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ORIGINAL ARTICLE

Lipid profiling of follicular fluid from women undergoing IVF: Young poor ovarian responders versus normal responders

THAIS CATALDI[1], FERNANDA BERTUCCEZ CORDEIRO[1], LÍVIA DO VALE TEIXEIRA DA COSTA[1], EDUARDO JORGE PILAU[4], CHRISTINA RAMIRES FERREIRA[3], FÁBIO CESAR GOZZO[2], MARCOS NOGUEIRA EBERLIN[3], RICARDO PIMENTA BERTOLLA[1], AGNALDO PEREIRA CEDENHO[1] & EDSON GUIMARÃES LO TURCO[1]

[1]*Department of Surgery, Division of Urology, Human Reproduction Section, Sao Paulo Federal University, Sao Paulo, SP, Brazil,* [2]*Dalton Mass Spectrometry Lab, Institute of Chemistry, University of Campinas, UNICAMP, Campus Universitario Zeferino Vaz SN, Campinas, SP, Brazil,* [3]*ThoMSon Mass Spectrometry Laboratory, Institute of Chemistry, University of Campinas, UNICAMP, Campus Universitario Zeferino Vaz SN, Campinas, SP, Brazil, and* [4]*Departament of Chemistry, University of Maringá, Jardim Universitário Maringá, Maringá, PR, Brazil*

Abstract
This study identified possible lipid biomarkers in follicular fluid from women with poor ovarian response. These biomarkers indicate pathophysiological pathways and have potential diagnostic applications. An observational case-control study of young women undergoing ovarian stimulation for in-vitro fertilization was conducted. The participants were categorized into a poor ovarian response group and a normal ovarian response to stimulation group. All of the women underwent the same ovarian stimulation protocol, and follicular fluid was collected after ovarian aspiration. Analyses were performed using matrix-assisted laser desorption/ionization mass spectrometry. Principal component analysis and Volcano plots were used to describe follicular fluid classification models based on the lipid profiles. A total of 10 lipids were differentially expressed between the study and control groups. Of these lipid ions, three belonged to the phosphatidylcholine subclass and were present in higher concentrations in the control group. The other seven differential lipids were present in the study group and classified into four lipid subclasses: phosphatidylethanolamines, phosphatidylglycerols, phosphatidylinositols, and diacylglycerols. These distinctive lipids may be involved in hormonal responses and oocyte development processes and may be useful as biomarkers for therapeutic intervention in women with poor ovarian response.

Keywords: *Poor ovarian response, in-vitro fertilization, follicular fluid, lipidomics, MALDI-MS*

Introduction

The success of in-vitro fertilization (IVF) and other assisted reproductive technologies critically depends on the success of ovarian stimulation protocols to obtain high-quality oocytes. It is critical to individualize hormonal stimulation according to aspects such as the chronological age, ovarian reserve, and endocrine status of the patients (Kligman & Rosenwaks, 2001).

In assisted reproduction, poor ovarian response (POR) poses a challenge regarding ovarian stimulation and oocyte quality and quantity an disassociated with low pregnancy rates (Padhy et al., 2010).

According to the Bologna criteria, POR is diagnosed using minimal criteria of which at least two of the following three features are present: (i) advanced maternal age ($\geq$ 40 years); (ii) abnormal ovarian reserve test; and/or (iii) a previous episode of POR ($\leq$ 3 oocytes for stimulation). Furthermore, only two episodes of POR after maximal stimulation are sufficient to classify a patient as a poor ovarian responder (Ferraretti et al., 2011).

Women designated as poor ovarian responders usually present secondary clinical features, such as low levels of serum estradiol E2 ($\leq$ 500 pg.L^{-1}) and/or few mature oocytes (MII) ($<$ 4 dominant follicles) on the day of human chorionic gonadotropin (hCG) administration. Women with POR have significantly reduced pregnancy rates (approximately 2–4%) and constitute approximately

Correspondence: Edson Guimarães Lo Turco, DVM, PhD, Department of Surgery, Division of Urology, Human Reproduction Section, Sao Paulo Federal University, Prof. Joao Arruda Street, 168 ap 52, Zip Code 05012-000, Sao Paulo, SP, Brazil. Tel: + 55(11) 7971-0809. Fax: + 55(11) 5576-4611. E-mail: edsonlt@gmail.com

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9–24% of women undergoing IVF with standard ovarian stimulation (Aghahosseini et al., 2011).

Several hypothesis have been proposed to explain POR, including insufficient follicular blood flow (Battaglia et al., 2000), cytokine and growth factor network dysfunctions (Neulen et al., 2001; Salmassi et al., 2005), and the presence of ovarian auto-antibodies (Luborsky et al., 2002); however, none of these propositions is well-understood.

Lipidomics is an area of study that allows the characterization of molecular species of lipids and their biological role in cellular metabolism (Lagarde et al., 2003). Initially, lipids were considered important mainly for their roles as membrane components or energy reserves. However, with the development of a lipidomics approach, the knowledge of these molecules has expanded, introducing more information about their role in the cellular microenvironment (Roberts et al., 2008). This knowledge has emerged from the development of increasingly sensitive, selective analytical techniques, particularly those based on mass spectrometry (MS) (Roberts et al., 2008; Ejsing et al., 2009). Among these techniques, matrix-assisted laser desorption/ionization MS (MALDI-MS) (Karas & Hillenkamp, 1988) has been successful in studying the compositions of lipids and other crucial biological molecules. MALDI-MS has also allowed the direct analysis of complex, unfractionated samples (Fuchs et al., 2010) and of intact organs, such as a single embryo (Ferreira et al., 2010).

A good strategy to analyze oocyte quality may be based on the lipid biomarkers present in the follicular fluid (FF) recovered with the oocytes, as this fluid represents the oocyte microenvironment (Schweigert et al., 2006). In the present study, we compared MALDI-MS lipid fingerprints in the FF of young poor responder women in comparison with normal responders. Multivariate and univariate statistics were used to indicate prospective biomarkers for predicting ovarian response after hormonal stimulation. These biomarkers may be useful in therapeutic interventions for women with poor ovarian response.

Materials and methods

The study was performed at the Center for Research in Urological Sciences at São Paulo Federal University. This prospective study received Institutional Review Board approval from the São Paulo Federal University Research Ethics Committee.

This study was conducted throughout the year 2010. Eighteen FFs were collected from women who underwent IVF treatment using intracytoplasmic sperm injection (ICSI).

The study group consisted of six FF samples from young poor ovarian responders (the POR group); a group composed only of women who fitted the European Society of Human Reproduction and Embryology (ESHRE) consensus (considering that all patient presented ≤ 3 oocytes for stimulation and a previous episode of poor response) (Ferraretti et al., 2011). The control group (normal ovarian responders, the NR group) consisted of 12 FF samples from normal responders who had no evidence of female infertility and had achieved a pregnancy. In these cases, the couple sought treatment mainly for male fertility issues (a requirement for at least 3 million sperm.ml^{-1} and over 4% strict morphology) (Kruger et al., 1986).

In the study, only women who are 35 years old and younger were considered in order to provide a more accurate result for young poor ovarian responders. In other words, for both groups, the inclusion criteria were a maximum age of 35 years old, and all embryo transfers were performed on Day 3. Exclusion criteria were patients with a history of polycystic ovary syndrome, cancer, endometriosis, and/or other gynecologic factors leading to infertility. In order to improve the poor responders group definition, we included women with less than four follicles present in both ovaries on the day of hCG administration and low antral follicle count (<5 follicles) in the early follicular phase, while normal ovarian response was defined as 5–15 follicles on that day.

For both groups, a minimal semen quality of 5 million sperm.ml^{-1} with at least 4% normal morphology under strict analysis (Kruger et al., 1986) was necessary to exclude potential seminal alterations that could influence in pregnancy. The sperm analysis was performed during the cycle itself using a prepared sample. Clinical diagnosis and pathological reports for all of the patients were obtained from São Paulo Federal University, São Paulo, Brazil.

Ovarian stimulation, oocyte collection, and FF sampling

For all patients, controlled ovarian stimulation was achieved using exogenous recombinant gonadotropins (225 IU/day of rFSH - Gonal-F, Merck-Serono, Darmstadt, Germany) starting on cycle Day 2. When the leading follicle reached 13 mm in diameter, endogenous Luteinizing Hormone (LH) release was suppressed using a GnRH antagonist analog (250 μg/day of cetrorelix acetate [Cetrotide®]; Merck-Serono, Darmstadt, Germany) until the day of hCG administration. When the leading follicle reached 17 mm in diameter, a total dose of 250 mg of hCG (Ovidrel®; Merck-Serono, Darmstadt, Germany) was administered. Ultrasound-guided transvaginal oocyte retrieval was performed 35 h after hCG administration using a 16-gauge needle. The oocytes were isolated from the FF for evaluation and culture.

The remaining FF from each patient was pooled, centrifuged for 10 min at $800 \times g$ to remove residual cells and stored at -20°C until lipid analysis. Oocytes were incubated in culture medium (SSM Ivine Scientific—Santa Ana, CA) for 3 h until the ICSI procedure. Fertilization was evaluated at 18-h post injection and embryo quality at 48 and 72 h. Embryos with the highest morphology scores were transferred to the patients' uterus, with an average number of 2.8 embryos for the NR group and 1.75 embryos for the POR group.

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Lipid extraction

Lipids were extracted based on the Bligh and Dyer protocol (Bligh & Dyer, 1959). Briefly, 300 μL of FF was placed in a microtube, and 375 μL of chloroform and 750 μL of methanol were added and the homogeneous mixture vortexed for 2 min. The polar and apolar phases were separated by addition of 150 μL of water and 187.5 mL of methanol. The mixture was vortexed for 1 min and centrifuged at 3,000xg for 1 min. The lower phase containing the lipids was recovered and transferred to a clean microtube, which was left open at room temperature for 6 h until the solvent gets evaporated.

Mass spectra acquisition and data processing

The lipid extract was resuspended in 10 μL of chloroform, and 1 μL was deposited onto a MALDI sample plate. 2,5-dihydroxybenzoicacid in methanol (DHB 0.5 M, Sigma-USA) was used as the organic matrix for the MALDI-MS.

Mass spectra were acquired using a quadrople-Time of flight Premier mass spectrometer (Synapt G1, Waters, Manchester, UK) equipped with a 200-Hz solid state laser in the *m/z* range of 700–1200 in the reflectron mode using the positive ion mode ionization. The operating conditions were 250 a.u. (laser energy), 10 kV (sample plate), and laser irradiation consisting of diverse shots over a 45-s time period.

The MALDI-MS raw data were imported to the MarkerLynx Applications Manager Version 4.1 (Waters, Manchester, UK). The parameters used were *m/z* range 700–1200 Da, mass tolerance 0.03 Da, isotopic peak exclusion, and noise elimination level at 10.0 ion counts, and the minimum intensity was set to 15% of the base peak intensity. A table of relevant *m/z* values for both experimental groups was exported to Microsoft Excel, and the ions normalized using the ratio of the constitutive ion that was present in each spectrum of the *m/z* 782.5676) MS analysis. To obtain consistent differential variables, the resulting matrix (patient *versus* ion intensity) was further reduced by removing peaks with missing values (ion intensity = 0) above 50% in both groups.

Statistical analysis

The MALDI-MS data sets were analyzed and validated separately using univariate and multivariate statistics. The statistical analysis was performed using the online software Metaboanalyst (http://www.metaboanalyst.ca/MetaboAnalyst/faces/Home.jsp) and a matrix constructed with a list of detected ions. All variables were normalized in the software by Pareto scaling.

A principal component analysis (PCA) was conducted to visualize the lipid differences between the NR and POR groups after means centering and unit variance scaling.

Table I. IVF performance data during the cycle in Poor Responders and Normal Responders.

	Group				
	NR		POR		
	Means	SD	Means	SD	P value
Age years	31.42	1.730	31.83	2.639	0.691000
Basal FSH IU/ml	6.61	1.88	6.65	1.680	0.968000
Endometrial thickness	11.20	.520	12.40	1.556	0.278000
Number of follicles	9.42	2.712	3.00	1.265	0.000054
Number of retrieved oocyte	5.83	2.517	2.50	1.225	0.007871
Oocytes fertilizated	3.00	1.944	1.75	1.708	0.284800
Number of transferred embryos	2.80	1.573	1.75	.957	0.154370

In addition to the multivariate approaches, the univariate method (Student's *t*-test and fold change) were performed to measure the significance of each lipid in distinguishing the POR and NR groups. The fold change threshold (x10) and a significance under 0.1% were adopted. To assess whether the lipid species highlighted in the loading scores were statistically significant for classifying FF, a volcano analysis was performed.

The lipids obtained via MALDI-MS analysis were identified using web-based resources, such as the Human Molecular Database (http://www.hmdb.ca/).

Results

Initially, the groups were evaluated in terms of clinical data (Table I), including the number of follicles, oocytes retrieved, and the percentage of MII. These results demonstrated that the study group had a statistically significant difference in the number of follicles and oocytes retrieved compared with the control group. Furthermore, in the control group, 5 of the 12 patients became pregnant, representing a pregnancy rate of 42% and in the POR group, two of the six patients became pregnant, representing a pregnancy rate of 33%.

A total of 286 ions were observed in the lipid fingerprint spectrum acquisition in the positive ion mode. Figure 1 shows the representative mass spectra for each group. The mass spectra gave low signal/noise ratios, and the most ions were concentrated in the region of *m/z* 800–900.

PCA is a statistically unsupervised method commonly used for reducing the dimensionality of a multivariate data set while retaining most of the original information content.

To explore the natural interrelation between the NR and the POR group, PCA was used for the MALDI–MS data analysis, with data normalization which included the relative ion intensities and *m/z* values. PCA was performed on the dataset and indicated a trend of intergroup separation on the scores plot. Figure 2 shows a projection of a screen plot used to visualize the PCA results and indicates that the three first principal

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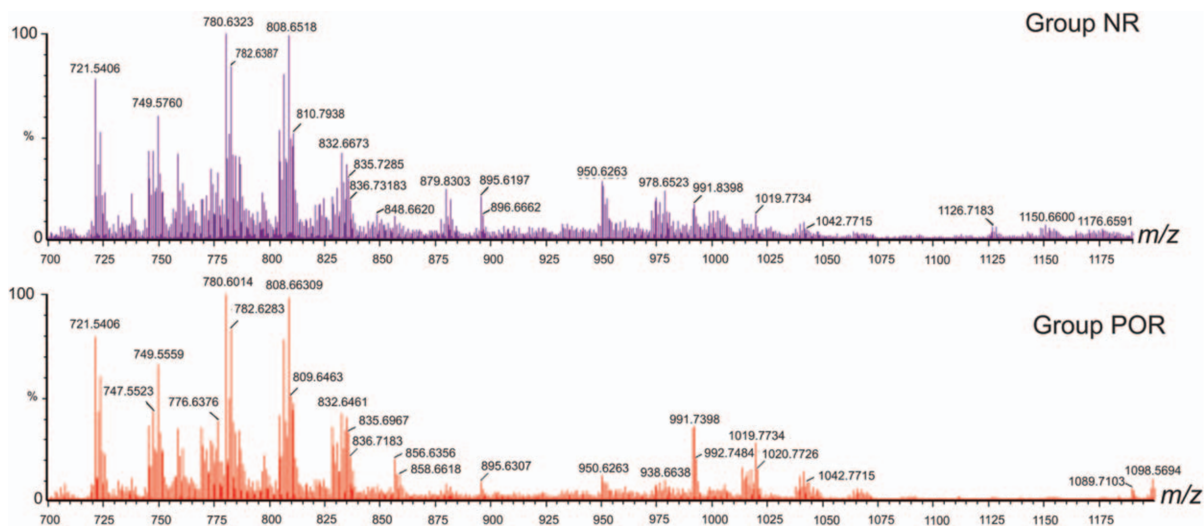

Figure 1. Typical MALDI-MS data for the POR and NR groups. The y-axis shows relative abundances whereas the x-axis shows m/z values. Note that cationic lipids are detected in their natural cationic forms whereas neutral lipids were detected in their protonated forms [M + H]+.

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components (PC1, PC2, and PC3) explained 87.6% of the total variance of the data, indicating the homogeneity of the data analyzed.

Figure 3 shows the 3-D score plot in which the positive value scores for PC1 efficiently separated the POR group from the NR group. The separation was indeed unequivocal, but two patients who were initially classified in the POR group were positioned in the NR group on this plot. This chemical classification via lipid profiles may be related to the fact that these two women became pregnant, despite their classification in the POR group.

A total of 10 overexpressed ions were observed (Figure 4). From these ions, Tables II and III were constructed with the ions (*m*/*z*), mass error in parts per million (ppm) and the lipid subclasses identified. Three of these ions, *m*/*z* 838.6785, 858.5918, and 726.5031, belong to the phosphatidylcholine (PC) subclass in protonated forms and were more abundant in the NR group. The ions corresponding to possible lipid biomarkers for poor responders came from four main lipid subclasses: phosphatidylethanolamines (PE), phosphatidylglycerols (PG), phosphatidylinositols (PI), and diacylglycerols (DAG). All of the lipids identified in this study belonged to the phospholipid subclass, which was expected because of the data acquisition conditions (DHB matrix and positive ion mode, Ferreira et al., 2010). The mass error in ppm was calculated for all of the lipids found and was considered only when the mass error was ≤ 50 ppm.

In addition to PCA, fold change threshold ($\times 10$) analysis and Student's *t*-test ($p < 0.001$) were performed. Only ions that were presented in both were considered for the Volcano plot. The results obtained from the Volcano plot were found to be in good agreement with those obtained from PCA.

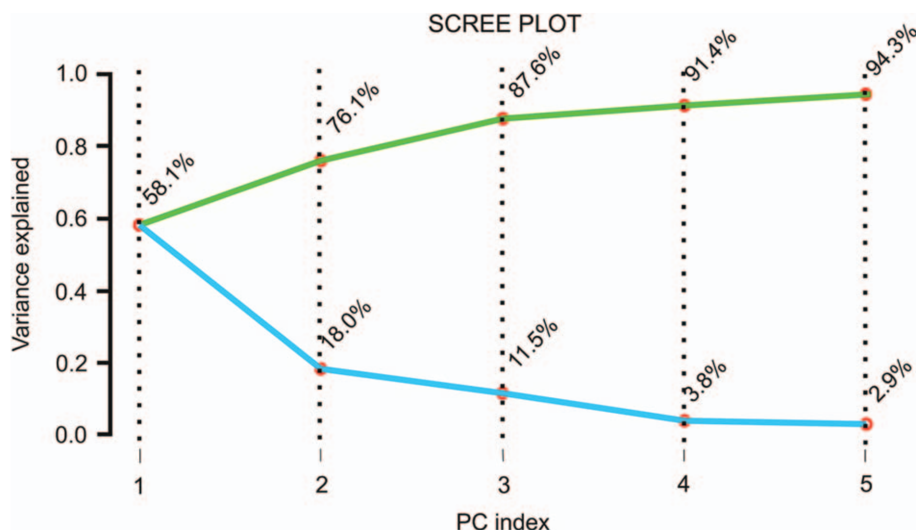

Figure 2. Scree plot shows the variance explained by PCs. The green line on top shows the accumulated variance explained; the blue line underneath shows the variance explained by individual PCs. The first three principal components explain as much as 87.6% of the total variance. It is clearly observed the grouping into two well-defined classes.

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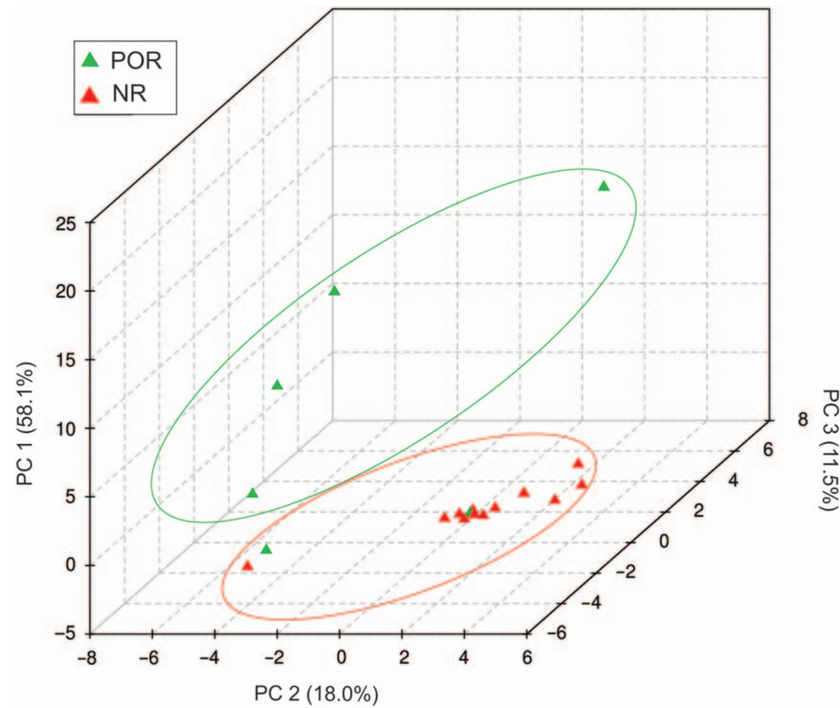

Figure 3. 3D score plot between the selected PCs. In y-axis we have PC1, in x-axis we have the PC2 and in the z-axis we have PC3, and the explained variances are shown in brackets. The green ellipse corresponds to the definition of poor responder group and the red ellipse indicates the normal population of patients who become pregnant.

These crucial lipids are known to be involved in numerous biological and molecular processes, including hormonal responses, oocyte production and quality, apoptosis, and cell proliferation.

Discussion

We investigated the FF of IVF patients using MALDI-MS with the aim of identifying lipid profiles and possible lipid biomarkers of young poor ovarian responders with high gonadotropin requirements, a reduced number of follicles, and few oocytes at retrieval (Kligman & Rosenwaks, 2001). Poor ovarian responders pose a specific challenge during assisted reproduction and represent a considerable proportion of the infertile population since poor response to IVF yields fewer oocytes and is associated with a poor pregnancy outcome (Padhy et al., 2010).

The patients included in this study were young women, when the condition of POR may be defined

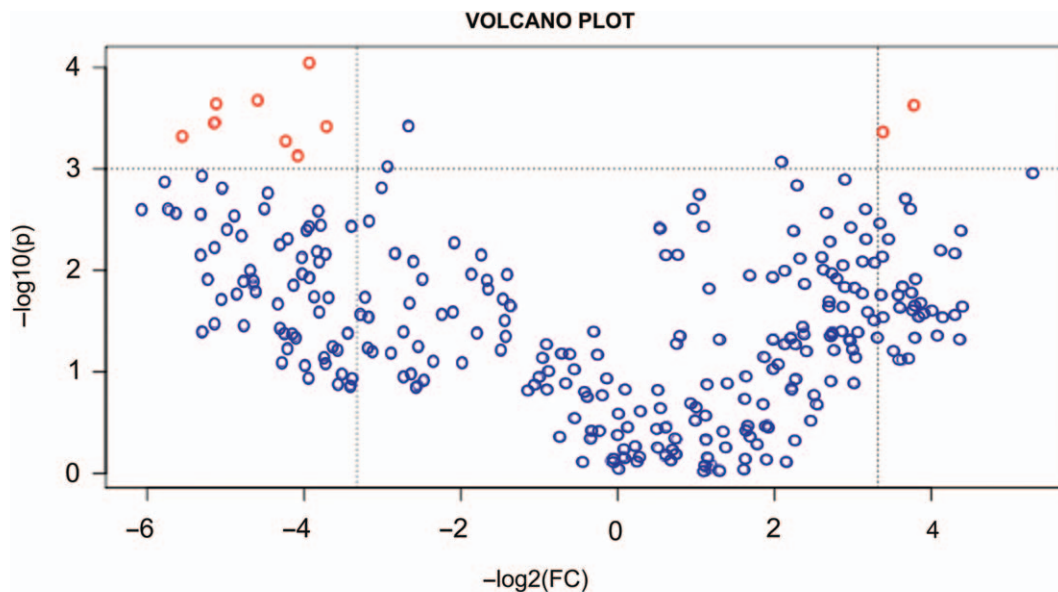

Figure 4. Most important ions selected by volcano plot (*red circles*). The fold change threshold (x) 10 and (y) Student's T-test ($p < 0.001$).

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Table II. Ions identified by volcano analysis in NR group. The ions were related to lipid subclass and mass error.

Peaks (*m*/*z*)	FC	p value	Lipid subclass	Error (ppm)	Molecular formula	Molecular structure
838.6785	13.839	0.00023367	PC	55	$C_{48}H_{88}NO_8P$	
858.5918	10.445	0.00043336	PC	10	$C_{50}H_{84}NO_8P$	
726.5031	0.059239	0.00075055	PC	5	$C_{40}H_{72}NO_8P$	

as premature ovarian failure, which can be caused by different factors and possibly lead to early menopause, demonstrating characteristics of ovarian senescence (Gleicher et al., 2009).

In addition to these characteristics, there are many other terms used in studies which make the comparison of results of treatments difficult (Tarlatzis et al., 2003; Devroey et al., 2009). Since there is no universal definition of poor responder and because of the need for a uniform definition, the Bologna criteria established that patients with a reduced number of oocytes retrieved would be defined as poor ovarian responders (POR). Other criteria associated with this medical definition of POR include the age and the number of antral follicles (Ferraretti et al., 2011).

In spite of the limited number of samples, rigorous selection criteria for samples were used as well as conservative statistical analysis (fold change 10X and $p < 0.001$) to make the conclusions more robust.

To the best of our knowledge, this study is the first to report the lipid profile, or lipid *fingerprinting*, of human FF samples from patients with poor response to controlled ovarian stimulation. The lipids identified have not been previously described in FF, nor have they

Table III. Ions identified by volcano analysis in POR group. The ions were related to lipid subclass and mass error.

Peaks (m/z)	FC	p value	Lipid subclass	Error (ppm)	Molecular formula	Molecular structure
834.4541	0.065653	8.9728e-05	PE	60	$C_{42}H_{80}NO_8P$	
811.4649	0.041478	0.00021038	PG	29	$C_{42}H_{77}O_{10}P$	
716.5332	0.028884	0.00022626	PE	14	$C_{39}H_{74}NO_8P$	
807.4682	0.02865	0.00034556	PI	41	$C_{41}H_{75}O_{13}P$	
739.5157	0.076214	0.00038075	DG	15	$C_{47}H_{72}O_5$	
844.4166*	0.021421	0.00047434	–	42	–	–
706.5328	0.053227	0.00052618	PE	8	$C_{38}H_{76}NO_8P$	

*This lipid subclass was not defined by HMDB database.

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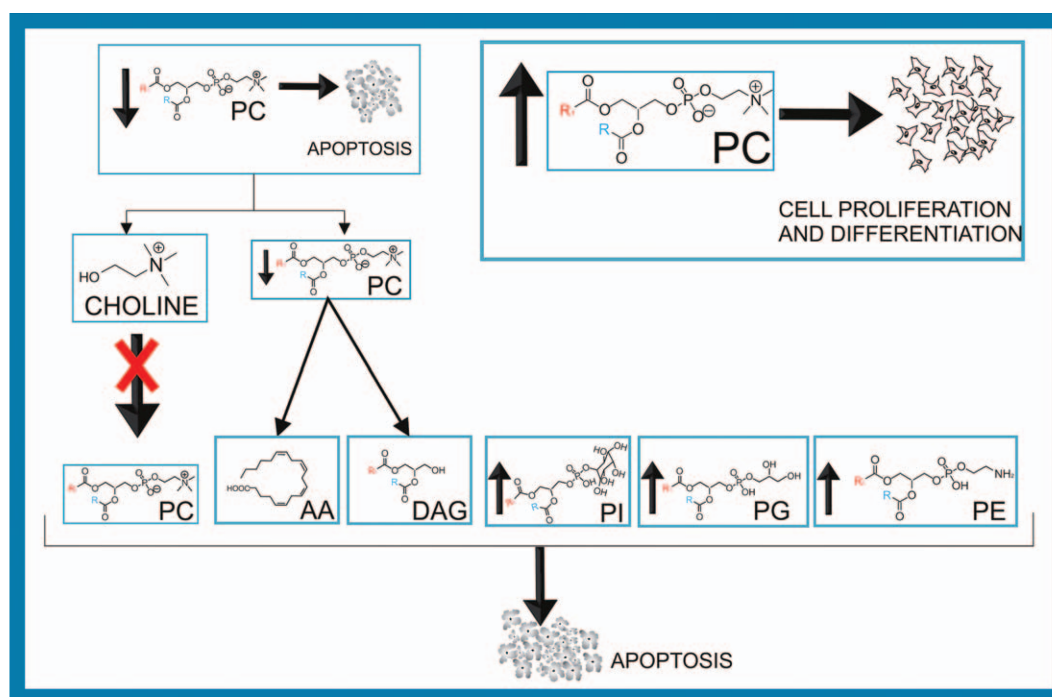

Figure 5. Proposed correlation between lipid subclasses found in POR and NR Groups. PC, phosphatidylcholine (NR group); and DAG, Diacylglycerol (POR group), PI, Phosphatidylinositol (POR group); PE, Phosphatidylethanolamine (POR group), and PG, phosphatidylglycerol (POR group). The AA, arachidonic acid was not observed probably because it presents a bad ionization in MALDI-TOF. The arrows indicate increase or decrease of each lipid subclass in FF.

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been associated with POR. Although the phospholipid pathways of cell proliferation and differentiation are well established, their involvement in cell death is much less understood (Bou Khalil et al, 2010). In Figure 5, we summarize a proposed mechanism by which those lipids are related, and through which they could play a putative role in FF.

PCs represent the major class of glycerophospholipids in eukaryotic cells and are involved in membrane structure and cellular signaling. PCs are synthesized by the CDP-choline pathway, and perturbations in PC homeostasis may cause a decrease in its synthesis and lead to cell death. The mechanisms involved in this process could be caused by defects in enzymes that participate in this pathway or by an increase of PC consumption, generating arachidonic acid (AA) and DAG (Cui & Houweling, 2002).

In the present study, PCs were increased only in the NR group, suggesting that the physiological processes of cellular proliferation and differentiation were occurring. The POR group showed less abundant PC ions, indicating that the process of cell proliferation in these women is insufficient. Indeed, evidence that a reduction of PC synthesis can initiate apoptosis was found in MT58 cells, which represent a mutant cell line in Chinese hamster ovaries (Cui et al., 1996).

Another lipid subclass found in this study was PI which was overrepresented in the POR group. PI is known to participate in membrane constitution and is an essential lipid in the metabolic processes. Abnormalities in myo-inositol and inositol phospholipid metabolism have been shown in numerous diseases, including diabetes, renal disorders, and cancer (Holub, 1986).

The DAG subclass can influence protein transport through the Golgi apparatus because DAG is required at the early stages of vesicle and tubule formation in this complex (Asp et al., 2009). Failures in the transport of proteins through the Golgi complex may be involved in the apoptosis mechanism. High levels of Fas and Fas ligand in the FF from poor ovarian responder women indicates that apoptosis is increased in these patients (Onalan et al., 2005)

In the POR group, PE was also observed. This class of phospholipids is involved in biological processes such as apoptosis and cell signaling and has a structural role in membranes. PE metabolism appears to be important for the heart because it is related to ischemia, which leads to sarcolemmal disruption (Vance, 2008). In addition, PE is required for contractile ring disassembly at the cleavage furrow of mammalian cells during cytokinesis (Emoto et al., 1997).

Finally, substantial differences in PG levels were also observed for the POR group. For PG synthesis, phosphatidylglycerol phosphate (PGP) is initially produced by glycerol-3-phosphate and CDP-diacylglycerol via PGP synthase (PGPS). PGP is subsequently dephosphorylated by PGP phosphatase (PGPP) into PG.

In hamster ovary cells (CHO), temperature-sensitive PGP synthase (PGPS, Ohtsuka et al., 1993a) activity was reduced by 99% but the levels of PG and cardiolipin (CL) were decreased to 90% and 70%, respectively. Concomitantly, reduced growth and alterations in mitochondrial morphology and function were observed (Ohtsuka et al., 1993b). When PGPS is overexpressed, PG synthesis is increased in CHO (Ohtsuka

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et al., 1993a). This alteration may be indirectly related to apoptosis, cellular process regulation, and oxidative stress because in animal tissues, PG functions as a CL biosynthesis precursor (Kawasaki et al., 1999).

In this study, we found a difference in glycerophospholipid (GP) ion abundance in FF samples through the direct lipidomics approach using MALDI-MS in the FF of POR and NR women.

The results were obtained from the analysis of a small number of patients because we applied strict group definition criteria to include young women (up to 35 years old) with a POR history. This experimental design provided a homogeneous group, as shown in Figure 2, in which the PC1 explained 50% of the data. Nonetheless, the prospective biomarkers proposed in this work need to be confirmed in a larger study.

In conclusion, alterations in the GP balance might impact ovarian hormonal responses. The present MALDI–MS study identified species from five glycerolipid subclasses (PC, PE, PI, DAG, and PG) as potential biomarkers for young poor ovarian responders. Our results suggest that changes in the expression or consumption rates of these lipids may function as an important diagnostic tool and may be closely related to alteration in ovarian response.

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