Robust metabolomics approach for the evaluation of human embryos from *in-vitro* fertilization

Cecilia Beatriz Figoli[a], Marcelo Garcea[b], Claudio Bisioli[b], Valeria Tafintseva[c], Volha Shapaval[c], Mariana Gómez Peña[b], Luz Gibbons[d], Fernando Althabe[d], Osvaldo Miguel Yantorno[a], Marcos Horton[b], Jürgen Schmitt[e], Peter Lasch[f], Achim Kohler[c]* & Alejandra Bosch[a,]*

[a.] *CINDEFI-CONICET, CCT La Plata, Facultad de Ciencias Exactas, UNLP, 1900 La Plata, Argentina*
[b.] *PREGNA Medicina Reproductiva, C1425 AYV Ciudad Autónoma de Buenos Aires, Argentina*
[c.] *Faculty of Science and Technology, Norwegian University of Life Sciences, 1432 Ås, Norway*
[d.] *IECS, Instituto de Efectividad Clínica y Sanitaria, C1414 Ciudad Autónoma de Buenos Aires, Argentina*
[e.] *Synthon GmbH, 69198 Schriersheim, Germany*
[f.] *Centre for Biological Threats and Special Pathogens (ZBS) Proteomics and Spectroscopy" Unit, Robert Koch-Institut, 13353 Berlin, Germany.*

*Corresponding author:
Alejandra Bosch, E-mail: bosch@quimica.unlp.edu.ar
Achim Kohler, E-mail: achim.kohler@nmbu.no

This Electronic Supplementary Material contains the following:

Fig. S1 Determination of oil contamination in 3-day-embryo–supernatant spectra.
Converting numerical into qualitative variables.
Table S1. Patient clinical data.
Heterogeneity analysis.
Fig. S2 Reproducibility levels of acquired spectra among the different wells in the ZnSe optical plate.
Fig. S3 Reproducibility among each batch of culture medium G1 Plus.
Fig. S4 Reproducibility among batches of culture medium G1 Plus.
Fig. S5 Heterogeneity of the spectra within classes.
Fig. S6 Spectral differences in the spectral region W2.
Fig. S7 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.
Fig. S8 Analysis of the age percentage distribution of women considering the defined age categories.

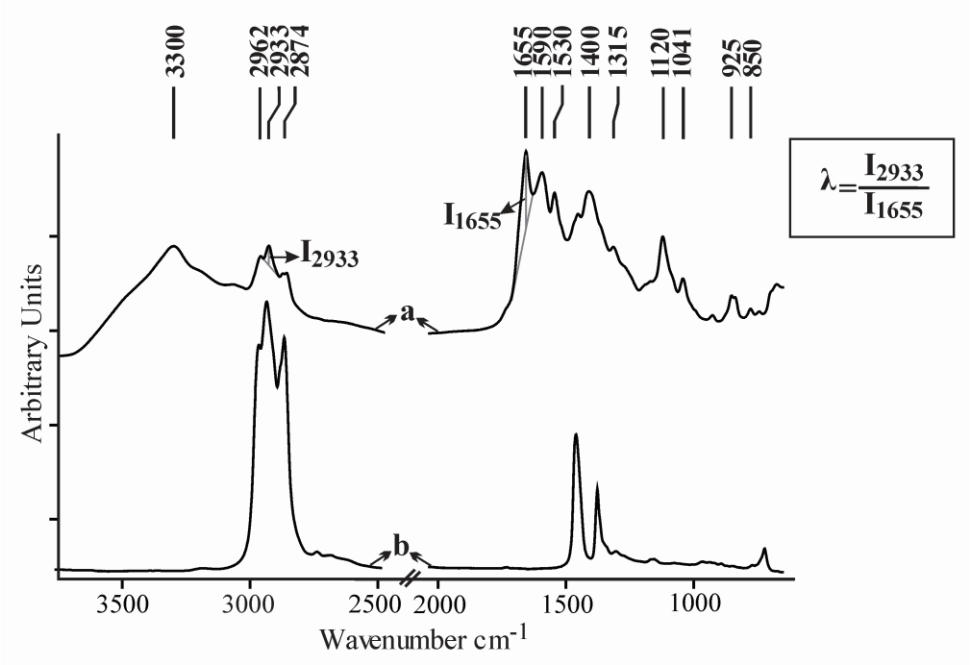

Fig. S1 Determination of oil contamination in 3-day-embryo–supernatant spectra. In the figure, the absorbance in arbitrary units is plotted on the *ordinate* as a function of the wavenumbers indicated in the *abscissa*. Spectrum a: FTIR spectrum of a 3-day-embryo supernatant contaminated with culture 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.

Converting numerical into qualitative variables.

Clinical data registered at the OpenClinica database such as mother's age, BMI, and smoking habits were converted from numerical variables into qualitative ones (Table S1):

The mother's age was categorized in 3 groups: Group 1, women older than 27 and younger than 35 years; Group 2, women between 35 and 40 years; and Group 3, women older than 40 years.

BMI was calculated in each individual by using the patient's height and weight information in the formula (weight in kg)/(height in m)^2 and then classified into 4 groups. Those patients with BMI values between 18.5 to 25.0 (normal weight), were included in Group 1, BMI values below 18.5 (underweight) were placed in Group 2, BMI values between 25.0 to 30.0 (overweight) corresponded to Group 3, and those with BMI values over 30.0 (obesity) were categorized in Group 4.

The information related to smoking habit provided by the mothers was divided into 5 categories: Group zero (0), women that never smoked; Group 1, ex-smokers that used to consume fewer than 10 cigarettes per day; Group 2, ex-smokers that used to smoke

more than 10 cigarettes per day; Group 3, woman smoking at the time of the IVF treatment at least 9 cigarettes per day; Group 4, active smokers of 10 to 19 cigarettes per day; and Group 5, smokers of 20 or more cigarettes per day.

Table S1. Patient clinical data.

Implantation classes	Metabolomic fingerprints classes	Age	Smoking habits	BMI	Embryo morphology
NIMP	NIF	3	1	1	3
NIMP	NIF	3	1	1	3
NIMP	NIF	2	5	1	1
NIMP	NIF	2	5	1	2
NIMP	NIF	2	1	1	2
NIMP	NIF	2	1	1	2
NIMP	NIF	2	1	1	1
NIMP	NIF	2	1	1	1
NIMP	NIF	2	0	nd	2
NIMP	NIF	2	0	2	2
NIMP	NIF	2	0	2	2
NIMP	NIF	2	0	4	1
NIMP	IF	2	0	4	1
NIMP	NIF	3	0	1	3
NIMP	NIF	2	0	nd	1
NIMP	IF	2	0	nd	2
NIMP	IF	1	4	1	2
NIMP	NIF	2	4	1	2
NIMP	NIF	2	4	1	1
NIMP	NIF	1	0	1	1
NIMP	NIF	1	0	1	2
NIMP	NIF	1	0	1	3
NIMP	IF	3	0	1	2
NIMP	NIF	3	0	1	2
NIMP	IF	2	3	1	2
NIMP	NIF	1	0	1	1
NIMP	NIF	1	0	1	1
NIMP	NIF	3	0	1	2
NIMP	NIF	2	0	1	1
NIMP	NIF	2	0	1	2
NIMP	NIF	1	0	1	2
NIMP	IF	1	0	1	2
NIMP	IF	3	0	1	1
NIMP	NIF	2	nd	nd	3
NIMP	IF	2	0	1	1
NIMP	IF	2	0	1	3
NIMP	NIF	2	0	1	1
NIMP	IF	3	0	nd	1
NIMP	NIF	1	0	1	2

NIMP	NIF	1	0	1	2
NIMP	NIF	1	0	1	2
NIMP	IF	1	3	1	2
NIMP	IF	1	3	1	2
NIMP	NIF	3	0	1	1
NIMP	IF	3	0	1	2
NIMP	NIF	3	0	1	1
NIMP	NIF	3	2	1	2
NIMP	IF	3	0	2	1
NIMP	IF	3	0	nd	3
NIMP	IF	2	0	1	3
NIMP	NIF	2	0	1	3
NIMP	NIF	2	0	1	1
NIMP	NIF	3	0	4	1
NIMP	NIF	3	0	4	1
NIMP	NIF	1	1	1	1
NIMP	NIF	1	1	1	1
NIMP	IF	2	0	1	1
NIMP	IF	2	0	1	2
NIMP	IF	2	0	1	1
NIMP	NIF	3	nd	nd	1
NIMP	NIF	3	0	1	2
NIMP	NIF	3	0	1	2
NIMP	NIF	1	2	1	2
NIMP	NIF	2	0	1	2
NIMP	NIF	3	4	1	2
NIMP	IF	1	0	1	2
NIMP	NIF	2	2	4	2
NIMP	IF	2	3	3	3
NIMP	NIF	2	0	1	2
NIMP	IF	1	nd	1	2
NIMP	NIF	3	0	1	1
NIMP	IF	2	1	1	2
NIMP	NIF	2	2	1	2
NIMP	IF	2	2	1	2
NIMP	IF	1	1	3	2
NIMP	NIF	1	1	3	2
NIMP	IF	2	3	3	3
NIMP	NIF	3	0	1	3
NIMP	IF	3	2	3	2
NIMP	NIF	3	2	3	2
NIMP	IF	2	nd	nd	2
NIMP	IF	2	nd	nd	2
NIMP	IF	2	nd	1	3
NIMP	IF	2	nd	1	2
NIMP	IF	2	nd	nd	2

NIMP	IF	1	0	1	2
NIMP	NIF	2	nd	1	2
NIMP	NIF	2	nd	4	3
NIMP	IF	1	0	1	3
NIMP	NIF	2	0	1	1
NIMP	NIF	1	2	1	1
NIMP	IF	2	1	1	1
NIMP	IF	1	0	1	3
NIMP	NIF	1	0	1	1
NIMP	NIF	3	2	3	2
NIMP	NIF	3	0	1	2
NIMP	NIF	2	0	1	2
NIMP	NIF	1	0	3	2
NIMP	IF	1	3	1	1
NIMP	NIF	2	nd	1	1
NIMP	IF	2	nd	1	1
NIMP	IF	3	5	3	2
NIMP	NIF	3	nd	1	3
NIMP	IF	3	0	nd	1
IMP	IF	2	0	1	2
IMP	IF	2	0	1	2
IMP	IF	1	0	1	2
IMP	IF	1	0	1	2
IMP	IF	1	0	1	1
IMP	IF	1	0	1	1
IMP	IF	2	6	1	2
IMP	IF	2	0	1	2
IMP	IF	2	0	1	2
IMP	IF	1	0	3	2
IMP	IF	1	0	1	2
IMP	IF	1	0	1	2
IMP	IF	2	3	1	1
IMP	IF	2	3	1	1
IMP	IF	1	1	1	1
IMP	IF	1	1	1	2
IMP	IF	2	0	1	1
IMP	IF	2	0	1	1
IMP	IF	1	0	3	2
IMP	IF	1	0	3	1
IMP	IF	2	0	1	2
IMP	IF	2	0	1	2
IMP	IF	1	0	1	2
IMP	IF	1	0	1	2
IMP	IF	2	1	4	2
IMP	IF	2	1	4	2

nd: no data available

Heterogeneity analysis. The unsupervised hierarchical-cluster-analysis was used for the evaluation of the heterogeneity of IMP, NIMP, IF and NIF. As previously reported1 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).[2–4] 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 (ESI Fig. S5†). These results support the analysis of the spectral-data internal structure performed by principal-component analysis.

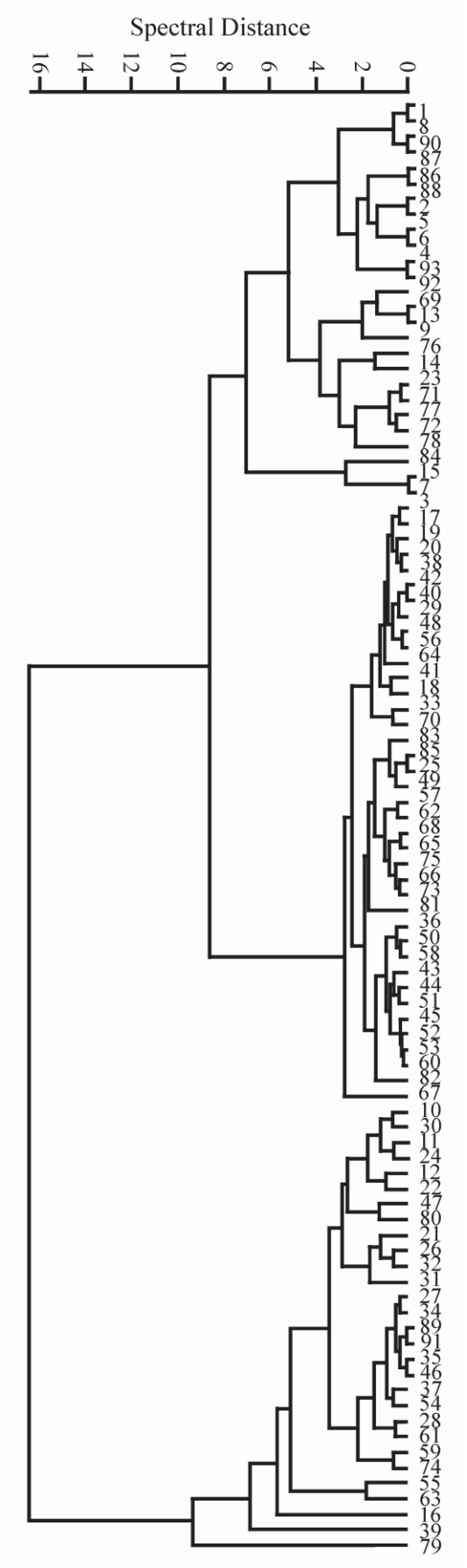
Spectral Distance
16
14
12
10
8
6
4
2
0
1
8
90
87
86
88
2
5
6
4
93
92
69
13
9
76
14
23
71
77
72
78
84
15
7
3
17
19
20
38
42
40
29
48
56
64
41
18
33
70
83
82
85
25
49
57
62
68
65
75
66
73
81
36
50
58
43
44
51
45
52
53
60
82
67
10
30
11
24
12
22
47
80
21
26
32
31
27
34
89
91
35
46
37
54
28
61
59
74
55
63
16
39
79

Fig. S2 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.

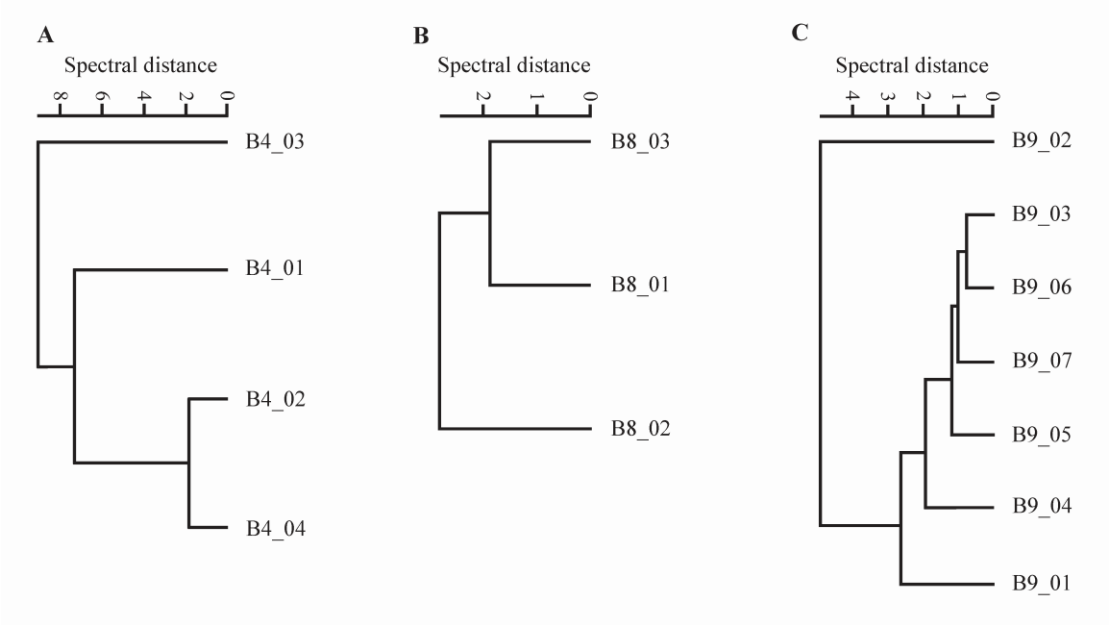

Fig. S3 Reproducibility among each batch of culture medium G1 Plus. Reproducibility levels among three different batches of G1 Plus culture medium. Panel A, Batch 4; Panel B, Batch 8; Panel C, Batch 9. The spectral distances were calculated through the use of the preprocessed procedure *A* in the spectral 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

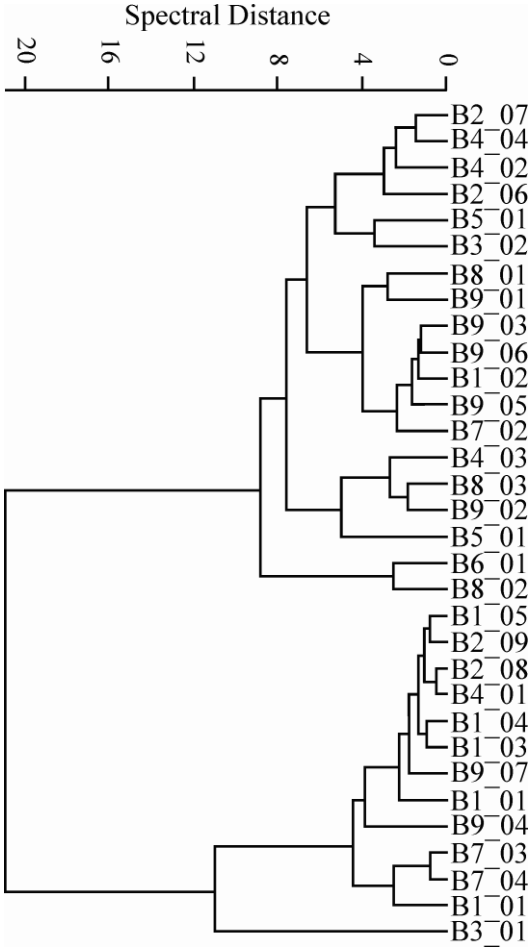

Fig. S4 Reproducibility among batches of culture medium G1 Plus. The spectral distances were calculated through the use of the preprocessed procedure *A* in the spectral ranges 3000–2800 cm^{-1}; 1800–1550 cm^{-1}; 1500–1250 cm^{-1}, and 1200–900 cm^{-1}. The fusion values in dendrograms were obtained 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

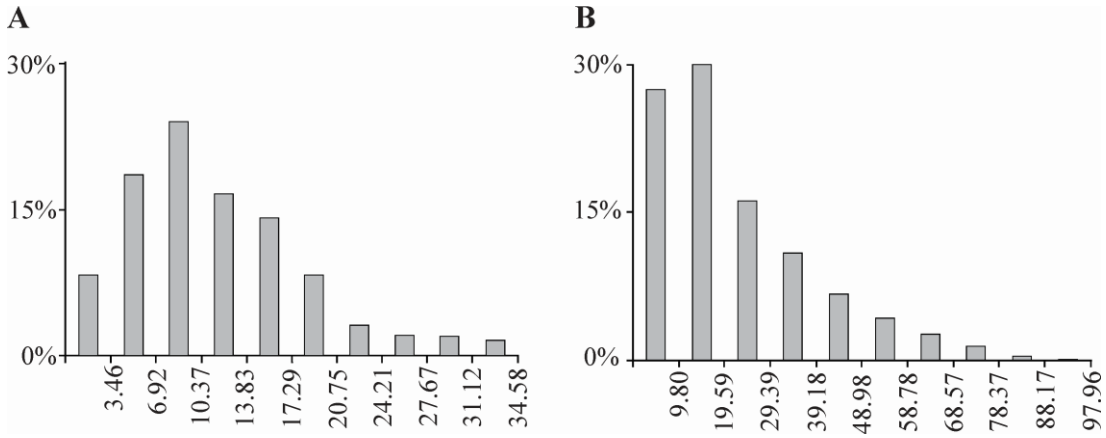

Fig. S5 Heterogeneity of the spectra within classes. Distribution of spectral-distance values of spectra acquired from 3-day-embryo supernatants belonging to IMP (Panel A) and NIMP (Panel B). In the two panels, the percent distribution of each spectral distance indicated on the *abscissa* is plotted on the *ordinate*.

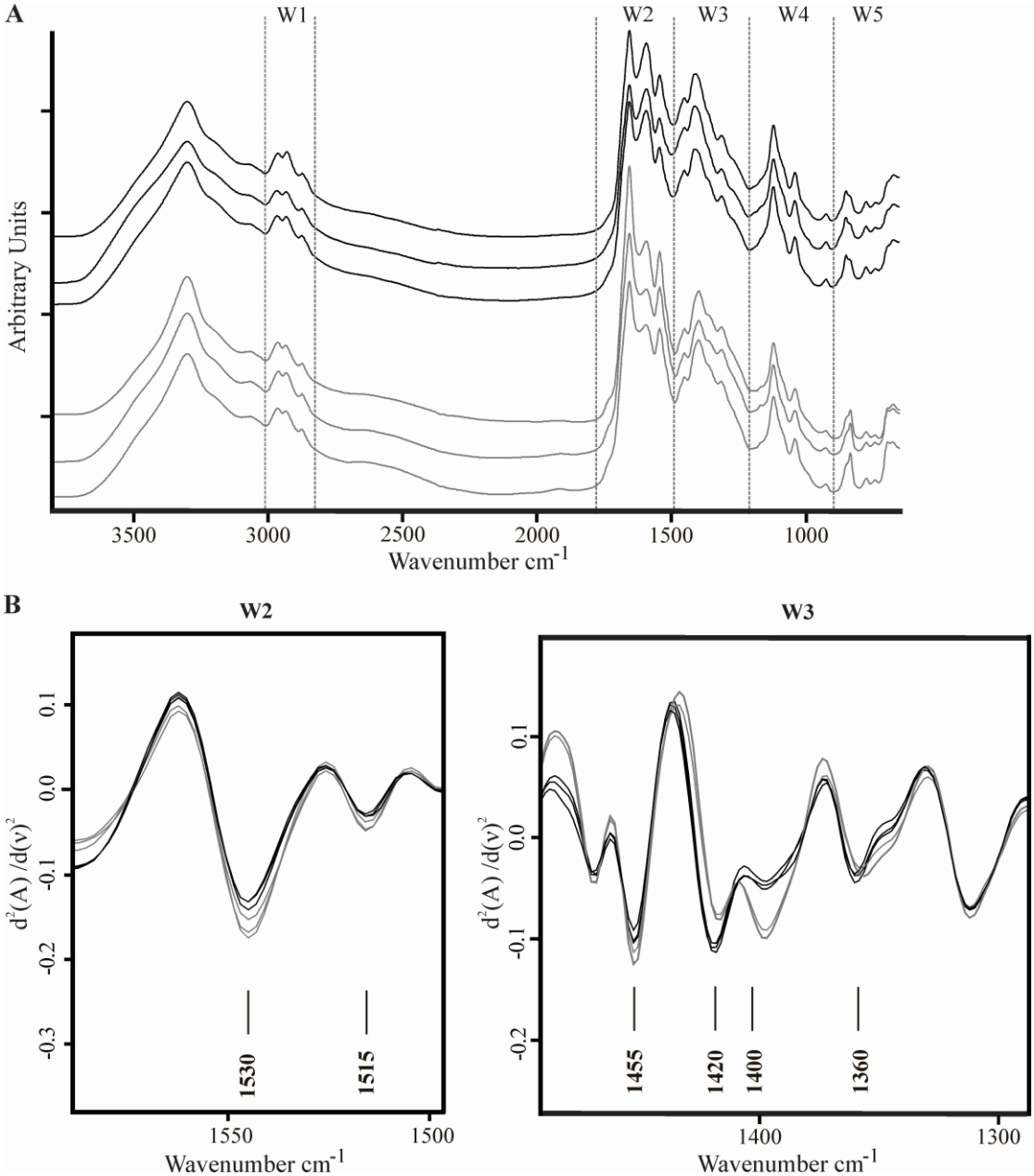

Fig. S6. Panel A: FTIR spectra of three embryos of IF class and three of NIF class (black and grey lines, respectively). Panel B: Discriminative features of the vector-normalized second-derivatives of the three spectra of the IF (black line) and NIF (gray line) classes in W2 and W3 spectral regions. The second derivative of the spectra is plotted on the *ordinate* as a function of the wavenumber on the *abscissa*.

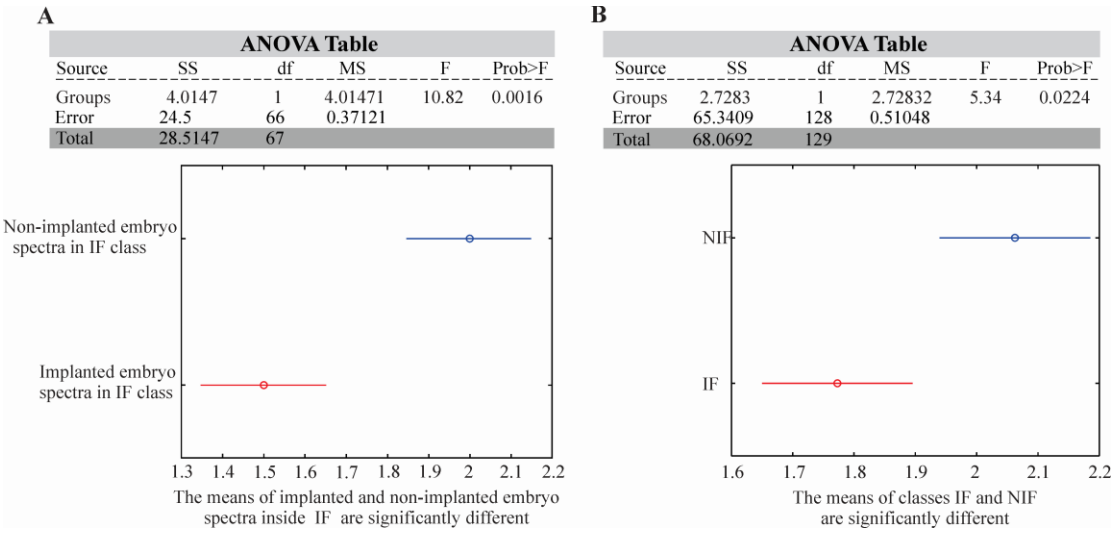

Fig. S7 Panel A: One-way ANOVA evaluating maternal age in the IF class (p <0.05). The statistical analysis demonstrated that within this IF class the age of the mothers was significantly different between the class IMP and the class NIMP embryo spectra, with the mothers being older in class NIMP. Panel 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.

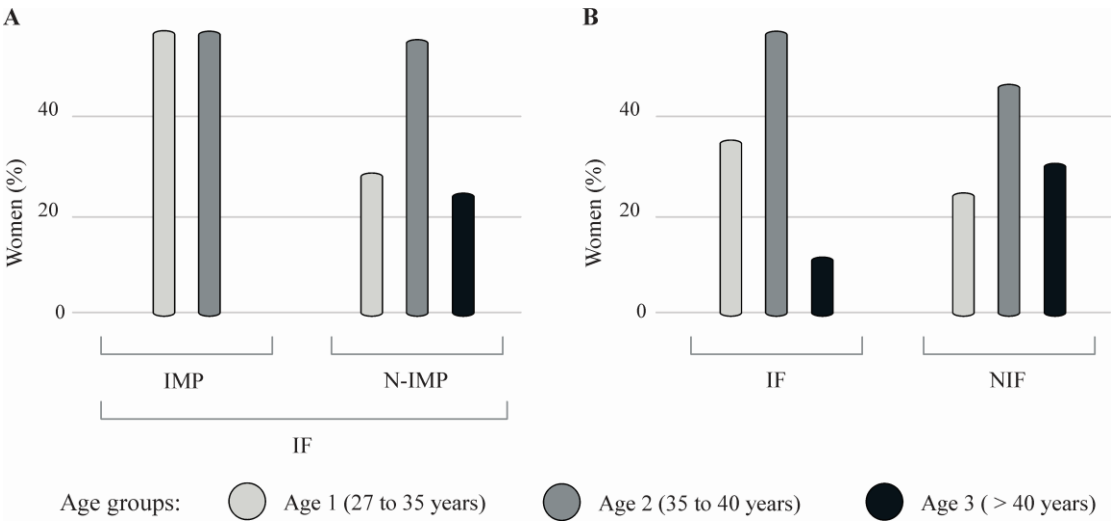

Fig. S8 Analysis of the age percentage distribution of women considering the defined age categories. Panel A: Maternal-age analysis between samples from implanted embryos (IMP) and nonimplanted embryos (NIMP) of the IF class. Panel B: Maternal-age analysis between samples from the IF and NIF classes. 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).

References

1 M. Wenning and S. Scherer, *Appl. Microbiol. Biotechnol.*, 2013, **97**, 7111–7120.

2 C. A. Rebuffo-Scheer, J. Dietrich, M. Wenning and S. Scherer, *Anal. Bioanal. Chem.*, 2008, **390**, 1629–1635.
3 A. Bosch, A. Miñán, C. Vescina, J. Degrossi, B. Gatti, P. Montanaro, M. Messina, M. Franco, C. Vay, J. Schmitt, D. Naumann and O. Yantorno, *J. Clin. Microbiol.*, 2008, **46**, 2535–2546.
4 D. Helm, H. Labischinski, G. Schallehn, D. Naumann, G. Schallehn~ and D. Naumann', *J. Gen. Microbiol.*, 1991, **137**, 69–79.