Non-invasive metabolomic analysis using a commercial NIR instrument for embryo selection

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Received: 08.02.2013
Review completed: 13.04.2013
Accepted: 11.06.2013

Access this article online

Quick Response Code:

Website: www.jhrsonline.org

DOI: 10.4103/0974-1208.117174

ABSTRACT

CONTEXT: Metabolomics was introduced in human *in vitro* fertilization (IVF) for noninvasive identification of viable embryos with the highest developmental competence. **AIMS:** To determine whether embryo selection using a commercial version of metabolomic analysis leads to increased implantation rates (IRs) with fetal cardiac activity (FCA) compared with morphology evaluation alone. **SETTING AND DESIGN:** Randomized controlled trial from April to December 2010 at a private IVF unit. The study was terminated prematurely due to the market withdrawal of the instrument. **MATERIALS AND METHODS:** IVF patients ≥18 and ≤43 years with ≥4 × 2PN were randomly allocated to metabolomic analysis combined with embryo morphology (ViaMetrics-E; metabolomics + morphology group) or embryo morphology alone (morphology group). Cycles with frozen embryos, oocyte donations, or testicular biopsy were excluded. **STATISTICAL ANALYSIS:** Categorical and continuous data were analyzed for statistical significance using 2-tailed Fisher's exact test and *t*-test, respectively. Statistical significance was accepted when $P < 0.05$. **RESULTS:** A total of 125 patients were included in the study; 39 patients were allocated to metabolomics + morphology group and 86 patients to morphology group. Patients were stratified according to the day of embryo transfer (Days 2, 3, or 5). IRs with FCA were similar for Days 2 and 3 transfers in both groups. For Day 5 transfers, IRs with FCA were significantly higher in the metabolomics + morphology group (46.8% vs. 28.9%; $P = 0.041$; 95% confidence intervalp [CI]: 1.09-34.18). Pregnancy and live births rates were similar for Days 2, 3, and 5 in both groups. The study was terminated early following the voluntary market withdrawal of ViaMetrics-E in December 2010. **CONCLUSIONS:** Metabolomic analysis using the commercial near-infrared (NIR) instrument does not appear to have a beneficial effect on pregnancy and live births, with improvement in IR with FCA for Day 5 transfers. However, no solid conclusions can be reached due to the lack of adequate study power. ClinicalTrials.gov Identifier: NCT01490515

KEY WORDS: Embryo selection, implantation rates, metabolomics, near-infrared, noninvasive

INTRODUCTION

Accurate, quick, and non-invasive identification of a single embryo with the highest reproductive potential remains a challenge in the *in vitro* fertilization (IVF) laboratory. The use of morphological characteristics to assess the quality of preimplantation embryos has been related with increased pregnancy and implantation rates (IRs).[1] However, morphological evaluation of embryos has been shown to have limited value in predicting true developmental competence and implantation ability.[2-4] This limitation indicates the need for additional technologies in human embryo viability assessment.

Several non-invasive methods aiming to determine embryo viability have been developed, including proteomics,[5] birefringence imaging,[6] embryo morphokinetics,[7] expression of cumulus cells,[8,9] measurement of respiration rate,[10] amino acid turnover,[11-13] soluble human leukocyte antigen-G,[14] pyruvate uptake,[15] and glucose uptake.[16,17] However, the majority of these methods are either expensive, require dedicated equipment and technical staff, or do not produce results quickly enough to be used within the time frame of clinical IVF.

Metabolomics is the systematic study of the complete array of small-molecule metabolites

that represent the functional phenotype in a biological system, and attempts to quantify metabolites associated with physiologic and pathologic states using spectral and analytical approaches.[18] Metabolomics was recently introduced in human IVF for noninvasive metabolomic profiling of embryo culture media.[19-23] The technologies studied include proton nuclear magnetic resonance,[21] Raman and near-infrared (NIR) spectroscopy,[19,20,22,24] and all showed a correlation with embryo viability and pregnancy outcome. In addition, noninvasive metabolic profiling of spent media from preimplantation embryos was recently proposed as a feasible method for the detection of aneuploidies using mass spectrometry and nuclear magnetic resonance spectroscopy.[25]

It was shown that the metabolomic profile is different in viable and nonviable embryos and that embryos of the same morphology also differ in their metabolic activity.[22] Therefore, noninvasive metabolic assessment may provide an additional objective criterion as an adjunct to morphology for embryo assessment before transfer, leading to improved pregnancy and IRs. Although numerous proof of principal studies have been reported, limited randomized controlled trials (RCT) data have been published to date comparing noninvasive metabolomic analysis versus morphology alone for embryo evaluation. In two studies, using a non-commercialized version of the NIR instrumentation, no additional benefit was found when using this technology to rank the metabolic profile of similar embryos of good morphology prior to single embryo transfer (SET) on Days 5[26] and 3.[27] A slight advantage was, however, reported when performing Day 2 SET.[26]

The aim of the study was to compare the novel noninvasive metabolomic profiling and the traditional method of morphology assessment for embryo selection in terms of implantation and pregnancy rates in a clinical IVF program. In addition, we evaluated the implementation of the ViaMetrics-E instrument in routine clinical operation.

MATERIALS AND METHODS

Study design and patient population

This is a single center prospective RCT, performed from April to December 2010 using a commercial version of the Viametrics-E instrumentation. The study was terminated prematurely in December 2010 due to the voluntary market withdrawal of Viametrics-E due to variability in the instrumentation.

IVF patients aged between 18 and 43 years, with at least four fertilized oocytes (2PN≥4) qualified to enter the study [Figure 1]. Frozen-thawed cycles and cycles with oocyte donation or testicular biopsy were excluded from the study. Patients were allocated to embryo selection using either metabolomic analysis combined with embryo morphology (metabolomics + morphology group) or embryo morphology only (morphology group). We used an unequal randomization (ratio metabolomics + morphology: Routine morphology = 1:2) in order to reduce trial costs,[28] as the study was self-funded and part of the cost of the sample cells would have to be covered by the patients. Allocation to the metabolomics + morphology or morphology groups was performed on the day of fertilization assessment (Day 1 postoocyte retrieval) by a study nurse using sealed envelopes following a computer-generated random allocation sequence. Neither patients nor doctors were blinded to the treatment assigned. Patients underwent embryo transfer on either Days 2, 3, or 5 according to the clinical protocol and previous history. The study was approved by our institutional ethics review board. An informed consent was obtained from all patients included in this study.

Ovarian stimulation and *in vitro* fertilization

Patients underwent ovarian stimulation using either a long gonadotropin-releasing hormone (GnRH) agonist protocol or a flexible GnRH antagonist protocol, as previously described.[29] Oocyte retrieval was performed 35-36 h after the human chorionic gonadotropin (hCG) injection by transvaginal ultrasound-guided double lumen needle aspiration. Oocytes were fertilized by IVF or intracytoplasmic sperm injection (ICSI) and fertilization was assessed 16-20 h postinsemination. Fertilized oocytes were individually cultured for 2, 3, or 5 days in 25 µl drops of sequential culture media (ISM1/BlastAssist; Origio, Denmark). Control culture media were incubated during this period under the same conditions. Embryo transfer using ultrasound guidance was performed 2, 3, or 5 days after oocyte retrieval, depending on patient history and embryo characteristics. Luteal phase support with 600 mg of micronized progesterone (Utrogestan Laboratoires Besins-International S.A., France) was initiated 2 days after oocyte retrieval.

Embryo selection based on noninvasive metabolomic profiling

In patients allocated to the metabolomics + morphology group, all embryos of similar good morphology from each patient's cohort were metabolically evaluated using Viametrics-E and ranked on the day of embryo transfer according to their Viability Score. Briefly, 10 µl of media samples of each embryo were loaded into specialized spectrometer-compatible sample cells and placed in a temperature equilibration chamber (21°C). After 4 minutes, sample cells were placed in an indium-gallium-arsenide array-based 512 element NIR spectrometer with a wavelength of 920-1675 nm (ViaMetrics-E, Molecular Biometrics, USA) for spectral analysis. Measurements of each sample and corresponding controls were repeated three times. Specific biomarkers, corresponding to unique functional groups of

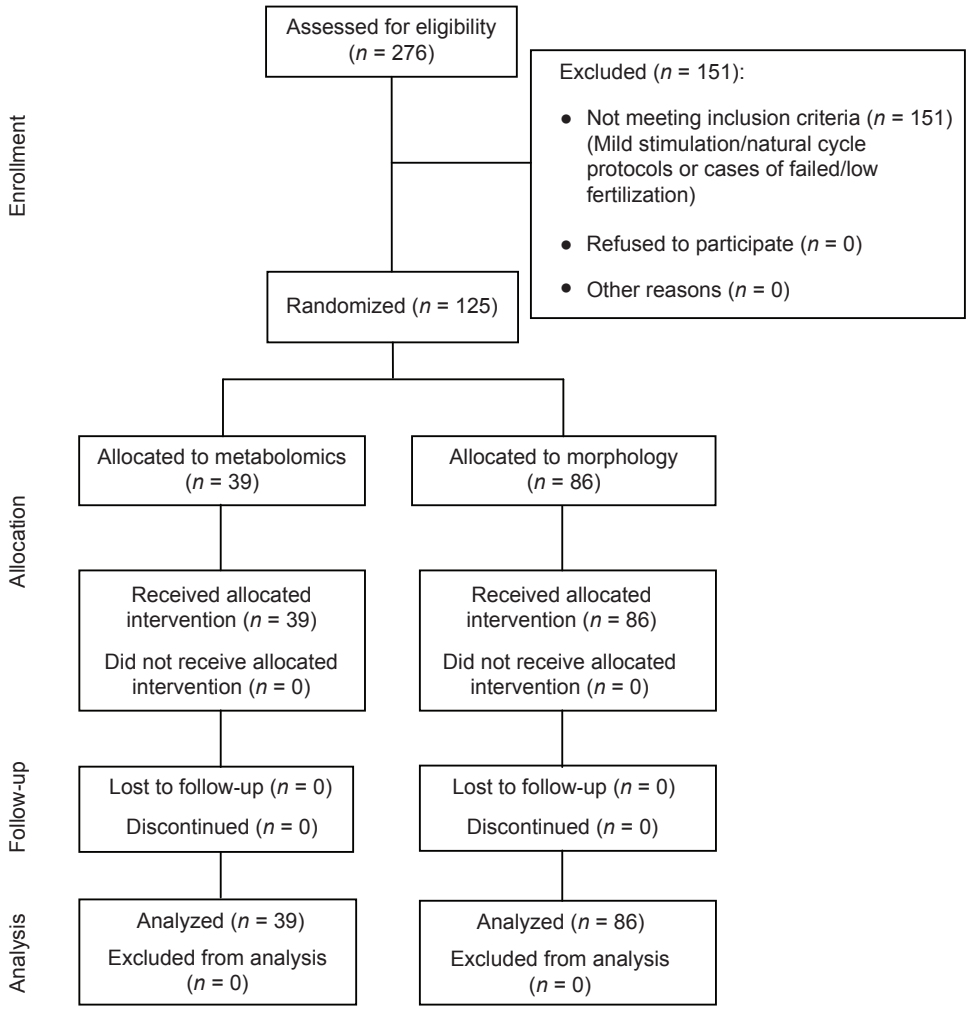

Figure 1: Consort diagram of study

molecules had been previously identified from training samples[22] by Molecular Biometrics Inc. Therefore, spent medium of a particular embryo generates a spectrum due to vibrations of specific functional groups including N–H, C–H, O–H, and S–H. The intensity of the light absorbed can be determined by analysis of the resulting spectra and is directly proportional to the concentrations of molecules, such as albumin, lactate, pyruvate, glutamate, and glucose, in the sample.[26] The unique metabolomic profile of each embryo was then quantified into a Viability Score, using a wavelength selective algorithm specific for Days 2, 3, or 5 transfers. The algorithms were preprogrammed into the commercial version of the instrumentation. The resulting Viability Score is a quantitative measurement of an embryo's potential to establish a pregnancy with fetal cardiac activity (FCA). The acceptable range for the Viability score was from 0 (lowest score) to 1 (highest score). When the viability score fell outside this range (<0 or >1), the corresponding embryo was either cryopreserved or discarded depending on the morphology grade allocated. One to four embryos with the highest-ranking viability scores were selected for transfer either on Days 2, 3 or 5, taking into account national legislation, patient's age and previous and current cycle characteristics. In split IVF/ICSI cases, embryos with the best viability scores were selected for transfer, irrespective of fertilization method. All patients in the metabolomics + morphology group underwent embryo transfer (ET).

Embryo selection based on morphology assessment

In patients allocated to the morphology group, embryo selection was based solely on morphological characteristics. Cleavage-stage embryos were assessed based on morphological criteria (number, size and shape of blastomeres, degree of fragmentation, multinucleation, appearance of cytoplasm), and were categorized in four grades [grade 1 (highest) to grade 4 (lowest)]. Embryos with 2-4 cells on Day 2 and embryos with 6-8 cells on Day 3 and <20% fragmentation were regarded as good quality embryos (grades 1 and 2).[30] In the case of blastocysts, degree of expansion, inner cell mass, and trophectoderm were graded according to a scoring system previously described.[31] One to four embryos with the highest morphology grade were selected for transfer either on Days 2,

3, or 5, taking into account national legislation, patient's age and previous and current cycle characteristics. In split IVF/ICSI cases, embryos with the best morphology scores were selected for transfer, irrespective of fertilization method. All patients in the morphology group underwent ET.

Outcome measures

Since multiple embryos were transferred, the primary outcome measure was ongoing IRs with FCA (number of fetal sacs with cardiac activity/number of embryos transferred at 12 weeks of gestation).

Secondary outcome measures included positive hCG rates, clinical pregnancy rates (presence of gestational sac with FCA at 7 weeks of gestation), and ongoing pregnancy rates (presence of gestational sac with FCA at 12 weeks of gestation), as well as incidence of multiple pregnancy, biochemical pregnancy (positive hCG test not reaching clinical pregnancy), and clinical miscarriage (clinical pregnancy not reaching ongoing pregnancy).

Statistical analysis

Categorical and continuous data were analyzed for statistical significance using 2-tailed Fisher's exact test and *t*-test, respectively. Statistical significance was accepted when $P < 0.05$. Multiple regression analysis was used to determine statistical effects of baseline patient characteristics and embryological data on the primary endpoint (IRs with FCA).

After setting the baseline implantation with FCA rate at 20% based on our Unit's results, and the detectable difference between groups at 10%, assuming an alpha level of 0.05, based on previously published data,[26] it was calculated that 231 and 462 patients (ratio 1:2) were required in the metabolomics + morphology and morphology group, respectively, in order to achieve a 0.80 power.

RESULTS

The study included patients randomized from April 2010 to December 2010 due to the voluntary market withdrawal of ViaMetrics-E. Due to the premature termination of the study, the final patient population was n = 39 in the metabolomics + morphology group and n = 86 in the morphology group, not reaching the sample size for achieving adequate power.

Baseline patient characteristics and embryological data are summarized in Table 1.

Ongoing IRs with FCA were similar for Day 2 transfers (19% vs. 39.2%; Table 2) and Day 3 transfers (20.6% vs. 24.4%; Table 3), in the metabolomics + morphology and morphology groups, respectively. For Day 5 transfers, IRs with FCA were significantly higher in the metabolomics + morphology group (46.8% vs. 28.9%; P =0.041; 95% confidence interval [CI]: 1.09-34.18; Table 4).

Positive hCG, clinical pregnancy, ongoing pregnancy, and live births, as well as multiple pregnancy rates and biochemical

Table 1: Baseline patient characteristics and embryological data

	Metabolomics group (*n*=39)	Morphology group (*n*=86)
Age (years)	34.5±4.7	35.7±4.4
BMI (kg/m^2)	25.1±5.1	25.3±4.2
Duration of infertility (years)	3.5±2.1	4.0±3.6
Previous IVF attempts	1.2±1.6	1.5±1.9
Basal FSH (IU/l)	7.6±2.5	8.2±2.7
Oocytes retrieved	17.2±7.3	15.1±6.0
IVF/ICSI/IVF+ICSI (*n*)	8/20/11	12/58/16
Fertilized oocytes (2PN)	9.9±4.0	8.4±4.1
Embryos transferred	2.6±0.7	2.9±0.6
Day of ET (Day 2/Day 3/Day 5) (*n*)	7/12/20	27/25/34

Values are expressed as mean±standard deviation, unless otherwise stated. There is no statistical significance in any of the parameters between the two groups *t* test. BMI=Body mass index; IVF=*In vitro* fertilization; FSH=Follicle-stimulating hormone; ICSI=Intracytoplasmic sperm injection; ET=Embryo transfer

Table 2: Implantation and pregnancy rates for Day 2 transfers

(%)	Metabolomics group (*n*=7)	Morphology group (*n*=27)	*P*
Implantation with FCA	4/21 (19.0)	20/51 (39.2)	0.168
Implantation	6/21 (28.6)	22/51 (43.1)	0.296
Positive hCG	5 (71.4)	17 (63.0)	1.00
Clinical pregnancy	4 (57.1)	16 (59.3)	1.00
Ongoing pregnancy	3 (42.9)	12 (44.4)	1.00
Live births	3 (42.9)	12 (44.4)	1.00
Multiple pregnancy	1 (14.3)	5 (18.5)	1.00
Twin pregnancy	1 (14.3)	4 (14.8)	1.00
Triplet pregnancy	0	1 (3.7)	1.00
Biochemical pregnancy	1/5 (20)	1/17 (5.9)	0.411
Clinical miscarriage	1/4 (25%)	4/16 (25)	1.00

FCA=Fetal cardiac activity; hCG=Human chorionic gonadotropin

Table 3: Implantation and pregnancy rates for Day 3 transfers

(%)	Metabolomics group (*n*=12)	Morphology group (*n*=25)	*P*
Implantation with FCA	7/34 (20.6)	19/78 (24.4)	0.809
Implantation	8/34 (23.5)	25/78 (32.1)	0.499
Positive hCG	7 (58.3)	19 (76)	0.443
Clinical pregnancy	6 (50)	14 (56%)	1.00
Ongoing pregnancy	5 (41.7)	11 (44%)	1.00
Live births	4 (33.3)	9 (36%)	1.00
Multiple pregnancy	2 (16.7)	5 (20)	1.00
Twin pregnancy	2 (16.7)	4 (16%)	1.00
Triplet pregnancy	0	1 (4%)	1.00
Biochemical pregnancy	1/7 (14.3)	5/19 (26.3)	1.00
Clinical miscarriage	1/6 (16.7)	3/14 (21.4)	1.00

FCA=Fetal cardiac activity; hCG=Human chorionic gonadotropin

pregnancy and clinical miscarriage were similar for Days 2, 3, and 5 transfers in both patient groups [Tables 2-4]. Twin and triplet rates appeared higher in the metabolomics + morphology group compared with the morphology group, but the differences were not statistically significant [Table 4].

In the metabolomics group, a total of 196 embryos were analyzed and 102 embryos were transferred. The mean number of embryos analyzed per patient was 4.9 ± 1.2. Viability score fell outside the acceptable range in two instances (one embryo with Viability Score < 0 and one embryo with Viability Score > 1), which were cryopreserved. There was no correlation between viability score and morphology grade for embryos on Day 2 ($r = 0.031$, $P = 0.861$), Day 3 ($r = -0.126$, $P = 0.306$), and Day 5 ($r = 0.020$, $P = 0.858$).

Multiple regression analysis showed no significant effect of patient age, body mass index (BMI), basal follicle-stimulating hormone (FSH), years of infertility, number of previous IVF attempts, and number of embryos transferred on IRs with FCA [Table 5].

DISCUSSION

Several proof-of-principle and validation studies on noninvasive metabolomic analysis of embryos have been published using proton nuclear magnetic resonance,[21] Raman, and NIR spectroscopy[19,20,22,24] and all showed a correlation with embryo viability and pregnancy outcome. This is one of the first randomized controlled studies comparing IVF outcomes after embryo selection based on noninvasive metabolomic profiling versus traditional morphology assessment, and the first to use a commercial version of the ViaMetrics-E instrumentation, as distinct from two other RCTs,[26,27] which involved earlier noncommercial versions.

Our results indicate a significant increase in ongoing IRs with positive FCA following embryo selection based on noninvasive metabolomic analysis combined with morphology on Day 5, but not on Days 2 and 3. In two previous studies, using a noncommercialized NIR version and SET, no beneficial effect on implantation and pregnancy rates was found when using this technology to rank the metabolic profile of similar embryos of good morphology on Day 5[26] and Day 3,[27] while a slight advantage was reported when performing Day 2 SET.[26]

In the present study, the increased IRs in the metabolomics + morphology group for Day 5 transfers suggest that the commercial NIR instrument may have enhanced the identification of blastocysts with the highest developmental competence. However, the fact that pregnancy and live birth rates were similar, despite the higher IRs in the metabolomics + morphology group on Day 5, may be explained by the higher, although nonsignificant increase in twin and triplet rates in the metabolomics + morphology group, combined with higher clinical miscarriage rates in the morphology group. Interestingly, the publication of Hardarson *et al.*, showed that the NIR instrument was least effective on Day 5 transfers.

Our initial experience with the commercial version of the NIR instrument showed that Viametrics-E was easily implemented in routine clinical practice and was not disruptive to everyday laboratory procedures. The method yielded the viability score for each assayed embryo in a rapid and straightforward manner, facilitating the real time clinical decision of embryo selection.

Our data show that the Viability Score was not correlated with embryo morphology grade on Days 2, 3, and 5, corroborating previous studies reporting that the viability score is independent of morphology grades for Days 2 and 3 embryos[22] and for Day 5 blastocysts.[32] Moreover, it has been shown that 75.4% of embryos with the best morphology did not have the highest viability score.[27] Therefore, it is suggested that metabolomic profiling of embryos may indeed provide an objective secondary level of assessment as an adjunct to embryo morphology.[22]

Table 4: Implantation and pregnancy rates for Day 5 transfers

(%)	Metabolomics group (*n*=20)	Morphology group (*n*=34)	*P*
Implantation with FCA	22/47 (46.8)	26/90 (28.9)	0.0405
Implantation	22/47 (46.8)	31/90 (34.4)	0.196
Positive hCG	16 (80)	24 (70.6)	0.533
Clinical pregnancy	13 (65)	19 (55.9)	0.576
Ongoing pregnancy	12 (60)	15 (44.1)	0.398
Live births	11 (55)	15 (44.1)	0.574
Multiple pregnancy	7 (35)	8 (23.5)	0.530
Twin pregnancy	6 (30)	7 (20.6)	0.517
Triplet pregnancy	2 (10)	1 (2.9)	0.548
Biochemical pregnancy	3/16 (18.8)	5/24 (20.8)	1.00
Clinical miscarriage	1/13 (7.7)	4/19 (21.1)	0.624

FCA=Fetal cardiac activity; hCG=Human chorionic gonadotropin

Table 5: Multiple regression analysis

	b	*P* value
Age (years)	−0.756	0.371
BMI (kg/m^2)	−0.022	0.673
FSH (IU/l)	−0.099	0.797
Years of infertility	−0.551	0.645
Previous IVF attempts	−3.194	0.079
Number of embryos transferred	4.836	0.389

Multiple regression analysis with dependent variable being implantation rates with FCA (primary outcome), and independent variables being patient age, BMI, FSH, years of infertility, number of previous IVF attempts, and number of embryos transferred. *b* is the value of the coefficient and *P* value shows the significance of its entrance in the logistic regression equation. None of the parameters had a significant effect on the primary outcome. BMI=Body mass index; IVF=*In vitro* fertilization; FSH=Follicle-stimulating hormone

The present study was terminated prematurely in December 2010, before achieving statistical power, following the voluntary market withdrawal of ViaMetrics-E due to variability in scores and a susceptibility of the algorithms to noise. Briefly, it was reported by the manufacturer that some of the commercial instruments showed variation in repeatability within the instrument, but more concerning was a larger problem with reproducibility between instruments. This translated to individual algorithms created on master instruments being vulnerable to noise and other factors when loaded on other instruments. In effect, it may have been that although some benefits were seen in our clinic, they may not have been transferable to other clinics using different instruments. The present study set out to describe our Unit's experience using the commercial NIR instrument of ViaMetrics-E. It shows that despite the aforementioned drawbacks of the instrument, the philosophy and the principles behind the method are valid and interesting to investigate. This is the only report on the use of ViaMetrics-E, which shows that the use of Viametrics-E was not harmful, but also gave higher IRs for blastocyst transfers on Day 5. There is no question that a new version should be developed, which will require stringent validation before commercial launch.

Indeed, larger studies using SET have been conducted using prototype NIR instruments to assess the precision and reproducibility of the Viability Score, and to evaluate the efficacy of the method in identifying the single best embryo of a patient's cohort. Two of those SET studies have failed to show a difference in implantation, clinical and ongoing pregnancy rates using metabolomic profiling of spent embryo culture media.[26,27] In light of our data, showing higher IRs on Day 5 with multiple embryo transfers, this could be an indication that score variability may increase the chance of failure to identify the most viable embryo in a SET scenario. The following possibilities exist: (i) That this particular instrument was less susceptible to noise and that the algorithm was more likely to examine the correct spectral profile and/or (ii) that when transferring multiple embryos the instrument had a greater chance to have measured the correct signal in one of those embryos when compared with the more stringent test of having to get the score correct every time with a SET.

In conclusion, our study suggests that metabolomic analysis using the commercial Viametrics-E does not appear to have a significant beneficial effect on pregnancy and live birth rates, with a marginal improvement in IRs with FCA for Day 5 transfers. A limited number of patients were included in the study due to the early withdrawal of the current instrument, and therefore the data presented here should be viewed with caution. It is anticipated that reduction of score variability and improvement of the instrument platform may yet provide a feasible system for the rapid, noninvasive assessment of embryos prior to transfer. At the time of writing, an upgraded commercial version of this technology is awaited, which will require further stringent assessment and validation, in a clinical setting with SET.

ACKNOWLEDGMENTS

The authors wish to thank G. Iliadis and K. Anagnostara for embryology work, G. Stavropoulou for patient coordination, and R. Carousou for secretarial support.

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How to cite this article: Sfontouris IA, Lainas GT, Sakkas D, Zorzovilis IZ, Petsas GK, Lainas TG. Non-invasive metabolomic analysis using a commercial NIR instrument for embryo selection. J Hum Reprod Sci 2013;6:133-9.

Source of Support: Nil, **Conflict of Interest:** None declared.