Day-3 embryo metabolomics in the spent culture media is altered in obese women undergoing in vitro fertilization

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Objective: To determine whether the global metabolomic profile of the spent culture media (SCM) of day-3 embryos is different in obese and normoweight women undergoing in vitro fertilization (IVF).
Design: Prospective cohort analysis.
Setting: IVF clinic.
Patient(s): Twenty-eight young, nonsmoking women with normoweight, nonsmoking male partners with mild/normal sperm factors undergoing a first IVF attempt for idiopathic infertility, tubal factor infertility, or failed ovulation induction: obese ovulatory women (n = 12); obese women with polycystic ovary syndrome (PCOS; n = 4); normoweight ovulatory women (n = 12).
Intervention(s): Fifty μl of SCM collected from two day-3 embryos of each cohort.
Main Outcome Measure(s): Metabolomic profiling via ultrahigh performance liquid chromatography coupled to mass spectrometry of SCM from a total of 56 embryos.
Result(s): The untargeted metabolomic profile was different in obese and normoweight women. Partial least squares discriminant analysis resulted in a clear separation of samples when a total of 551 differential metabolites were considered. A prediction model was generated using the most consistent metabolites. Most of the metabolites identified were saturated fatty acids, which were detected in lower concentrations in the SCM of embryos from obese women. The metabolomic profile was similar in obese women with or without PCOS.
Conclusion(s): The metabolomic profile in the SCM of day-3 embryos is different in normoweight and obese women. Saturated fatty acids seem to be reduced when embryos from obese patients are present.
Clinical Trial Registration Number: NCT01448863. (Fertil Steril® 2015;103:1407–15. ©2015 by American Society for Reproductive Medicine.)
Key Words: Day-3 embryos, female obesity, metabolomics, saturated fatty acids, spent culture media

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Obesity (defined as a body mass index [BMI] greater than 30 kg/m^2) is continuing to rise globally, with a current prevalence in adults of more than 20% to 30% in most developed countries (1). Female obesity has been clearly related to poor reproductive outcomes in both natural and assisted conception (2–8). However, the exact mechanisms by which oocytes, embryos, or endometrial receptivity may be affected are not clearly known.

Regarding endometrial receptivity, some studies performed using the ovum donation model have shown an impaired outcome in obese recipients. In fact, the largest retrospective analysis performed in a single institution,

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which included 9,587 first cycles of ovum donation, showed a significant reduction in implantation, pregnancy, clinical pregnancy, and live-birth rates when the BMI of the recipient was higher than 30 kg/m^2 (8). The same research group observed that endometrial gene expression during the window of implantation was different in obese and normoweight women, especially when polycystic ovary syndrome (PCOS) was associated (9).

However, no consensus concerning embryo quality has been reached. There are some studies showing alterations in several aspects of embryo morphology in obese women, while others have not detected any difference with respect to normoweight controls (10–13). In fact, the largest single-center study on the effect of female BMI on IVF outcome published to date (5) demonstrated that implantation, pregnancy, and live-birth rates were reduced in obese women, but no significant correlation was found between female BMI and embryo quality on day 2 or 3 of embryo development after considering more than 81,000 oocytes and their corresponding embryos. In addition, when embryo morphology was more dynamically assessed using time-lapse analysis up to day 3 of development, no differences between obese and normoweight infertile women were detected either (11), although one recent study reported that embryos develop to the morula stage faster in overweight women (13).

Therefore, the alteration of embryo quality in obese women may be functional rather than morphologic. In this way, the metabolites consumed from, or secreted into, the culture media in which the embryo is developing may differ in obese and normoweight women, indicating that the embryos derived from these patients have a different metabolomic status. In fact, Leary et al. (13) have recently shown some metabolic alterations in the spent culture media from supernumerary blastocysts donated for research by overweight women. We determined whether the global metabolomic profile in the spent culture media of day-3 embryos is different in obese and normoweight women undergoing IVF and also considered the association with polycystic ovary syndrome (PCOS).

MATERIALS AND METHODS

Study Population

Two groups of women were analyzed and compared. The first group consisted of young ($\leq$38 years old) obese (BMI $>$ 30 kg/m^2) women undergoing their first IVF cycle due to ovulation induction (scheduled intercourse or intrauterine insemination) failure, tubal factor infertility, or unknown infertility etiology. All had a normal uterus as indicated by two-dimensional ultrasound and hysterosalpingography, as well as three-dimensional ultrasound in some cases. Both ovaries were present with an adequate antral follicular count (AFC; at least five antral follicles per ovary). No endometriosis or hydrosalpinx was detected in any of the women as indicated by ultrasound, hysterosalpingography, and clinical symptoms. None of the patients smoked, and all the male partners were normoweight, had no smoking habit, and presented with normozoospermia or mild sperm factors (more than 50 million spermatozoa with progressive motility before sperm preparation, or 2 million spermatozoa with progressive motility afterward).

The obese patient group included two subgroups: group A (n = 12), ovulatory with regular menses (every 21–35 days); and group B (n = 4) with PCOS as defined by the Rotterdam criteria (14). The control group (group C; n = 12) consisted of young (<35 years old), normoweight (BMI from 20 to 24.9 kg/m^2) oocyte donors. The male partner of the corresponding recipient was also normoweight, without a smoking habit, and had normozoospermia or a mild sperm factor, similar to those from the obese group.

All the women included in the study underwent the same controlled ovarian hyperstimulation (COH) with a short antagonist protocol, recombinant follicle-stimulating hormone (FSH) and human chorionic gonadotropin (hCG) triggering. Briefly, 150–300 IU/day of recombinant FSH (Gonal-F; Merck-Serono) was administered according to the woman's age, basal hormone values, ovarian pattern identified by ultrasound, and BMI. Gonadotropins were administered from day 3 of menstruation. Serial transvaginal ultrasound examinations and serum estradiol determinations were initiated on day 5 of the COH and repeated every 48 hours to monitor the ovarian response. From day 6 of COH until the day of hCG administration, a 0.25-mg dose of gonadotropin-releasing hormone (GnRH) antagonist (Cetrotide; Merck-Serono) was given daily. We administered hCG (Ovitrelle; Merck-Serono) subcutaneously when at least two leading follicles had reached a mean diameter of 18 mm. No previous contraceptive pill was given in any cases.

We excluded any cases in whom a low or high ovarian response occurred (<5 or >20 oocytes retrieved, respectively), or when the estradiol concentration was more than 3,000 pg/mL or fell by $\geq$20%, or more than 1 ng/mL of progesterone was detected on the day of hCG administration. Intracytoplasmic sperm injection (ICSI) was performed for fertilization. Women with fewer than two ongoing day-3 embryos were also ruled out. These embryos were then transferred, frozen, or left to reach the blastocyst stage for transfer or freezing.

Considering all the previously described inclusion and exclusion criteria, 12 women were accepted into group A, 4 into group B, and 12 into group C. Demographic (age, parity, and menstrual pattern) and anthropometric (weight, height, BMI, waist, hip, and waist-to-hip [WHR] ratio) parameters were recorded in these 28 patients, as well as their AFC. In the obese group, hormone and metabolic determinations (FSH, LH, estradiol, testosterone, androstenedione, insulin, glycemia, and antimüllerian hormone) were also evaluated in fasting conditions on days 2 to 4 of menstruation (either spontaneously achieved or induced by progesterone treatment with Utrogestan, 200 mg/day for 5 days [SEID]).

The study was approved by the institutional review board and ethics committee. It is identified at ClinicalTrials.gov with identifier NCT01448863.

Metabolomic Study

Cook Sidney IVF Cleavage Medium was used in this study. Microdrops of this media (50 μL) were in contact with IVF embryos from day 0 (after ICSI) to day 3 (72 hours). On day 3 of

embryo development, 50 μl of spent culture media was collected from the two best embryos in each cohort according to their morphologic criteria (Supplemental Fig. 1, available online). Therefore, 24 samples were taken for analysis in groups A and C, respectively, and 8 samples in group B, for a total of 56 embryos from 28 patients. In addition, one or two culture blanks per patient were also assessed (Supplemental Fig. 1, available online). The same procedure was used to collect these matched control media samples, consisting of microdrops of media cultured in the same conditions but without embryos. We analyzed 12 blanks for normoweight, 15 for obese, and 6 for obese PCOS patients.

The samples were centrifuged, frozen (at −80°C) and stored until the analysis was performed. The global metabolomic profile of the three study groups was compared by ultrahigh performance liquid chromatography coupled to mass spectrometry (UPLC-MS/MS).

Sample Preparation

The samples were defrosted, vortex mixed, and centrifuged. A 40-μL aliquot was then diluted with 150 μL of methanol and vortex mixed. Subsequently, the samples were centrifuged at 13,000 rpm at 4°C for 15 minutes, after which the supernatants were collected and transferred to microtubes for speed-vacuum drying. Finally, the samples were reconstituted with 50 μL of chromatographic mobile phase (water/methanol, 1:1 v/v), containing internal standards (i.e., phenylalanine-d5 4 μg mL^{-1}, reserpine 1 μg mL^{-1}, and leucine enkephalin 2 μg mL^{-1}); they were then centrifuged for a further 5 minutes and transferred to vials for UPLC-MS/MS global metabolic profiling.

UPLC-MS/MS System

Chromatographic analysis of samples was performed in an Acquity UPLC chromatograph using an Acquity UPLC HSS T3 (100 × 2.1 mm, 1.8 μm) analytical column from Waters Corporation. Autosampler and column temperatures were set to 4°C and 40°C, respectively, and the injection volume was 5 μL. A gradient elution with a total run time of 14 minutes was performed at a flow rate of 500 μL min^{-1} as follows: initial conditions of 100% of mobile phase A (ultrapure water containing formic acid (0.1%, v/v)) were maintained for 1 minute, followed by three linear gradients from 0 to 15% of mobile phase B (acetonitrile containing formic acid (0.1%, v/v)) for 2 minutes; from 15% to 50% of phase B for 3 minutes; and from 50% to 95% of phase B for 3 minutes; isocratic conditions of 95% phase B were held for 3 minutes; and finally a 0.5-minute gradient was used to return to the initial conditions (i.e., 100% of phase A), which were held for 1.5 minutes.

Mass spectrometry detection was performed using a QTOF SYNAPT HDMS spectrometer (Waters). The following electrospray ionization parameters were selected in the positive V mode (ESI+) with an extended dynamic range: capillary and cone voltages were set at 3.2 kV and 20 V; source and desolvation temperatures were set at 120°C and 380°C, respectively; flow rates of the cone and nebulization gases were set at 50 and 800 L h^{-1}, respectively. Full scan data were collected in the time-of-flight (TOF) MS mode from 50 to 1,000 mass to charge ratio (m/z) with a scan time of 0.1 seconds. A lock spray interface was used to maintain mass accuracy during the analysis. To this end, a 50 pg mL^{-1} solution of leucine enkephalin (m/z 556.2771) in ultrapure water/acetonitrile (1:1, v/v) containing formic acid (0.1%, v/v) was infused postcolumn for 150 ms every 10 seconds using an isocratic pump at a flow rate of 40 μL min^{-1}. MassLynx 4.1 (Waters) UPLC-MS/MS software was used for data acquisition.

Data Analysis

Raw UPLC-TOFMS chromatograms were converted into the .netCDF format using DataBridge (Waters). Peak detection, integration, and alignment of .netCDF data was performed using an in-house R-script across all samples, blanks, and quality controls (QC), providing a raw data matrix X0 (119 × 3111), with samples in rows and features in columns. The peak table was imported into MATLAB for data analysis. We detected 2,120 variables in blanks, and 440 with an RSD (relative standard deviation) greater than 25% in the QC samples were removed from the data set, leaving a total of 551 variables for further analysis.

Partial Least Squares Discriminant Analysis Modeling

Supervised orthogonal partial least squares discriminant analysis (PLS-DA) was performed using the nonlinear iterative partial least squares (NIPALS) algorithm, a maximum number of latent variables of 9, and autoscaling for X-block pretreatment. The y vector containing the class labels (i.e., 1 or 0 for {obese and obese-PCOS} and normoweight samples, respectively) was mean-centered.

Results were obtained from a PLS-DA model {normoweight} versus {obese and obese-PCO} using a calibration set including 34 samples from 19 patients and 542 variables. The sample set included replicates from the same patient. To avoid providing overoptimistic results during cross-validation of the PLS model, 19 CV-groups of samples were defined based on the patient to avoid the presence of samples from the same individual in the calibration and test sets. The number of misclassified samples (NMC = FalsePositives + FalseNegatives) was used to evaluate the model performance and to select the number of latent variables.

Nine variables from spiked internal standards were removed from the data set. After this, 171 differentiating variables were selected using a VIP (variable importance in the projection) score threshold value of 1. The VIP score is a predictor that summarizes the importance for the projections to find h LV, because the average of the squared VIP scores equals 1, and the "greater than one" rule is generally used as a variable selection criterion (15). VIP scores were calculated for all the variables included in our model, and those showing a VIP value greater than 1 (171 variables) were included for further PLS-DA analysis.

A second PLS model was generated using this subset of variables and the same parameters. The predictive

performance of the model was estimated from the results obtained from the validation set.

Identification of Metabolites

The molecular formula of selected metabolites was estimated based on the *m*/*z* values of the molecular ion and its isotopic profile. After identification of the molecular formula, the Human Metabolome Database (HMDB, http://www.hmdb.ca), the MassBank (http://www.massbank.jp), and the Lipid Maps (http://www.lipidmaps.org/) open databases were used to identify the putative metabolites, applying a mass tolerance of ±5 mDa or 5 ppm. Data preprocessing, and multivariate analysis was performed in R-Studio version 0.97.312 and MATLAB 2012a (Mathworks Inc.) using the PLS Toolbox 6.7 (EVRI) and in-house MATLAB scripts.

Statistics

Descriptive variable values were compared between normoweight, obese non-PCOS, and obese PCOS women. Data were presented as mean or proportion together with the 95% confidence interval (CI 95%). The results were analyzed using the Mann-Whitney *U* test for pairwise comparisons of quantitative variables, and the chi-square and Fisher's tests for comparison of categorical variables. $P<.05$ was considered statistically significant. Statistical analysis was performed using the Statistical Package for the Social Sciences 17.0 (SPSS Inc.). For the calculations of CI 95% of proportions or risk ratios we used Macro !CIP for SPSS. Multivariate analysis was used in the untargeted metabolomic study to discover the differential metabolites found in order to create a predictive tool, using principal component analysis (PCA) and (PLSDA) models.

RESULTS

As shown in Table 1, women in the control group (normoweight oocyte donors) were statistically significantly younger and slimmer, and had a lower BMI. Obese PCOS women had longer intermenstrual periods, exhibited larger waist circumferences, and showed higher WHR than the other two groups. In addition, they also presented a higher AFC and basal serum androstenedione concentrations. The LH and AMH values were higher but not statistically significantly different when compared with the other two groups. Obese non-PCOS women showed a lower ovarian response, with a reduced number of mature oocytes, embryos, and ongoing day-3 embryos obtained from them.

We found and quantified 3,111 analytes in the spent conditioned media from the embryos from the three different groups; 2,120 that were also detected in the blanks and 440 analytes with an RSD higher than 25% in the QC samples were removed from this data set, leaving only 551 that were considered for further analysis. These analytes were used to develop a PLS-DA model in which samples which came from normoweight women as well as those from obese women were grouped together (Fig. 1A). However, despite the fact that we found a trend toward a higher difference between samples coming from normoweight women and those from obese-PCOS women, no statistically significant

TABLE 1

Descriptive parameters of the study groups.

Parameter	Group A Obese no PCOS (n = 12)	Group B Obese PCOS (n = 4)	Group C Donors (n = 12)
Age (y)	34.9 (32.9, 36.9)	33.3 (26.9, 37.5)	26.8 (23.7, 29.9)[a]
Menstrual pattern (d)	29.5 (28.2, 30.7)	51.7 (0, 107.7)[a]	28.1 (27.3, 28.9)
Weight (kg)	88.1 (81.6, 94.5)	95.2 (90.9, 99.4)	60.3 (55.1, 65.6)[a]
Height (m)	1.6 (1.6, 1.7)	1.7 (1.6, 1.8)	1.6 (1.6, 1.7)
BMI (kg/m^2)	33.6 (31.9, 35.4)	34.3 (30.0, 39.0)	22.7 (21.4, 23.9)[a]
Waist (cm)	96.7 (91.7, 101.6)	107.0 (98.7, 115.3)[a]	
Hip (cm)	117.3 (110.9, 123.7)	117.0 (113.6, 120.4)	
WHR	0.83 (0.79, 0.86)	0.91 (0.85, 0.97)[a]	
No. of AFC	12.7 (8.5, 16.9)	26.3 (17.2, 35.2)[a]	15.2 (13.2, 17.1)
FSH (mIU/mL)	6.2 (5.2, 7.2)	6.6 (2.3, 10.4)	
LH (mIU/mL)	3.9 (2.7, 5.1)	8.5 (0, 19.87)	
E_2 (pg/mL)	27.9 (20.3, 35.5)	33.1 (8.8, 57.4)	
Testosterone (ng/mL)	0.6 (0.4, 0.7)	0.6 (0.4, 1.1)	
Androstenedione (ng/mL)	1.9 (1.2, 2.5)	4.3 (3.3, 5.4)[a]	
Insulin (μU/mL)	9.1 (6.0, 12.1)	13.3 (0–27.3)	
Glucemia (mg/dL)	90.8 (84.9, 96.6)	89.0 (82.4, 95.6)	
AMH (pmol/L)	11.3 (5.8, 16.8)	30.6 (0, 79.5)	
No. of oocytes	11.7 (8.4, 14.9)	16.3 (0, 33.2)	12.8 (10.7, 14.8)
No. of oocytes MII	8.2 (5.9, 10.4)[a]	12.5 (10.6, 23.9)	11.3 (9.9, 12.8)
No. of embryos	5.3 (3.8, 6.7)[a]	9.8 (0.5, 18.9)	8.8 (7.1–10.5)
No. of day-3 embryos	4.2 (2.8, 5.5)[a]	9.8 (0.5, 18.9)	7.9 (5.9, 99)
No. of embryos transferred	1.7 (1.3, 2.0)	1.5 (0, 2.0)	1.7 (1.3, 2.0)
No. of embryos cryopreserved	1.8 (0.8, 2.8)	6.5 (0, 14.5)	2.8 (1.4–4.3)

Note: AMH = antimüllerian hormone; BMI = body mass index; E2 = estradiol; LH = luteinizing hormone; MII = metaphase 2; PCOS = polycystic ovary syndrome; WHR = waist–hip ratio.
[a] Statistically significant comparison.

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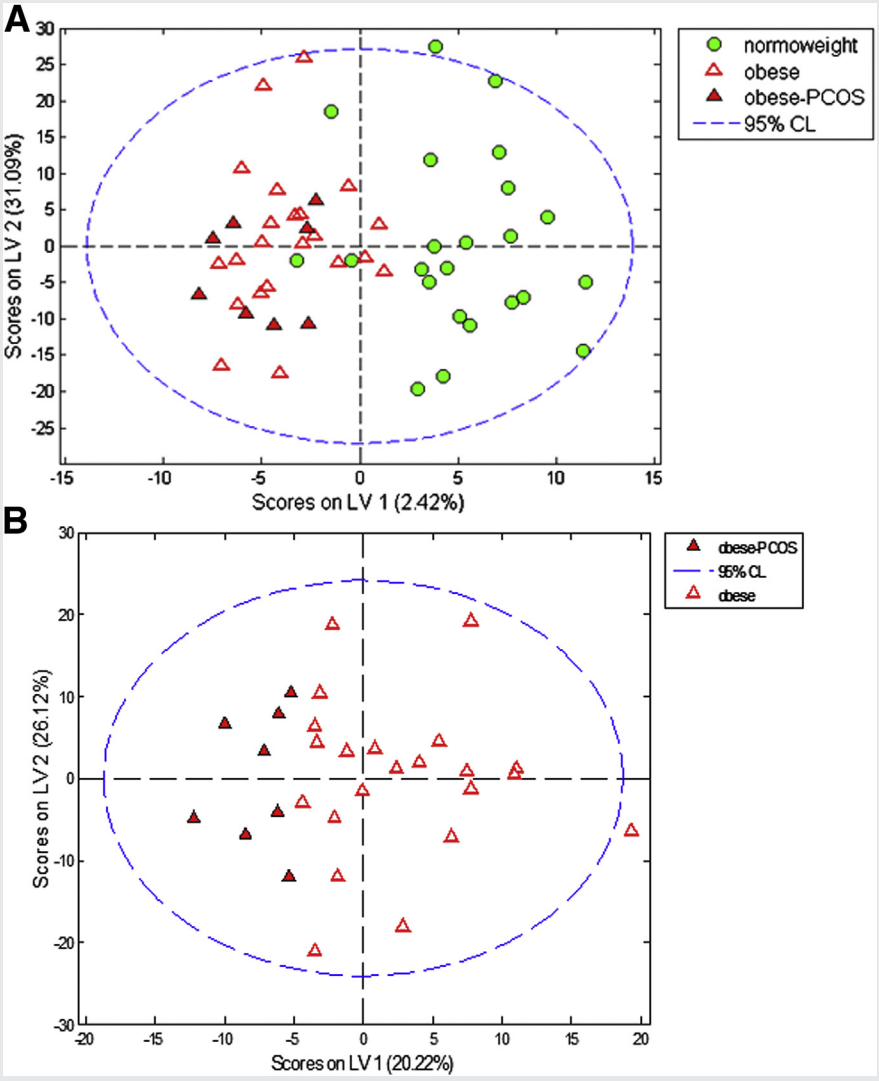
FIGURE 1



Partial least squares discriminant analysis of normoweight versus obese and obese-polycystic ovary syndrome (PCOS) samples. (**A**) Each point on the partial least squares discriminant analysis (PLS-DA) represents the 551 metabolites measured for each spent media sample analyzed. *Green circles* represent normoweight embryos, and *triangles* represent obese patient embryos (*red triangles* for obese PCOS, and *empty triangles* for obese non-PCOS patients). (**B**) PLS-DA of obese non-PCOS versus obese-PCOS samples. *Red triangles* represent obese PCOS, and *empty triangles* represent obese non-PCOS patients.

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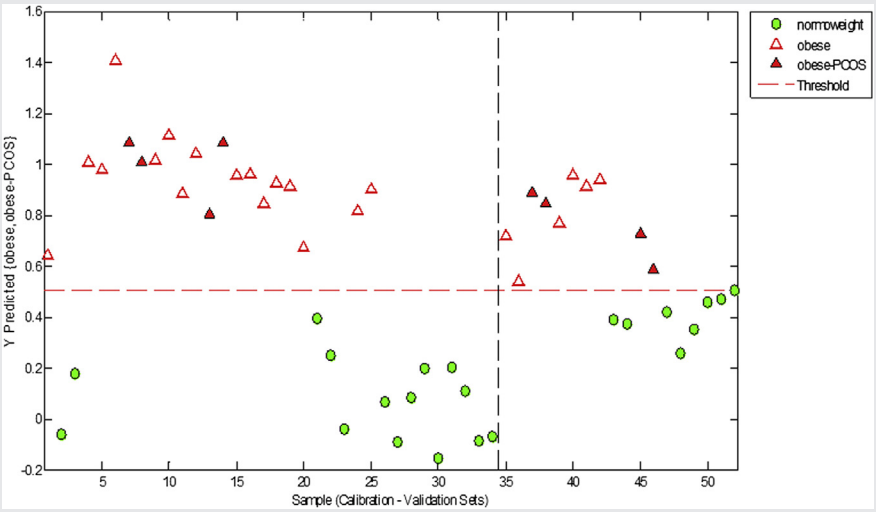
differences appeared. This is why we grouped together all the samples from obese women. We further confirmed this pattern when we analyzed obese versus obese-PCOS patients only (see Fig. 1B). A blank comparison among groups also showed no statistically significant differences (data not shown).

Considering the differences identified between groups, a PLS-DA regression model (predictive model) using the 171 variables with VIP scores higher than 1 was developed. This model was built using the 34 analyzed samples (20 obese and 14 normoweight) as a training set, and was then validated with another 18 samples: 8 normoweight and 10 obese. In this prediction model 87.8% of the samples (7 of 8) coming from normoweight women were identified correctly, as well as 100% of the samples (10 of 10) that came from obese women (Fig. 2).

From the data obtained from this model, we wanted to identify the metabolites that best separated these two populations (normoweight and obese groups). According to their exact mass and retention time, and by consulting the available databases (the HMDB, MassBank, and Lipid Maps), a hypothetical molecular formula was obtained, and in some cases these analytes could then be identified with a very low range of error. The quantification of these

FIGURE 2



Partial least squares discriminant analysis regression model of normoweight versus obese and obese-polycystic ovary syndrome (PCOS) samples. A total of 34 samples (20 obese and 14 normoweight) were used to calibrate the model (*left panel*), and 18 samples (7 normoweight, and 11 obese) were set aside for model validation (*right panel*). We correctly predicted 87.8% of the normoweight samples (7 of 8) and 100% of the obese samples (10 of 10).

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metabolites between normoweight and obese patients was also established. Table 2 shows the 30 metabolites with the highest VIP scores. From these 30 metabolites 13 were identified, and 9 of them were saturated fatty acids that were reduced in the spent culture media of embryos that came from obese women in comparison with the normoweight controls.

DISCUSSION

This study shows that the global metabolomic profile in the spent culture media of day-3 embryos is different in normoweight and obese women. Despite the fact that a trend was observed, we failed to demonstrate clear differences between obese PCOS and obese non-PCOS women.

Obesity is a general disease that affects all the tissues and organs in the body. The reproductive system is not an exception. In fact, many studies have demonstrated an impaired reproductive outcome in both obese men and women, and in both natural and assisted conception cycles (2, 5, 16–18). Several systemic hormonal and metabolic alterations have been described in obese patients as well as local disturbances at the level of the gonad, which may explain these described outcomes (19, 20). In addition, recent studies have shown that weight reduction or simply regular exercise may reverse these negative effects, thus increasing conception and ongoing pregnancy rates (21, 22). However, studies that have tried to ascertain the exact mechanisms by which oocytes, embryos, and endometrial receptivity are affected have not yet reached a consensus.

Some investigators have described morphologic differences in oocytes and/or embryos that come from obese women compared with normoweight women, but they fail to agree on the specific alterations observed. Others have shown similar morphologic parameters in normoweight and obese women, either by static assessment or dynamically using time-lapse analysis (5, 10–13). In fact, it has been claimed that the classic morphologic criteria commonly used in IVF laboratories to select the best embryos for transfer are not useful for determining the real damage that obesity exerts on gametes and embryos (5, 23), which has led to the development of new lines of research to address this issue.

Metabolomics studies the dynamic inventory of metabolites, using them as small molecular biomarkers to represent functional phenotypes in biological systems (24). Low-molecular-weight metabolites are final products of cell regulatory processes and can thus reflect biological changes in early embryo metabolism (25). Metabolism is intrinsic to the health of the embryo, and many studies have focused on identifying noninvasive metabolic markers associated with the developmental ability of embryos. This would allow researchers to obtain biochemical fingerprints that may be useful for biological classification or for developing specific diagnostic methods. Special interest has focused on oxidative phosphorylation, Na /K -ATPases, redox reactions, and amino acid metabolism (26–30). With regards to assisted reproduction, evaluating cultured embryos might highlight different metabolic markers and profiles, providing quantitative parameters that we may be able to link to their implantation capacity or might reflect their pathological status (31). In addition, this is a noninvasive technique based on sampling the metabolites secreted into, or consumed from, the culture media by embryos during in vitro development.

TABLE 2

The top 30 variables sorted by the highest variable importance in projection scores in our partial least squares discriminant analysis model.

Variable (mass/Rt)	Molecular formula	Error (mDa)	ID	Ratio mean (normoweight/obese)
261.9242–0.44	NA	NA	NA	0.85
146.0272–0.56	NA	NA	NA	3.20
218.211–4.87	[C12H28NO2]+	−0.4	[Isobutyl octanoate+NH4]+[a]	1.93[a]
334.2956–5.83	[C18H40NO4]+	1.4	[9,14-dihydroxy-octadecanoic acid+NH4]+[a]	2.26[a]
295.8785–0.44	NA	NA	NA	0.78
318.301–7.07	[C18H40NO3]+	0.9	[Hydroxy-octadecanoic acid+NH4]+	0.59
220.2192–4.86	NA	NA	NA	1.95
263.2432–4.97	ISOTOP. [C14H32NO3]+	0.3	[Hydroxy-tetradecanoic acid+NH4]+[a]	1.83[a]
262.2378–4.97	[C14H32NO3]+	0.3	[Hydroxy-tetradecanoic acid+NH4]+[a]	1.72[a]
696.8464–0.41	NA	NA	NA	1.41
442.8782–0.38	NA	NA	NA	1.50
306.2646–5.05	[C16H36NO4]+		[4,12-dihydroxy-hexadecanoic acid+NH4]+[a]	1.88[a]
703.9518–3.94	NA	NA	NA	1.76
150.0092–0.46	[C4H5N3OK]+	−2.2	NA	0.88
307.2682–5.05	ISOTOP. [C16H36NO4]+		[4,12-dihydroxy-hexadecanoic acid+NH4]+[a]	1.77[a]
246.2424–5.71	[C14H32NO2]+	−0.5	[trymethylundecanoic+NH4]+[a]	1.60[a]
185.1153–4.8	[C8H18O3Na]+	<0.1	NA	1.52
563.4292–9.73	NA	NA	NA	1.47
284.2209–4.97	[C14H31NO3Na]+	−1.0	[Hydroxy-tetradecanoic acid+NH4-H+Na]+[a]	1.57[a]
636.9962–5.04	NA	NA	NA	0.27
147.0768–0.51	[C5H11N2O3]+	−0.2	[Glutamine+H]+	0.68
183.0768–0.51	NA	NA	NA	0.69
832.832–0.41	NA	NA	NA	1.24
158.0811–3.71	NA	NA	NA	3.55
163.1079–0.32	[C6H15N2O3]+	−0.3	[Hydroxylysine+H]+	1.35
218.1135–0.51	[C8H16N3O4]+	−1.0	NA	0.65
126.093–0.37	[C7H12NO]+	−0.3	NA	0.82
290.2697–6.49	[C16H36NO3]+	1.0	[Hydroxy-hexadecanoic acid+NH4]+	0.72
764.198–5.04	NA	NA	NA	0.26
290.2691–5.77	[C16H36NO3]+	1.0	[Hydroxy-hexadecanoic acid+NH4]+[a]	1.58[a]

Note: Each variable is defined by its exact mass and retention time. When available, the molecular formula and identification is provided. The ratio mean between both groups (normoweight versus obese) is also shown. Positive values higher than 1 indicate more abundance in normoweight embryos whereas positive values <1 represent higher abundance on obese embryos. NA = not applicable.

[a] Indicates a lipid with positive values higher than 1.

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Different metabolomic parameters have been measured in developing embryos using noninvasive techniques. In 2007, Seli et al. (32) published the first study using a metabolomic approach to assess the viability of human embryos. In a follow-up study, viability markers were identified using a nuclear magnetic resonance (NMR) metabolic platform which identified statistically significant differences in glutamate concentrations in the spent-media from embryos that resulted in pregnancies and/or births and those that ended in implantation failure (33). Other recent studies have identified genes, proteins, and metabolomic profiles that can detect which oocytes or embryos are viable, and have used these characteristics to measure their implantation capacity (34–36).

We decided to use a metabolomic approach by UPLC-MS/MS because it is currently the most sensitive technique to detect analytes differences in the spent media samples. This technique has now been used even for single-cell analysis (37). In our analyses, which used the same culture media for all of our experiments, a different global metabolomic profile was observed between spent media from embryos coming from normoweight and obese groups. In fact, samples from these two groups tended to group separately in the PLS-DA model. Samples from obese-PCOS women tended to group further away from those of normoweight controls, meaning that embryos from this obesity subgroup had an even more disturbed metabolism than those derived from non-PCOS obese women, although perhaps due to the small sample size of this group, this difference was not statistically significant. Therefore, we grouped obese PCOS and non-PCOS women together before using this data to develop the predictive model. The most representative metabolites found between the obese and normoweight groups were used to create a predictive model using PLS-DA regression. This model was then validated using a separate data set and showed high prediction values, meaning that we could identify the origin of the sample with a high degree of accuracy.

Metabolites are characterized by a specific mass and retention time, but most of them are not currently found in the appropriate online databases. In this study a hypothetical molecular formula could be obtained for 13 out of the 30 metabolites with a high VIP score. Nine out of the 13 metabolites that we identified were saturated fatty acids, and these were statistically significantly increased in samples from embryos coming from normoweight women in comparison with those from obese women.

Fatty acids are widely accepted as potential metabolic substrates for oocytes and early embryos (38). Furthermore, it has been recently shown (39) that nonesterified fatty acids

are retained by bovine oocytes before and during meiotic maturation, and that modification of the fatty acid composition in bovine IVM medium led to significant changes in how well the resulting embryos tolerated cryopreservation (40). In one of very few reports to examine the human lipid uterine fluid composition, Vilella et al. (41) found that fatty acids were indeed present in human uterine secretions and could be good predictors of implantation.

There is only one very recently published study which assesses embryo metabolomics in obese women (13). In this study, glucose consumption was reduced, amino acid metabolism was modified, and endogenous triglyceride levels were increased in blastocysts from overweight women. However, this was a retrospective study in which very few patients were obese (BMI $\geq$ 30 kg/m^2), and the blastocysts analyzed came from supernumerary embryos that had been donated for research and thus were not the best ones in the cohort.

In our present study only obese women were included prospectively, following very strict selection criteria. Moreover, good quality embryos (the best two from each cohort) were analyzed. These specific selection criteria were the reason why not many obese patients (n = 16) could be included in our study. Nevertheless, the number of obese women was higher than those considered in the metabolic study published by Leary et al. (13) in which only 2 obese and 5 overweight women were assessed in the CORE research project. Despite this, there are some similarities between our findings and those obtained by Leary et al.; they suggest that the metabolic changes they described for their blastocysts may be a consequence of an abnormal oocyte environment in women with excess weight. One of these alterations, the reduction of glucose consumption by blastocysts, may be due to increased β-oxidation, which causes a reduction in glycolysis via elevated cytosolic citrate levels which inhibit phosphofructokinase. Although our study pertains to day-3 embryos and thus a stage previous to that of the Leary study, we speculate that the reduction in saturated fatty acids released to the culture media that we observed from embryos that came from obese women may be similarly related to increased β-oxidation.

The main limitation of our study was the small sample size of patients included (especially in the obese-PCOS group) due to the strict inclusion criteria that we applied. Therefore, larger sample sizes will be required to confirm or refute our findings and to more definitively determine whether the spent culture media of embryos from obese-PCOS and obese-non-PCOS women present a different metabolomic profile. Another limitation of our study was that the control group did not comprise infertile women, so we cannot discard the specific role of infertility itself in the results obtained. Similarly, the younger age of the control group may have had an influence. Thus, a further analysis including infertile normoweight women in the control group would also be advisable.

In summary, this study showed that the spent culture media from embryos that came from obese women had a different metabolomic profile compared with controls; more specifically, there was a statistically significant reduction in the saturated fatty acid content. This metabolic alteration may explain some differences in embryo function that could be responsible for the poorer results observed in women with weight excess after assisted reproduction, although further studies with larger sample sizes are needed to confirm these findings.

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SUPPLEMENTAL FIGURE 1

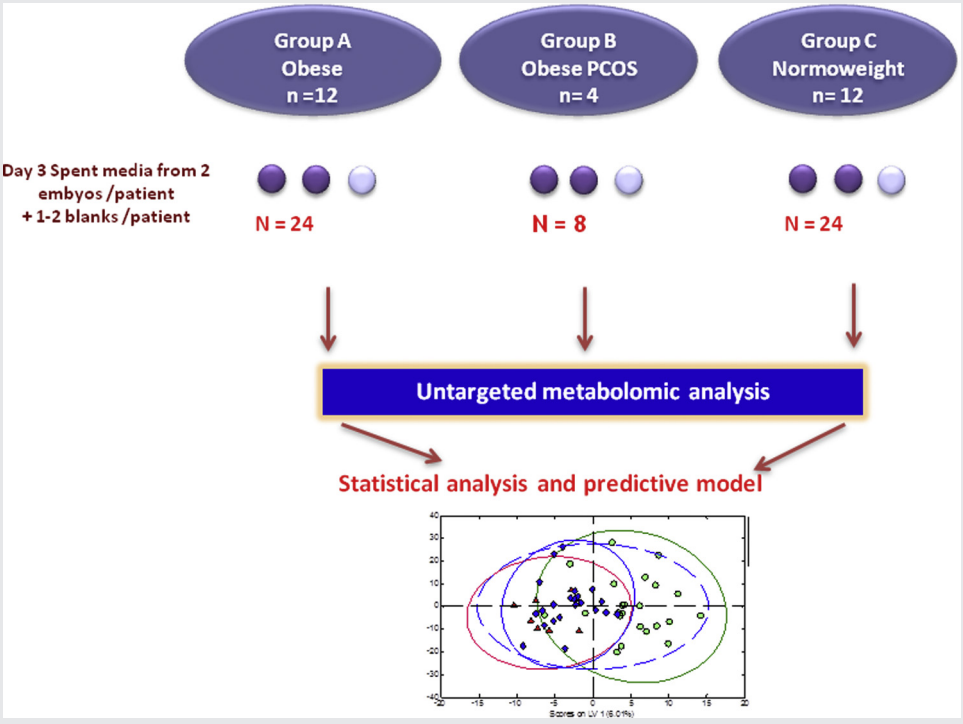

Diagram of the study design.

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