ROBUST METABOLOMICS APPROACH FOR THE EVALUATION OF HUMAN EMBRYOS FROM IN-VITRO FERTILIZATION

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SUPPLEMENTARY INFORMATION INCLUDES:

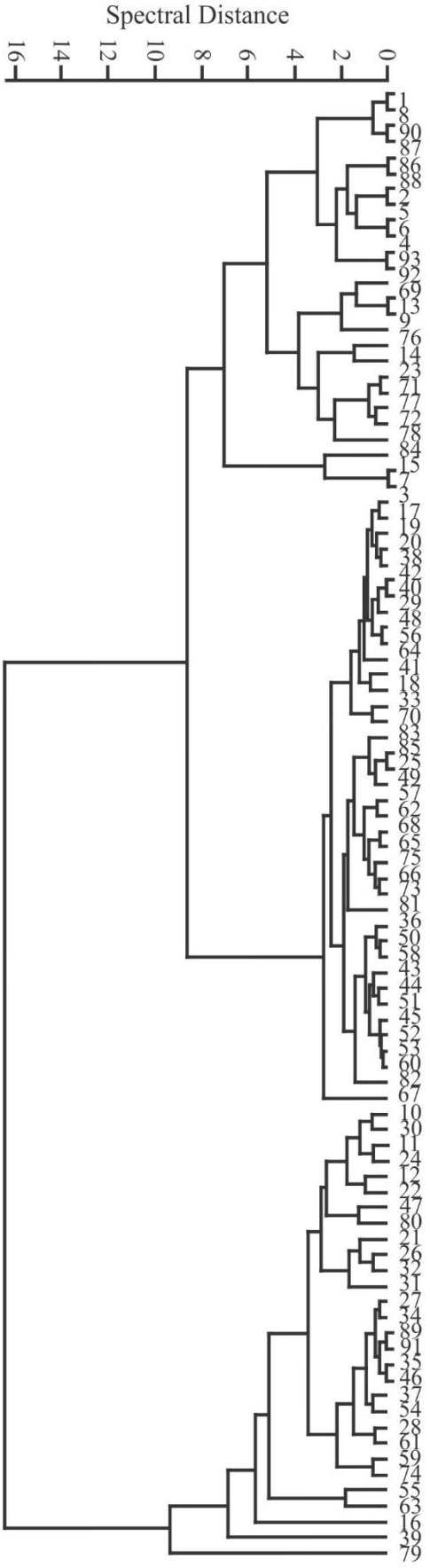
SUPPLEMENTARY NOTE 1

SUPPLEMENTARY FIGURES 1 TO 8

Supplementary Note 1: Heterogeneity analysis of IMP and NIMP spectra. The unsupervised hierarchical-cluster-analysis was used for the evaluation of the heterogeneity of IMP and NIMP. As previously reported[46] and detailed in the Materials and Methods section, the spectral variances in each data set were determined as the average ± 2 standard deviations of the so-called spectral distance (D)[52,66,67]. This parameter corresponds to a dissimilarity measurement equal to (1 – r) x 1000, with r being Pearson's product-moment–correlation coefficient. The spectral distances were calculated through the use of the preprocessed procedure *B* in the spectral ranges 1500–1800 cm^{-1} and 730–1280 cm^{-1}. The fusion values in

dendrograms were obtained by means of the average linkage (OPUS versions 7.0 Bruker Optics GmbH, Ettlingen, Germany).

The heterogeneity obtained for IMP (D ± SD = 11.70 ± 7.00) was smaller than that obtained for NIMP (D ± SD = 21.98 ± 16.97). This analysis indicated that IMP spectra were quite homogenous, while those from NIMP were highly heterogeneous (Supplementary Fig. 4). These results support the analysis of the spectral-data internal structure performed by principal-component analysis.


Spectral Distance
16
14
12
10
8
6
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2
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8
90
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86
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93
92
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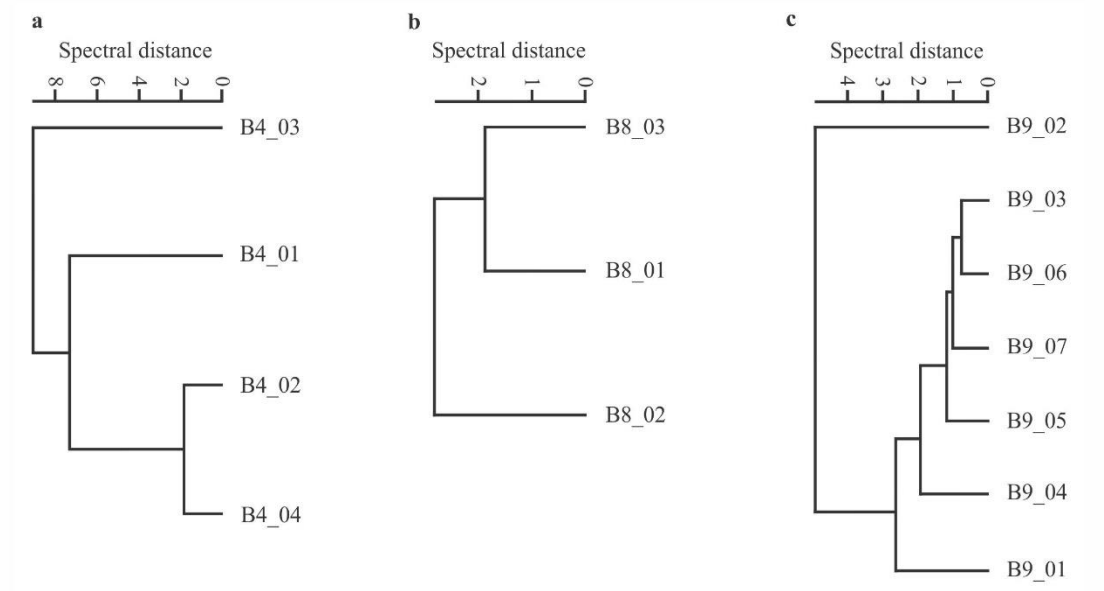
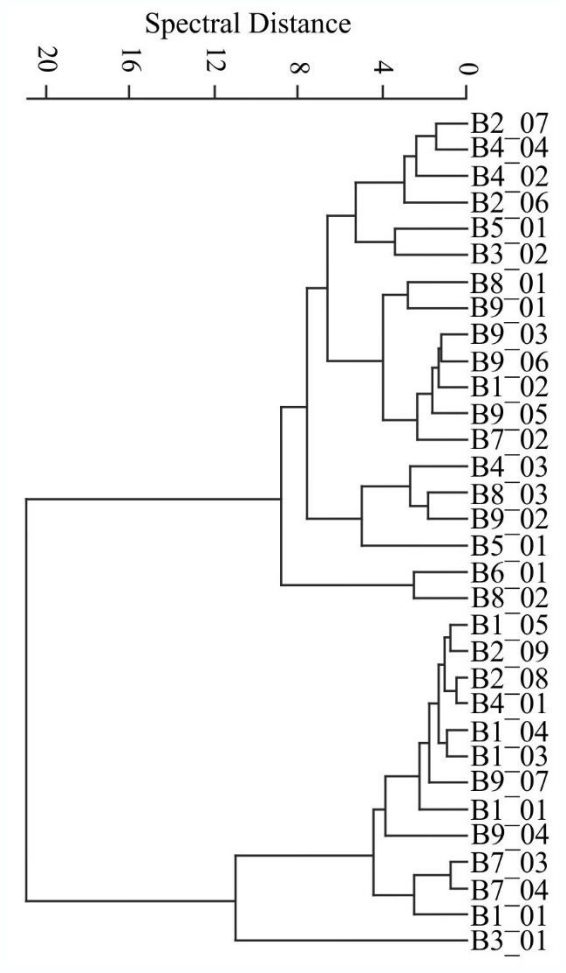
Supplementary Fig. 1 Reproducibility levels of acquired spectra among the different wells in the ZnSe optical plate. The spectral distances were calculated through the use of the preprocessed procedure *A* in the spectral ranges 3000–2800 cm^{-1}, 1800–1550 cm^{-1}, 1500–1250 cm^{-1}, and 1200–900 cm^{-1}. The fusion values in dendrograms were obtained by means of the average linkage (OPUS software 7.0, Bruker, Optics, Germany). The spectral distance measured for this independent replicate was 6.30 ± 5.26. The analysis indicated that the spectral quality was not affected by the sample desiccation that occurred during the time period required for the measurements. Three wells of the ZnSe microtiter plate remained empty. One well (A1) was used to measure the spectrum background and the other poaition were measured empty in order to stabilize the air circulation of the equipment.



Supplementary Fig. 2 Reproducibility among each batch of culture medium G1 Plus. Reproducibility levels among three different batches of G1 Plus culture medium. Panel a, Batch 4; Panel b, Batch 8; Panel c, Batch 9. The spectral distances were calculated through the use of the preprocessed procedure *A* in the spectral ranges

3000-2800 cm^{-1}, 1800 cm^{-1}, 550 cm^{-1}, 1500–1250 cm^{-1}, and 1200–900 cm^{-1}. The fusion values in dendrograms were obtained by means of the average linkage (OPUS software 7.0, Bruker, Optics, Germany). The widest spectral distances measured for these culture-medium batches were 7.08 ± 3.29 (Panel a), 2.50 ± 0.94 (Panel b) y 3.34 ± 2.53 (Panel c), respectively. The reproducibility obtained within each batch of G1 Plus culture medium exhibited a low level of variability.

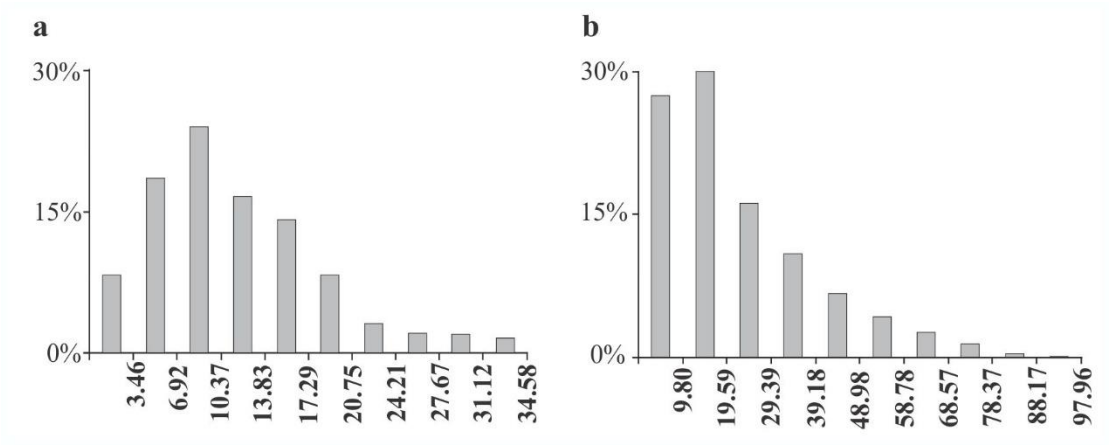
B: batch



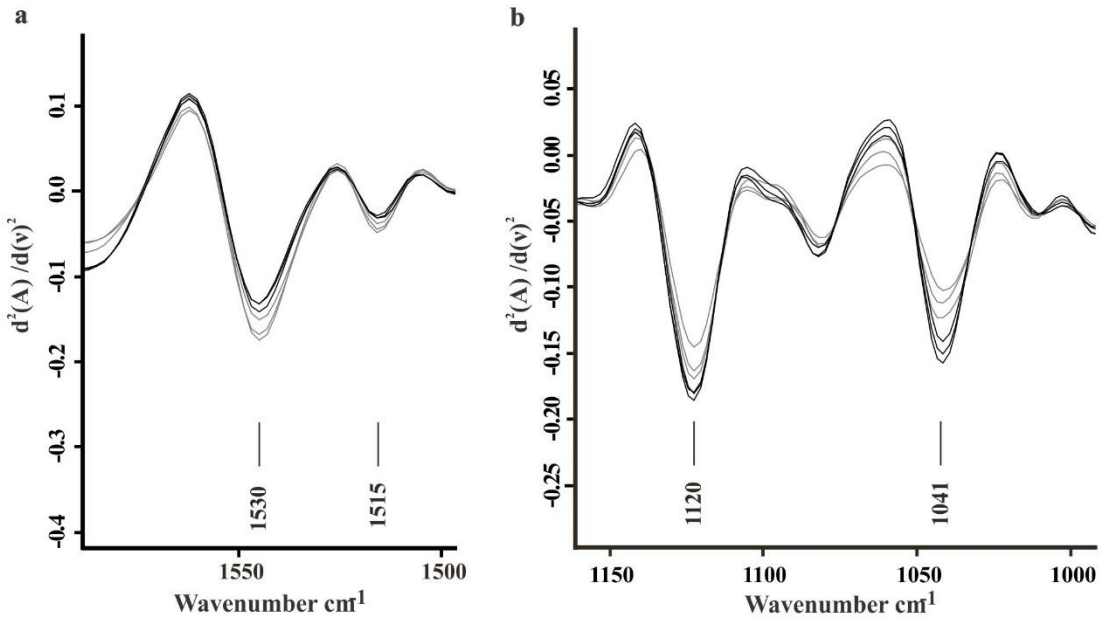
Supplementary Fig. 3 Reproducibility among batches of culture medium G1 Plus. The spectral distances were calculated through the use of the preprocessed procedure *A* in the spectral ranges 3000–2800 cm^{-1}; 1800–1550 cm^{-1}; 1500–1250 cm^{-1}, and 1200–900 cm^{-1}. The fusion values in dendrograms were obtained by means of

the average linkage (OPUS software 7.0, Bruker, Optics, Germany). The widest spectral distance measured for analysis of the variance of these G1 Plus culture medium batches was 9.31 ± 6.60. This analysis indicated that a high level of reproducibility was observed, while no significant spectral differences were recorded for more than 15 different batches of G1 Plus culture medium assayed.

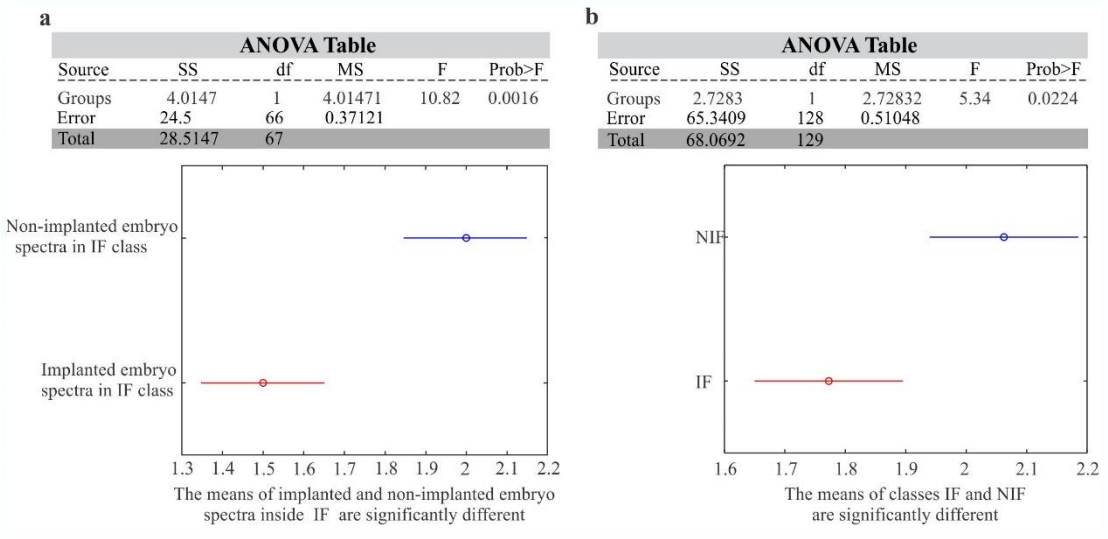
B: batches



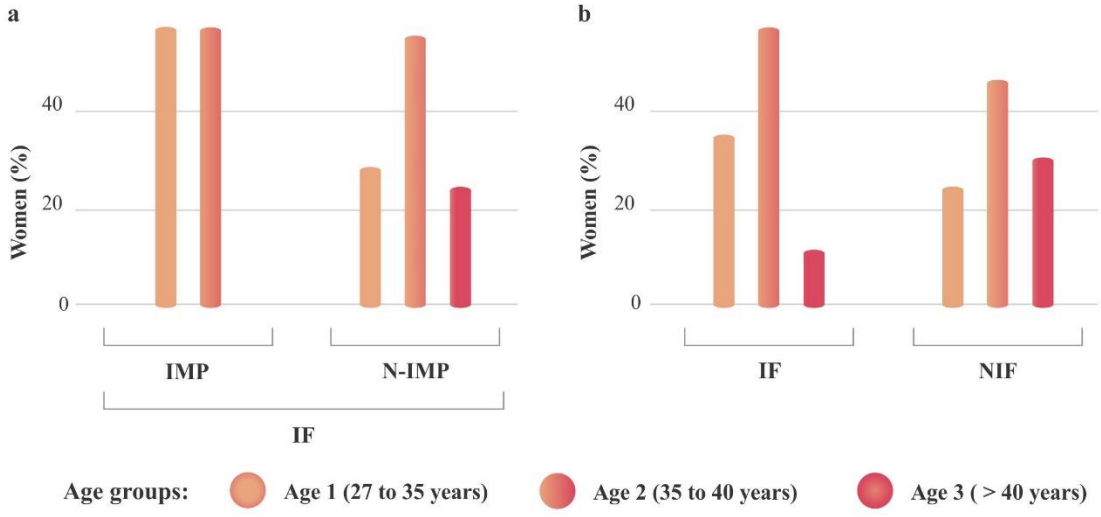
Supplementary Fig. 4 Heterogeneity of the spectra within classes. Distribution of spectral-distance values of spectra acquired from 3-day-embryo supernatants belonging to IMP (Panel a) and NIMP (Panel b). In the two panels, the percent distribution of each spectral distance indicated on the *abscissa* is plotted on the *ordinate*.



Supplementary Fig. 5 Spectral differences in the regions W2 and W4. In both panels of the figure, the second derivative of the spectra is plotted on the *ordinate* as a function of the wavenumber on the *abscissa*. Panel a: Vector-normalized second-derivatives of the three spectra of the IF (black line) and NIF (gray line) classes at 1530 cm^{-1} associated with amino acids. Panel b: Vector-normalized second-derivatives of the three spectra of the IF (black line) and NIF (gray line) classes at 1120 cm^{-1} and 1041 cm^{-1} assigned to the C–O stretching in the carboxylic group of lactate.

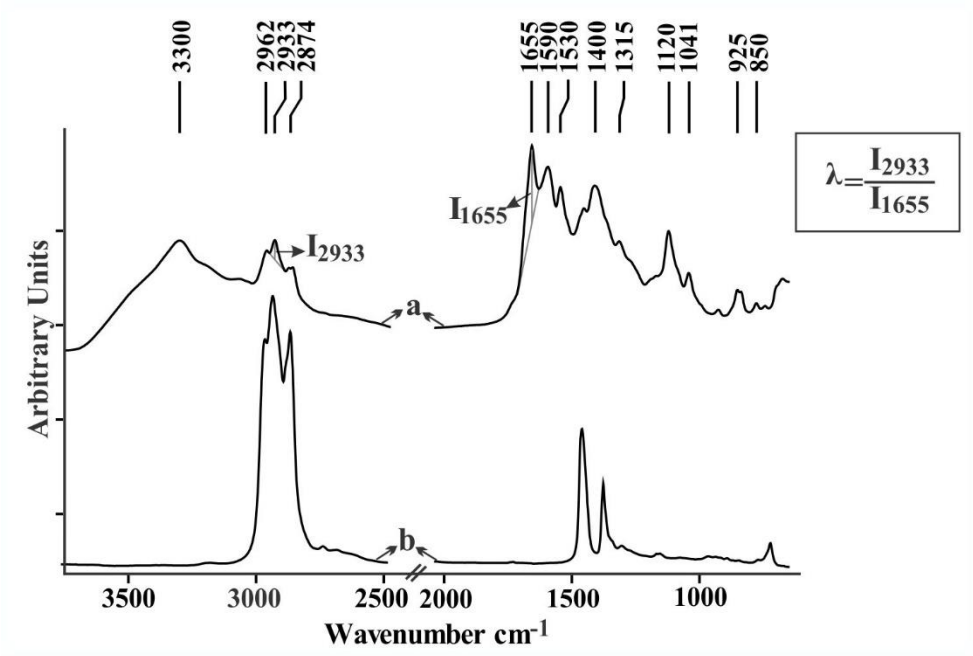


Supplementary Fig. 6 Panel a: One-way ANOVA evaluating maternal age in the IF class (p <0.05). The statistical analysis demonstrated that within this IF class the age of the mothers was significantly different between the class-IMP- and the class-NIMP–embryo spectra, with the mothers being older in class NIMP. Panel b: One-way ANOVA comparing maternal ages and metabolomic-fingerprint classes (p <0.05). The analysis indicated that the embryo-supernatant samples classified as NIF were from older mothers than those classified as IF.



Supplementary Fig. 7 Analysis of the age percentage distribution of women considering the defined age categories. Panel a: Maternal-age analysis between

samples from implanted embryos (IMP) and nonimplanted embryos (NIMP) of the IF class. Panel b: Maternal-age analysis between samples from the IF and NIF classes. In the two panels, the percent of the women within a given age class is plotted on the *ordinate* with respect to the transferred embryos having the implantation outcomes. Age Groups: Age 1 (27 to 35 years), Age 2 (35 to 40 years), Age 3 (>40 years).



Supplementary Fig. 8 Determination of oil contamination in 3-day-embryo–supernatant spectra. In the figure, the absorbance in arbitrary units is plotted on the *ordinate* as a function of the wavenumbers indicated in the *abscissa*. Spectrum a: FTIR spectrum of a 3-day-embryo supernatant contaminated with culture oil. Spectrum b: FTIR average spectrum of the oil. The intensity of the $–CH_2$ stretching of lipids at 2933 $cm^{–1}$—used as a marker band for the oil content—and the amide-I band at 1655 $cm^{–1}$—an internal standard for the total material—can be used to determine the relative amount of oil present in the spectra of embryo supernatants.