# Noninvasive metabolomic profiling as an adjunct to morphology for noninvasive embryo assessment in women undergoing single embryo transfer

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**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 analyzed 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 ($\geq$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 with 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 $\geq$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. (Fertil Steril® 2010;94:535–42. ©2010 by American Society for Reproductive Medicine.)

**Key Words:** Noninvasive embryo assessment, single embryo transfer (SET), near infrared (NIR), spectroscopy, metabolomic profiling, in vitro fertilization (IVF)

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).

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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 patients 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 8 of 10 transferred embryos to implant and 2 of 3 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 noninvasive 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 noninvasive 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 noninvasive 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 an 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 hours 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 $E_2$ 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 (Suprecur; Aventis Pharma, Tokyo, Japan). Follicular aspiration was performed 34–35 hours 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 hours after hCG injection using standard IVF or intracytoplasmic sperm injection (ICSI) procedures.

At 16–18 hours after insemination (day 1), each oocyte was examined for evidence of fertilization and placed into individual droplets of 25 $\mu$L of HTF media at VUMC, and 20 $\mu$L 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 4 cell and $\geq$7-cell embryo, respectively, with less than 5% fragmentation, whereas a grade B embryo was a day 2 and day 3 embryo with 4 cells and $\geq$7 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 labeled