos to achieve pregnancy (Figure 1). This association has been a long-standing area of research, in particular in relation to the metabolism of energy substrates[25,26]. Our findings further strengthen the idea and demonstrate significant clinical implications by establishing that determinants of the culture media metabolome show a quantitative association with the reproductive potential of the embryo and that a higher viability index may reflect a higher likelihood of pregnancy.

Next, we tested whether metabolomic profiling provides an additional level of information about embryo viability independent of morphology. We found that viability indices calculated by metabolomic profiling of embryo culture media do not correlate with morphological grades of day 2 or day 3 embryos (Figure 2). This finding is consistent with the report by Vergouw et al.[23], using the same data set of embryo media, that did not find a correlation between the viability index and the number of blastomeres or percent fragmentation, and an earlier study in mouse by Gardner and Lane[27], which stated that morphology alone cannot discriminate between sibling embryos. Interestingly, Gardner and Lane[27] have previously shown that morphologically identical mouse blastocysts could be better identified using metabolic criteria as "viable" before transfer.

The benefit derived from embryo grading systems in determining embryo(s) to transfer is well established[28,29]. However, although it is a valuable tool, morphological embryo assessment has many recognized pitfalls. This has been well documented in studies showing that assessment on day 3 fails in more than 50% of the time to identify embryos that will go on to form blastocysts[30,31]. In the current study, we went on to ask whether metabolomic profiling used as an adjunct to morphology may improve our ability to identify the embryo(s) that are most likely to result in pregnancy. In our study, where the transferred embryos were selected based on morphology alone, we found

that embryos with a viability index ≥0.3 were more likely to result in pregnancy compared to embryos with a viability index <0.3 (Figures 3 and 4A,B). These findings suggest that the availability of a secondary, more objective form of analysis to morphology would allow greater discrimination between embryos of similar morphology, and potentially lead to an improvement in implantation rates. Although our observations are encouraging, a randomized prospective trial design will need to be adopted to establish the true value of metabolomic assessment as an adjunct to morphology in increasing implantation rates.

Near-infrared and Raman are vibrational spectroscopies. One advantage of the vibrational spectroscopic approach is that whole sample matrices can be monitored non-invasively. Likewise, a spectral signature provides detailed multicomponent information about the sample from one measure, decreasing the time and amount of sample necessary for analysis. This has led numerous researchers to examine the use of both Raman and NIR for metabolomic assessment of blood constituents and tissue composition[17]. In the present study, we were able to analyse individual samples in approximately 1 min, using 7-µl of media. This was a lower volume compared to our previous report where we used approximately 15-µl for analysis[19].

We have previously tested both NIR and Raman spectroscopies for the metabolomic profiling of embryo culture media with comparable success[19,20]. Both modes of vibrational spectroscopic analyses are associated with unique advantages. For example, although vibrational modes of molecules are highly specific in Raman spectroscopy allowing better constituent identification from background, signal intensity is higher with NIR spectroscopy. In the current study, we used NIR spectroscopy as our aim is to develop a technology that is clinically applicable, and NIR technology is more cost-efficient compared to Raman. Nevertheless, it is conceivable that in the future, a combination of these two vibrational spectroscopies or additional technologies may provide better insight into the analysis of embryo culture media sample composition.

In women undergoing IVF, embryo transfers are most commonly performed on days 2, 3, or 5 after retrieval. In the current study we analysed spent embryo culture media samples collected after SET on day 2 (KLC) or day 3 (VUmc). Although metabolic activity shows significant similarities between days 2 and 3 (cleavage stage) embryos, the additional 24 h culture in day 3 samples could potentially affect the metabolomic profile of the culture environment. Therefore, specific and independent models (different from the previously published ones) were developed for each developmental stage to achieve the best possible separation. In addition, the data points determined by GA to generate a predictive model for day 2 and day 3 NIR spectra differed from

each other and reflected the metabolomic activity specific for the developmental stage and culture length of the embryo. When we cross-tested the model developed for the culture media of day 2 embryos to assess the reproductive potential of day 3 embryos, it was not predictive (data not shown). Therefore, metabolomic models specific to the developmental stage of the embryo rather than a global algorithm may be necessary for optimal assessment of reproductive potential.

Multiple types of commercial media exist for the culture of human embryos in IVF laboratories. These media are all developed based on the metabolic needs of human embryos[32] and although they are largely believed to be similar, their complete formulas are protected under patent laws and are not available to the public. A valid and widely applicable methodology for the evaluation of embryo culture media should be able to detect the changes associated with embryo viability in different types of culture media. We have previously shown that a model developed in one type of media may effectively assess reproductive potential of embryos cultured in a different type of media[19,20]. In the present study, we normalized the spectrum of each sample to that of a control sample cultured under the same conditions without an embryo to eliminate the effect of formulary differences among different types of culture media on metabolomic parameters. Nevertheless, metabolomic profiling in relation to different types of commercial culture media still needs to be further validated.

The current study extends our previously published findings by establishing robust metabolomic models that are independent of morphological grade, and may provide an adjunct to morphology for non-invasive assessment of embryo viability. Our findings validate the concept that a different metabolism is apparent in viable and non-viable embryos and that embryos of the same morphology may differ in their metabolic activity. Therefore, this study introduces metabolomic profiling as a rapid and non-invasive technology that can provide an objective assessment aiding in the decision of which embryo(s) to transfer. The ability to use this technology as an adjunct to morphology for embryo assessment may provide a second tier of assessment before embryo transfer. Most important, an improved understanding of the reproductive potential of embryos will aid in identifying the embryos that are most likely to result in a pregnancy, and allow more accurate decisions to be made about the number of embryos to be transferred. This, in turn, may reduce the likelihood of multiple gestations while maintaining or even increasing PRs. Although the SET data provided in this article is substantial in number, further studies will be necessary to determine the true value and limitations of the use of metabolomics in IVF.

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