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REPRODUCTIVE PHYSIOLOGY AND DISEASE

Sperm lipidic profiles differ significantly between ejaculates resulting in pregnancy or not following intracytoplasmic sperm injection

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Abstract

Although assisted reproduction techniques involve the use of semen samples, there is little scientific methodology applied when selecting sperm. To select the most appropriate spermatozoa, first we need to define the optimal molecular characteristics. Sperm lipids may contribute to sperm function, thus our aim was to compare the lipidic profiles of sperm samples used in intracytoplasmic sperm injection cycles that ultimately led to a pregnancy with those that did not.

Spermatozoa from infertile patients after intracytoplasmic sperm injection (group non-pregnant, $n = 16$; vs. group pregnant, $n = 22$) were analyzed for lipid composition using ultra-high performance liquid chromatography coupled to mass spectrometry, by means two platforms for measuring fatty acyls, bile-acids, lysoglycerophospholipids, glycerolipids, cholesteryl-esters, sphingolipids, and glycerophospholipids. Lipid levels were compared using a univariate test and multivariate analyses after logarithmic transformation.

We detected 151 different lipids in the sperm samples, 10 of which were significantly increased in sperm samples from the NP group, ranging from 1.10- to 1.30-fold change. These were primarily ceramides, sphingomyelins and three glycerophospholipids, a lysophosphatidylcholine, and two plasmalogen species. Additionally, 2-Monoacylglycerophosphocholine were also found in higher levels in non-pregnant group.

Our results describe the composition of sperm lipids linked to optimal sperm function, opening new possibilities for the development of male fertility diagnostic tools and culture media formulations to improve sperm quality and enhance reproductive results. Given that lipids compose the majority of the sperm plasma membrane, this information is also useful in designing new sperm selection tools that will allow for the selection of the best spermatozoa.

Keywords Sperm · Lipids · Assisted reproductive technology · UHPLC-MS

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Introduction

Approximately 14% of couples attempting to conceive are infertile and seek out assisted reproduction techniques (ARTs) after experiencing infertility [1]. Male factor is responsible for about 50% of infertility cases, although the diagnosis is complex due to a lack of diagnostic tools and because the analytical techniques available to estimate male fertility neglect several relevant molecular features involved in sperm physiology [2]. Basic sperm analysis procedures recommended by the World Health Organization (WHO) [3] are commonly used worldwide to determine sperm quality. The results provide overall information about the fertile capacity of the ejaculate, assist in the selection of appropriate therapies, and may also indicate whether additional or functional tests are needed. These measurements and decision-making thresholds vary substantially among centers and are frequently based on institution-specific criteria and previous experience [2]. The WHO sperm analysis guidelines have significant limitations, however, primarily due to the fact that "normal" sperm quality does not necessarily mean "fertile" and the results do not accurately predict a natural pregnancy or assisted pregnancies [4, 5]. Additionally, there is increasing evidence that male fertility/infertility and sperm normal/aberrant physiology are determined by multiple factors [2, 6], necessitating the use of numerous molecular biomarkers to precisely assess male reproductive potential. Such biomarkers could also be used to identify the most promising ejaculates or spermatozoa to select for it use in reproduction treatments.

To overcome these limitations [3], efforts are underway to identify sperm biomarkers correlated with a greater probability of reproductive success. These include methods designed to detect aneuploidies, DNA fragmentation, gene expression profiles (transcriptomic profiles), epigenetics, molecules involved in sperm function and structure, oxidative stress, hyaluronic acid receptors, ubiquitin, platelet activating factor, and proteomic or metabolic profiles of fertile and infertile sperm [2, 7]. Other studies have focused on the importance of lipids in several spermatozoa functions. For example, docosahexaenoic acid plays an important role in membrane integrity, motility, viability, and morphology of sperm. When added as a dietary supplement, this fatty acid is also associated with an increase in fertilization ability and semen quality in fresh and post-thawed spermatozoa [8]. Therefore, the sperm surface can vary or be remodeled through the addition of exogenous lipids or through interactions with the sperm microenvironment, promoting improved sperm quality or preparation for fertilization. These lipids are of special interest as fertility markers and therapeutic targets not only due to their functions in sperm, but also because they are sensitive to external and environmental signals, many of which can be replicated in culture media [8].

It is of great interest to the scientific community to identify the lipids most closely related to the reproductive success in a broad and comprehensive manner. Characterization of molecules involved in an array of biological processes can be accomplished through lipidomics approaches [9] extending beyond DNA/RNA to amino acids (proteins), carbohydrates (sugars), and lipids (fats) through lipidomics [10]. Yet, few studies have described the sperm lipidomic profile and have been limited to generic metabolic compounds [8, 11–16]. Consequently, limited information is available regarding human seminal plasma lipid composition and its relevance to fertility. Gupta et al. [12] detected 10 metabolites involved in determining male infertility, including glycerophosphocholine, while Deepinder et al. [17] identified differences in glycerylphosphorylcholine and glycerylphosphorylethanolamine levels in sperm plasma between donors and infertile males. More recently, using two techniques (proton nuclear magnetic resonance and gas chromatography coupled to mass spectrometry), Paiva et al. [11] identified lipids including 2-methylglutarate, 2-hydroxy-3-methylvalerate, butyrate, caprate, O-acetylcarnitine, L-acetylcarnitine, and sn-glycero-3-phosphocholine in spermatozoa from patients undergoing routine semen analysis, among a myriad of metabolites.

These preliminary studies [8, 11–16] suggest that sperm plasma membrane lipids may be directly involved in mediating reproductive process, but so far, no study has detailed differences in lipidomic profiles between sperm samples able to successfully fertilize and result in pregnancy versus those unable to fertilize/achieve pregnancy. The aim of our study was to compare, using a wide lipidic profiling, sperm samples that achieved a pregnancy via intracytoplasmic sperm injection (ICSI) cycles with sperm samples that failed to achieve pregnancy and to determine which sperm lipids may be involved in reproductive process.

Material and methods

Study population, sample collection, and study design

Thirty-eight semen samples from individuals whose partners underwent ICSI cycles either with their own or donated oocytes were included in this study. Sample size was established based on previous studies with a similar design [18–21], and was dependent upon the available economic resources to conduct the experiments given the fact that no previous data were available to pre-define the differences between groups. Moreover, no unique main outcome measurement was defined, given that the total lipidic profile was to be evaluated. Inclusion criteria for women using their own oocytes were that women were ≤ 38 years, on their first or second cycle, had no uterine anomalies, no recurrent miscarriages (≥ 2), and had a

BMI ≤ 30 kg/m^2, while couples undergoing oocyte donation were scrutinized for the same criteria except for age, and accepted at ≤45 years. We included males with more than one million spermatozoa after sample preparation and aliquots from each sample (300 μL) were used for the development of this study.

The study was prospective and analytical, and utilized nested cases and controls with all 38 patients, including 22 samples that achieved pregnancy and 16 that failed. Samples were collected and classified in two groups according to their ability to achieve pregnancy, paralleling previous research conducted by García-Herrero et al. [18–21]. Moreover, four culture medium samples (Sydney IVF Fertilization Medium, COOK Medical, Bloomington, IN, USA) were also analyzed to establish a limit of detection (LOD). Spermatozoa obtained after routine sperm selection procedures in the lab of each sample were afterwards analyzed by ultra-high performance liquid chromatography coupled to mass spectrometry (UHPLC-MS).

Ethical approval

Written informed consent was obtained from all patients and the study was approved by the Ethical Committee of IVI Valencia (#1209-C-108-RR).

Assisted reproduction treatments

Controlled ovarian stimulation

Standard controlled ovarian stimulation (COS) protocols were followed. Briefly, women received 1–2 months of treatment with oral contraceptives for cycle synchronization before controlled ovarian stimulation. For stimulation, we applied a gonadotropin-releasing hormone (GnRH) antagonist protocol in which 225 UI/day of recombinant follicle-stimulating hormone (FSH) (Gonal-F®; Merck, Madrid, Spain; Puregon®; MSD, Madrid, Spain) were administered by subcutaneous injection (s.c.) from day 2 to 3 of the cycle. When the leading follicle reached a size of 14 mm, daily s.c. administration of 0.25 mg of GnRH antagonist commenced (Cetrotide, Merck, Madrid, Spain). For final oocyte maturation, a single dose of 0.2 mL Triptorelin was administered intramuscularly (Decapeptyl®; IpsenPharma, Barcelona, Spain) when at least three or more follicles reached a mean size of ≥ 18 mm [22].

Recipient endometrial preparation in the oocyte donation program

The hormone therapy protocol we employed for oocyte recipients was described previously [23] and was conducted per clinical routines established in our centers. In brief, a baseline transvaginal scan was carried out before down-regulation to ensure that the uterus was normal. In the case of recipients who still had natural cycles, down-regulation was performed (Decapeptyl®; IpsenPharma, Barcelona, Spain) in the mid-luteal phase of the previous cycle with an intramuscular dose of 3.75 mg Triptorelin. Hormone therapy was initiated on days 1–3 of the following cycle, and doses of estradiol valerate (Progynova®; Schering-Plough, Madrid, Spain) were administered as follows: 2 mg/day for the first 8 days of treatment; 4 mg/day for the following 3 days; and then at least 6 mg/day until a pregnancy test was performed. On day 15, an ultrasound was performed to evaluate endometrial growth. The day after donation, when fertilization was assessed, 800 mg/day of intravaginally administered micronized progesterone (Progeffik; Effik Laboratories, Barcelona, Spain) were added to the regimen.

Sperm sample collection and preparation

Semen samples were collected by masturbation into sterile containers after 2–7 days of sexual abstinence. Following our standard operating procedure, after liquefaction of samples at 37 °C, 5% CO_2, for 10 min; semen volume was measured by pipet; sperm concentration and motility were determined using Makler® chamber (Sefi Laboratories, Tel Aviv, Israel); and morphology was determined on Diff-Quik (Biognost Ltd., Zagreb, Croatia) stained smears (200 sperm counted/slide) according to the WHO, fifth edition criteria [3]. All determinations were performed in duplicate and by two independent observers. Results were accepted when the differences between the two observations were less than 5%. Then, all samples were prepared according to the routine protocol for ICSI. All samples were prepared by swim-up. Briefly, raw ejaculates were diluted 1:1 with Fertilization Medium (Cook, IVF Medium Group). Then, they were pelleted at 400 g for 10 min, and the supernatants were discarded. This was followed by the careful addition of 0.5–1 mL of fresh medium without disturbing the pellet and incubation for 45 min at a 45° incline. After this period, the upper 0.1–0.5 mL was taken for the ICSI process. Aliquots from the same samples employed in ICSI were collected and frozen in liquid nitrogen without cryoprotectant medium and then stored at − 20 °C until reproductive results were known to assign the appropriate study group [19–21].

Ovum pick-up, ICSI cycles, and embryo culture

Follicles were aspirated and oocytes were washed in Gamete Medium (COOK™, Bloomington, IN, USA). After washing, oocytes were cultured in Fertilization Medium (Cleavage Medium, COOK™, Bloomington, IN, USA) at 5.5% CO_2 in air at 37 °C for 4 hours before

oocyte denudation. Oocyte denudation was carried out by mechanically pipetting 40 IU/mL of hyaluronidase in the same medium. Subsequently, ICSI was performed in a medium containing HEPES (Gamete Medium, COOK™, Bloomington, IN, USA) at 400× magnification using an Olympus IX7 microscope. Immediately after ICSI, the injected oocytes cycles were placed in Petri dishes (Falcon, Madrid, Spain) (drop culture) of culture media (Cleavage Medium, COOK™, Bloomington, IN, USA) under oil at 37 °C and 5.5% CO_2 in air (Heraeus, Heracell, Madrid, Spain). Embryos were incubated at 37 °C, 5.5% CO_2, and atmospheric O_2 and cultured individually until embryo day 3 (72 h after ICSI) in Cleavage Medium (COOK™, Bloomington, IN, USA). From day 3 to day 5, we used CCM Medium (Vitrolife, Goteborg, Sweden). Culture dishes included three washing drops of 100 μL and six culture drops of 50 μL, covered by 7 mL of mineral oil [18].

Embryo scoring and transfer

Successful fertilization was assessed at 16–19 h post-ICSI and confirmed by the presence of two pronuclei and two polar bodies. Embryo morphology was analyzed on day 2 (48 h post-ICSI) and day 3 (72 h post-ICSI) taking into account the number, symmetry, and granularity of the blastomeres, the type and percentage of fragmentation, the presence of multinucleated blastomeres, and the degree of compaction as previously described [24]. Based on this grading system, we selected embryos for transfer on day 3.

The number of embryos transferred was decided based upon embryo quality. Supernumerary embryos were frozen for future transfer using the standard vitrification technique in our clinic. The primary end point for this study was ongoing pregnancy confirmed by the presence of gestational sacs with fetal heartbeat by transvaginal ultrasound examination during week 12.

Lipidomic extraction procedures

Lipid extraction was accomplished by fractionating the cell samples into pools of species with similar physicochemical properties, using appropriate combinations of organic solvents. Proteins were precipitated and discarded from the lysed cell samples by adding methanol. After brief vortex mixing, the samples were spiked with chloroform. Both extraction solvents, which were added at the beginning of the lipid extraction procedure, were spiked with metabolites not detected in un-spiked cell extracts. As the applied analytical methods cover lipids from different classes, multiple internal standards covering most of those classes were included: tryptophan-d5(indole-d5), PC(13:0/0:0), non-esterified fatty acid (NEFA)(19:0) and dehydrocholic acid were spiked in the methanol and used as internal standards in the first UHPLC-MS analysis (platform 1); SM(d18:1/6:0), PE(17:0/17:0), PC(19:0/19:0), TAG(13:0/13:0/13:0), Cer(d18:1/17:0), and ChoE(12:0) were added to the chloroform and used as internal standards in the second UHPLC-MS analysis (platform 2). Samples were incubated at − 20 °C for 30 min, and after vortex mixing, two different phases were collected.

For analysis in platform 1, which included fatty acyls, bile acids, steroids, and lysoglycerophospholipid profiling, supernatants were collected after centrifugation at 16,000x*g* for 15 min, dried, reconstituted in methanol, re-suspended for 20 min, and centrifuged (16,000x*g* for 5 min) before being transferred to vials for UHPLC-MS analysis.

For platform 2, which included glycerolipids, cholesteryl esters, sphingolipids, and glycerophospholipid profiling, cell extracts were mixed with water (pH = 9). After brief vortex mixing, samples were incubated for 1 h at − 20 °C. After centrifugation at 16,000x*g* for 15 min, the organic phase was collected and the solvent removed. The dried extracts were then reconstituted in acetronitrile/isopropanol (50:50), re-suspended for 10 min, centrifuged (16,000x*g* for 5 min), and transferred to vials for UHPLC-MS analysis.

Additionally, two different types of quality control (QC) samples were used to assess data quality [25]. The QC samples are reference culture medium samples, which were evenly distributed over the batches and extracted and analyzed at the same time as the individual samples. The *QC calibration sample* was used to correct for the different response factors between and within batches and the *QC validation sample* was used to assess how well the data pre-processing procedure improved the data quality. For each of the two analytical platforms, randomized duplicate sample injections were performed, with each of the QC calibration and validation extracts uniformly interspersed throughout the entire batch run.

LC-MS analysis

Two UHPLC-time-of-flight (TOF)-MS-based platforms were used for optimal profiling of the lipidome [26]. Platform 1 was analyzed on an Acquity-LCT Premier XE system (Waters Corp., Milford, MA) and platform 2 on an Acquity-Xevo G2QTOF (Waters Corp., Milford, MA).

The retention time stability (generally < 6 s variation, injection-to-injection), mass accuracy (generally < 3 ppm for m/z 400–1000, and < 1.2 mDa for m/z 50–400) and sensitivity of the system throughout the course of the run were examined. The overall quality of the analysis procedure was monitored using five repeat extracts of the QC validation sample. Generally, the retention time stability was < 6 s injection-to-injection variation and the mass accuracy < 3 ppm for m/z

400–1200, and < 1.2 mDa for m/z 50–400. (MS data were acquired over an m/z range of 50–1000 for platform 1 and of m/z 50–1200 for platform 2). The average intrabatch %CV values were 11.12 for both platform 1 and 2.

Data pre-processing

All data were processed using the TargetLynx application manager for MassLynx 4.1 software (Waters Corp., Milford, MA, USA). A set of predefined retention time and mass-to-charge ratio pairs, *Rt-m/z*, corresponding to metabolites included in the analysis were fed into the program. Associated extracted ion chromatograms (mass tolerance window = 0.05 Da) were then peak-detected and noise-reduced in both the LC and MS domains such that only true metabolite-related features were processed by the software. A moving average smoothing method was applied for noise reduction. The LC-MS features were identified prior to the analysis, either by comparison of their accurate mass spectra and chromatographic Rt with those of available reference standards or, where these were not available, by accurate mass MS/MS fragment ion analysis, as described previously by Barr et al. [26]. Briefly, the identified ion features in the platform 1 included fatty acids, acyl carnitine, lysophospholipids, and oxidized fatty acids. The ion features considered for the following data analysis were the adducts [M-CO2H]-, [M-CO2H]-, [M-H]-, [M-CO2H]- and [M-H]- for NEFA, AC, oxidized fatty acids, monoacyl- and monoether-phosphatidylcholines, and monoacyl- and monoether-phosphatidylethanolamines or phosphatidylinositols, respectively. The platform 2 provided coverage over glycerolipids, cholesteryl esters, sphingolipids, and glycerophospholipids. The ion features considered for the following data analysis of these lipid classes were the adducts [M + H] + for diacyl- or monoether, monoacyl-phosphatidylcholines, phosphatidylethanolamines or phosphatidylinositols, Monohexosylceramides and sphingomyelins; [M + H-H2O] + for ceramides; [M + Na] + for diacylglicerols; and [M + NH4] + for cholesteryl esters and triacylglicerols.

Statistical analysis

Linear regression (internal standard corrected response as a function of sample injection order) was used to estimate in the QC calibration samples any intrabatch drift not corrected for by internal standard correction [27]. For all variables, internal standard corrected responses in each batch were divided by the corresponding intrabatch drift trend, such that the normalized abundance values of the study samples were expressed with respect to the batch averaged QC calibration serum samples (arbitrarily set to 1). After normalization, the concordance between duplicate sample injection response values was assessed. When coefficients of variation > 30% were found, corresponding sample injection data were returned for manual inspection of the automated integration performed by the TargetLynx software and modifications were made appropriately. Any remaining sample injection variable response zero values in the corrected dataset were replaced with missing values before averaging to form the final dataset that was used for study sample statistical analyses.

Data were expressed as means or proportions with their corresponding 95% confidence intervals (95%CI) or standard deviations (SD).

Quantitative variables were compared between groups using the Student's *t* test for independent samples when data were normally distributed (Shapiro-Wilk test). Univariate statistical analyses of lipidomic variables were performed calculating Wilcoxon signed-rank test *p* values for the comparison between samples from which a pregnancy was obtained versus those who failed when data did not follow a normal distribution.

For categorical data, chi-square tests were used to compare proportions between groups.

Diagnostic sensitivity and specificity were calculated and receiver-operating characteristic (ROC) analysis was constructed by plotting sensitivity against the false positive rate (1-specificity) of various cutoff values for predicting pregnancy. The area under the ROC curve (AUC) was calculated for the lipidomic parameters between groups. The value with the optimal combination of sensitivity and specificity was chosen as the cutoff value. The ideal screening test is one that approaches or reaches the upper left corner of the graph (100% sensitivity and 100% specificity). Positive and negative predictive values were also calculated.

A volcano plot was used to summarize both fold change and *t* test criteria. It is a scatter-plot of the negative $\log_{10}$-transformed *p* values from the *t* test against the $\log_2$ fold change. Metabolites with statistically significant differential levels according to the *t* test fell above a horizontal threshold line. Metabolites with large fold change values lie far from the vertical threshold line at $\log_2$ fold change = 0, indicating whether the metabolite is up or down-regulated.

The dimensionality of the complex data set was reduced to enable easy visualization of any metabolic clustering of the different groups of samples. This was achieved by multivariate data analysis, including the non-supervised principal components analysis (PCA) [28] and/or supervised orthogonal partial least-squares to latent structures (OPLS) approaches [29, 30].

For all tests, a *p* value less than 0.05 was considered statistically significant. All statistical analyses were performed using the SPSSv22 (SPSS Inc., Chicago, IL, USA) and using statistical software package R v.3.1.1 (R Development Core Team, 2011; http://cran.r-project.org) with MASS package, the latter for the lipidomic analysis.

Results

Demographics

The study groups (P and NP) were comparable since there are no statistically significant differences between the parameters and characteristics studied (Table 1).

Sperm lipidomic profile between samples able or unable to achieve pregnancy

A different UHPLC-MS method was used for each platform, and chromatographic separation and mass spectrometric detection conditions are summarized in Supplementary Table 1.

Multivariate analysis

We began grouping the samples using a PCA scores plot, comparing the sperm that achieved pregnancy (P) versus sperm that did not achieve a pregnancy (NP). Our PCA model analysis was generated using 22 P samples and 16 NP samples. Having validated the quality of the experiment we found that two samples were outside the Hotelling's T2 ellipse. Following Chauvenet's criterion, further inspection of the data relating to those samples showed that one of them had elevated levels of most of the lipid species, and thus this sample together with another one inside the ellipse but with elevated levels of most of the lipid species were removed from both the univariate and multivariate analysis. One removed sample was from group P, and the other from group NP.

Table 1 Patient characteristics

	Pregnant (P) (Mean ± SD)	Non-pregnant (NP) (Mean ± SD)	*p* value
N	21	15	
Female age (years)	38.6 ± 4.81	39 ± 4.42	0.79
BMI (kg/cm2)	22.5 ± 2.67	22.4 ± 4.1	0.91
Antral follicle	13.3 ± 3.64	11.4 ± 3.05	0.09
Mature oocyte (MII)	9.68 ± 5.01	8.86 ± 3.9	0.58
Fertilization rate (%)	79.5 ± 16.9	80.8 ± 16.4	0.81
Number of embryo transferred	1.86 ± 0.35	1.79 ± 0.43	0.57
Embryo quality-frequencies	Frequency	Frequency	Pr(z)*
A	2/21	3/15	0.91
A + A	8/21	4/15	0.69
A + B	2/21	0	
A + C	0	2/15	
B	1/21	0	0.69
B + B	4/21	2/15	0.19
B + C	2/21	0	
N/A	2/21	4/15	
Sperm characteristics	(Mean ± SD)	(Mean ± SD)	*p* value
Male age (years)	42.7 ± 5.04	43.5 ± 6.26	0.68
Volume (mL)	2.93 ± 1.02	2.39 ± 0.738	0.07
Progressive sperm (%)	41.5 ± 13.7	42.7 ± 15.3	0.81
Non-progressive sperm (%)	8.32 ± 5.18	5.92 ± 3.82	0.12
Inmotile sperm (%)	50.2 ± 14.8	51.8 ± 15.5	0.76
Total espermatozoa (mill)	148 ± 103	147 ± 102	0.97
Total progressive spermatozoa (mill)	60.3 ± 41.7	57.2 ± 40.1	0.82
Normal morphology (%)	4.38 ± 2.2	4.07 ± 2.59	0.72
Head anomalies (%)	90.9 ± 20.5	95.5 ± 3.57	0.33
Midpiece anomalies (%)	35.6 ± 12.2	30.6 ± 9.42	0.18
Tail anomalies (%)	13.8 ± 7.66	13.2 ± 7.56	0.84

Pearson's chi-squared test with simulated *p* value (based on 2000 replicates)

p value = 0.39

N number of participants, *BMI* body mass index (kg/cm^2), *MII* metaphase II, *A, B, C*, embryo quality categories

**Pr(z)* significant correlation with response variable

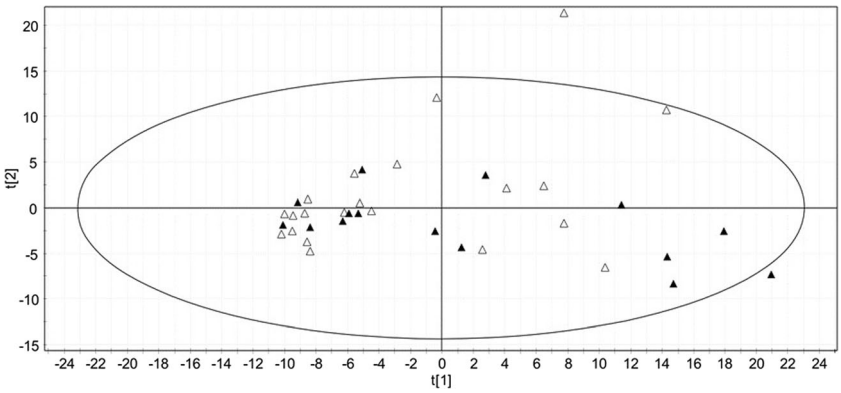

Fig. 1 Principal component analysis of sperm that achieved pregnancy using ICSI (P; white triangles) and did not achieve pregnancy (NP; black triangles) with ICSI. R2X(cum) = 0.708 and Q2X(cum) = 0.621 (two components)

After removing these outliers (both samples explained above), the PCA (Fig. 1) showed a random distribution of samples (group P = 21; group NP = 15) on the two first principal components t [1] and t [2], indicating that the global lipid spermatozoa content did not clearly separate according to pregnancy ability. The supervised OPLS model is not provided as it showed a negative predictive ability, indicating that it was not valid to separate the two groups using all the lipids found in our study. Multivariate statistical analyses after logarithmic or square root transformation were also applied; significant models were found.

Univariate analysis

Supplementary Table 2 shows the 151 different lipid species detected in the sperm samples. Ten of them were significantly overexpressed in sperm samples from the NP group ranging from 1.10 to 1.30-fold change and a p value < 0.05 (Table 2).

Among them, lipids found to be different between the NP and P groups included ceramides (Cer)(d18:1/22:0), Cer(d18:1/23:0), Cer(d18:1/24:0), and sphingomyelins (SM)(38:1), SM(d18:1/22:0), SM(42:1), and SM(d18:1/25:0). Additionally, 1 or 2-monoacylglycerophosphocholines PC(0:0/20:0), 1-ether, 2-acylglycerophosphocholines PC(O-22:0/20:4) and 1-ether, 2-acylglycerophosphoethanolamines PE(P-16:0/18:2) showed different levels between groups (Table 2).

All significantly different ceramides consisted of a sphingosine moiety of 18 carbons (sphing-4-enine) that is amide-linked to a long chain fatty acyl group. Several sphingomyelins were also found in higher levels in spermatozoa from which cycles failed, most of them also containing the sphing-4-enine moiety. Additionally, three glycerophospholipids, the

Table 2 Ejaculate lipid comparison between P and NP samples

		Group NP		Group P			ROC Analysis							
Lipid identification	Chemical formula	Mean	SD	Mean	SD	p value	Fold change	Area under the curve	Sens	Spec	PPV	NPV	Youden's index	Cutoff
Cer(d18:1/22:0)	C40H79NO3	6.04	5.44	2.83	3.38	*0.04*	2.14	0.71	0.87	0.52	0.57	0.85	0.39	1.67
Cer(d18:1/23:0)	C41H81NO3	0.39	0.37	0.17	0.16	*0.04*	2.35	0.70	0.87	0.55	0.59	0.85	0.42	0.12
Cer(d18:1/24:0)	C42H83NO3	2.02	1.99	0.89	0.96	*0.05*	2.27	0.70	0.40	0.95	0.86	0.68	0.35	2.82
PC(O-22:0/20:4)	C50H94NO7P	0.31	0.26	0.13	0.16	*0.01*	2.33	0.77	1.00	0.53	0.62	1.00	0.53	0.03
PE(P-16:0/18:2)		1.15	1.08	0.53	0.54	*0.05*	2.18	0.70	0.79	0.55	0.55	0.79	0.34	0.31
SM(38:1)		1.74	1.63	0.77	0.99	*0.04*	2.27	0.70	0.73	0.67	0.61	0.78	0.40	0.70
SM(d18:1/22:0)	C45H91N2O6P	0.95	0.90	0.39	0.51	*0.03*	2.42	0.72	0.73	0.67	0.61	0.78	0.40	0.40
SM(42:1)		1.45	1.34	0.59	0.76	*0.04*	2.45	0.72	0.73	0.76	0.69	0.80	0.50	0.63
SM(d18:1/25:0)		0.28	0.23	0.12	0.13	*0.04*	2.30	0.74	0.86	0.60	0.60	0.86	0.46	0.08
PC(0:0/20:0)	C28H58NO7P	0.07	0.06	0.03	0.04	*0.04*	2.29	0.71	0.93	0.45	0.54	0.90	0.38	0.01

p values reflect the values obtained in t tests for normally distributed samples or Wilcoxon tests for non-normally distributed samples. This table shows statistically significant differences in italics

SD standard deviation, *Sens* sensitivity, *Spec* specificity, *PPV* predictive values for positives, *NPV* predictive values for negatives, *Cer* ceramides, *CMH* monohexosylceramides, *ChoE* cholesteryl esters, *PC* phosphatidylcholines, *PE* phosphatidylethanolamines, *PI* phosphatidylinositols, *SM* sphingomyelins, *TG* triacylglycerols, *LPC* lysophosphatidylcholines, *LPE* lysophosphatidylethanolamines, *AC* acyl carnitines, *LPI* lysophosphatidylinositols

lysophosphatidylcholine PC(0:0/20:0), and two ether-linked glycerophospholipids PC(O-22:0/20:4) and the plasmalogen species PE(P-16:0/18:2) were significantly higher in spermatozoa without fertilizing ability. These differences may be related to the membrane structure; for instance, polyunsaturated ethanolamine plasmalogens are known to destabilize the lipidic bilayer. Moreover, inverted cone-shaped phospholipids, such as lysophosphatidylcholine, have only one acyl chain and also tend to form curved bilayers and non-bilayer phases. Changing the balance of bilayer-forming lipids with non-bilayer-inducing lipids can alter the membrane curvature and indirectly affect the properties of membrane proteins.

Considering the lipid classes classification, univariate statistical analyses showed higher levels of 2-monoacylglycerophosphocholine (glycerophospholipids) (Table 3) with a fatty acid esterified in the sn-2 position in NP spermatozoa compared to the fertile P between 46 lipid classes (detailed information can be found on Supplementary Table 3).

The volcano plot shown in Fig. 2 reflects the most significant metabolites, considered individually, for the comparison of NP vs. P spermatozoa, showing that the 10 lipids significantly overexpressed were found in group NP. The volcano plot highlights the most differentially detected individual metabolites, considering the different *p* values for each lipid (0.05 or 0.01) and the positive or negative fold change in NP vs. P sperm.

ROC curve analysis

When we evaluated the predictive power of single lipids and lipid classes, several molecules showed promising predictive abilities (Tables 2 and 3). Particularly, the predictive ability to forecast pregnancy was better for ceramides, with an area under the ROC curve of about 0.70, with a sensitivity of almost 90% for Cer(d18:1/22:0) and Cer(d18:1/23:0) and specificity of 0.95 for Cer(d18:1/24:0). The positive predictive value is 0.86 for the latter, although it had a limited Youden's index value as a way of summarizing the performance of a diagnostic test (Fig. 3).

A similar pattern was found for PC(O-22:0/20:4) and PE(P-16:0/18:2), SM(42:1), SM(d18:1/25:0), SM(38:1), and SM(d18:1/22:0).

Discussion

Sperm biomarkers are needed to improve ART treatments, which are not successful for every couple providing sperm and oocytes [31, 32]. Here, we describe sperm plasma membrane lipids related to reproductive process. Several unique lipids and lipid families were linked to sperm function in achieving reproductive process. Normally, ejaculated semen contains millions of motile cells, which are genetically unique due to the random selection of either maternal or paternal chromosomes or genetic recombination. Further modifications may occur in the genital tract and epididymal storage. A sperm selected for reproductive purposes may lead to success, while others may lead to failure. Diagnostic and/or selection techniques for sperm could impact live birth rates and the duration of controlled ovarian stimulation. Several molecular sperm quality characteristics have been reported in the literature [2, 6]. Yet, sperm research has been somewhat neglected since the introduction of ICSI, despite the likelihood that sperm diagnostic and selection methods affect ART success. Indeed, sperm are selected for microinjection in a subjective manner. An objective definition of characteristics of the best sperm would improve the selection process and, thereby, contribute to better outcomes.

Among cellular components with relevant sperm functions, one key structure in reproduction is the sperm plasma membrane, particularly but not exclusively due to its involvement in the fusion of the sperm cell and oocyte during fertilization as well as the complex changes sperm undergo throughout their lifecycle including maturation, capacitation, and acrosome reaction [33]. Sperm cells have different membranes compared to those present within somatic cells, which will undergo significant changes after leaving the testes, being organized into lateral regions of the sperm head surface and properly re-orientating their molecules throughout the male and female tract. In addition, a role in post-fertilization events cannot be ruled out [34]. Sperm membrane components, particularly lipids, are directly involved in core sperm function. The lipids from the sperm plasma membrane have a critical role in sperm-oocyte interaction and sperm preparation leading to successful fertilization [8] and contribute to the ability of the sperm to survive after freezing/thawing and to stabilize oxidative stress.

Although variable in mammalian species, the sperm plasma membrane is roughly built of 70% phospholipids, 25% neutral lipids, and 5% glycolipids [35], which is to some extent comparable to somatic cell types. Human sperm may present about 50% phosphocholine glycerides, 30% phosphoethanolamine glycerides, 12.5% SM, 3% phosphatidylserine, 2.5% cardiolipin, and about 2% phosphatidylinositol [35]. More interestingly, some lipid components may vary significantly between individual males but also among different ejaculates because they can vary during their maturation in the epididymis acquiring motility and recognitions of physicochemical signals abilities that will make them suitable for the reproductive success.

Although available information is limited, some older studies tried to find the relation between the lipid composition of sperm membrane and viability and functionality of spermatozoa, highlighting this relation [36]. In fact, these studies demonstrated the important role of sperm membrane composition

Table 3 Lipid classification among P and NP samples

			Group NP		Group P				ROC curve analysis						
Lipid class	Subclass	Abbreviated form	Mean	SD	Mean	SD	*p* value	Fold change	Area under the curve	Sens	Spec	PPV	NPV	Youden's index	Cutoff
Glycerophospholipids	2-Monoacylglycero phosphocholine	2-MAPC	0.07	0.06	0.03	0.04	*0.04*	2.29	0.71	0.93	0.45	0.54	0.90	0.38	0.01

p values reflect the values obtained in *t* tests for normally distributed samples or Wilcoxon tests for non-normally distributed samples. The table shows statistically significant comparisons in italics
SD standard deviation, *Sens* sensitivity, *Spec* specificity, *PPV* predictive values for positives, *NPV* predictive values for negatives

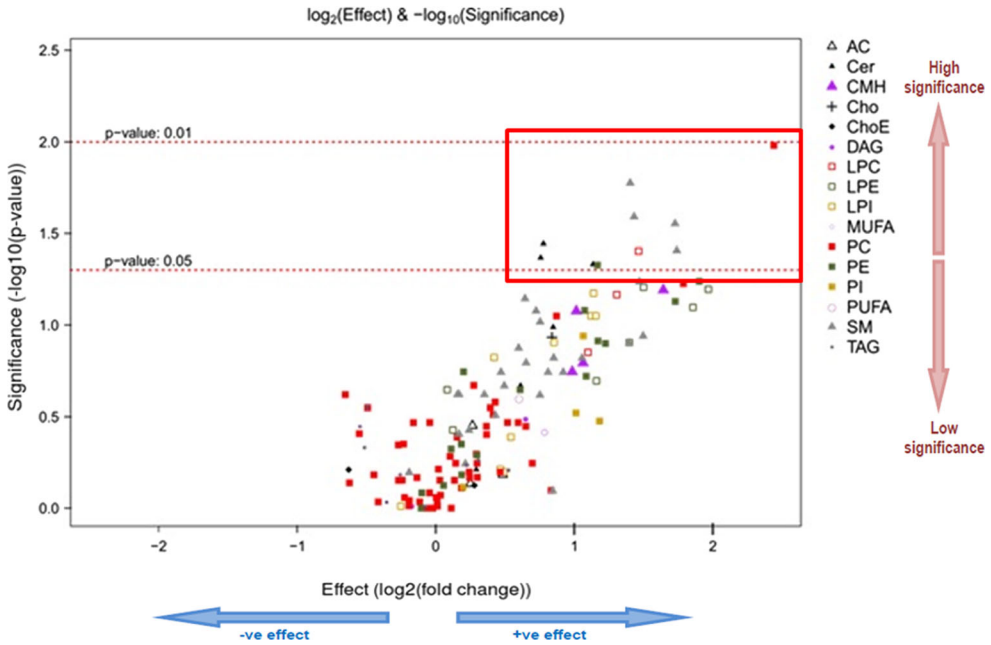

Fig. 2 Volcano plot comparing sperm that achieved pregnancy (P) or not (NP) using ICSI. This plot summarizes both fold change and *t* test criteria. It is a scatter plot of the negative *p* values transformed in $\log_{10}$ from the *t* test against the $\log_2$ fold change. Metabolites with statistically significant differential levels according to the *t* test fall above a horizontal threshold line. Metabolites with the largest fold change values fall farthest from the vertical threshold line at $\log_2$ fold change = 0. Red squares include 10 lipids significantly overexpressed (group NP). AC acyl carnitines, Cer ceramides, CMH monohexosylceramides, ChoE cholesteryl esters, DAG diacylglycerols, LPC lysophosphatidylcholines, LPE lysophosphatidylethanolamines, LPI lysophosphatidylinositols, MUFA monounsaturated fatty acids, PC phosphatidylcholines, PE phosphatidylethanolamines, PI phosphatidylinositols, PUFA polyunsaturated fatty acids, SM sphingomyelins, TAG triacylglycerols

and its lipid balance in sperm quality (sperm viability and functionality) [37–39]. Moreover, grade of oxidative susceptibility of polyunsaturated fatty acids could be related to oxidative damage that diminishes the sperm's ability to fertilize the oocyte. In this sense, we can find high amounts of polyunsaturated fatty acids in the sperm membrane. These fatty acids, besides improving sperm motility and membrane fluidity, make sperm an easy target for reactive oxygen species. In this way, the integrity and viability of spermatozoa is affected, introducing irreversible DNA damage and reducing sperm fertilization capacity [40]. In fact, a recent study [41] demonstrated that the loss of motility was gradual and highly correlated with the induction of lipid peroxidation. Therefore, this issue has become an important consideration in the causes of male infertility, given that defective spermatozoa are the main cause of this type of oxidative damage.

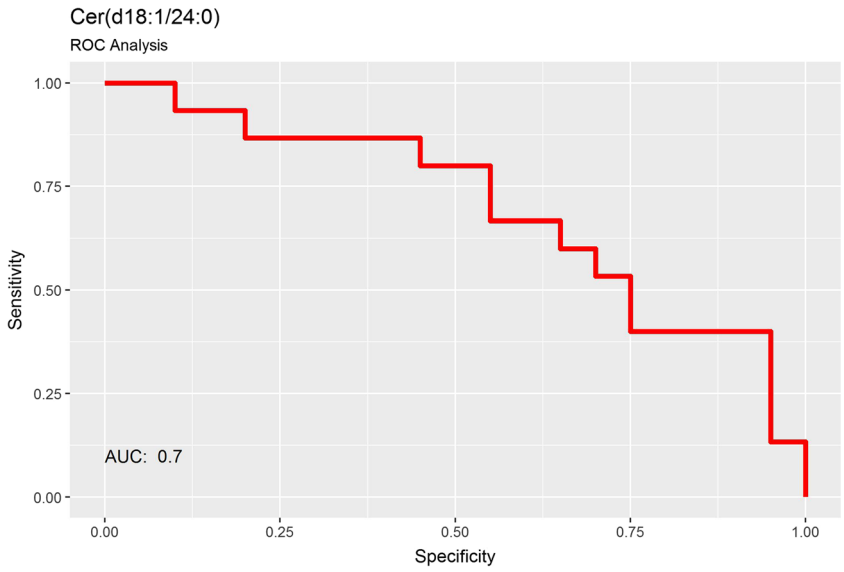

Fig. 3 Receiver-operator curve (ROC) analysis representing the predictive value of Cer(d18:1/24:0) in spermatozoa to forecast pregnancy achievement

On the other hand, human sperm cells, for instance, contain high amounts of cholesterol, and this seems to be related with the length of capacitation, since cholesterol is depleted from the plasma membrane in this process. Besides cholesterol, low amounts of desmosterol, cholesterol sulphate, and cholesteryl esters can be found [35]. Supplementation of these molecules in the culture media or diet has been demonstrated to protect sperm from cryodamage [42, 43]. For instance, the addition of cholesterol or cholesterol-loaded cyclodextrins [44] may prevent sperm deterioration [45–47] while maintaining fertility, as demonstrated in several species [48, 49].

This study identified 10 statistical significant individual lipids and 1 lipidic class overexpressed in NP group vs. P. Among them, ceramides have predictive ability to forecast pregnancy, specifically Cer(d18:1/24:0) and plasmalogens species and lysophosphatidylcholine. The lipids overexpressed in the NP group are known to destabilize the lipidic bilayer. Moreover, inverted cone-shaped phospholipids, such as lysophosphatidylcholine, have only one acyl chain and tend to form curved bilayers and non-bilayer phases. Changing the balance of bilayer-forming lipids with non-bilayer-inducing lipids can alter the membrane curvature and indirectly affect the properties of membrane proteins. Moreover, the fact that we did not find cholesterol in our results likely reflects that cholesterol is depleted from the plasma membrane in the capacitation process.

Although the study of ICSI cycles only could be a limitation, the results provide useful information for different reasons: (a) the composition of the membrane is the result of spermatogenesis, and we sought to identify any indicator of "abnormal spermatogenesis" that affects reproductive outcomes; (b) identifying sperm biomarkers associated with success in ICSI cycles can provide clues about why patients experience infertility, e.g., if a particular biomarker was related to or caused the failure in ART (where it was needed for fertilization); and (c) ICSI is the most used technique in our clinics, and if we find biomarkers of success, whatever their biological explanation, there is potential for application in many patients.

Although ultimately only one sperm will be responsible for oocyte fertilization, few techniques are available to evaluate the sperm that will be microinjected. A deep knowledge of sperm physiology will undoubtedly unveil the key components of sperm needed to help select the most successful spermatozoa. Future research will study the specific lipids relevant in reproduction and the determination of lipidic peroxidation, and improve strategies used to modulate their presence in spermatozoa to improve reproductive success. In fact, although we found that the global spermatozoa lipid content did not clearly separate according to pregnancy ability, the fact that most metabolites appeared in higher levels in spermatozoa that did not achieve pregnancy can be useful in designing new sperm selection tools, such as using magnetically activated cell sorting to select spermatozoa with specific lipids, to select those which achieve gestation or not.

In summary, our lipidomic study of spermatozoa samples differing in reproductive success revealed that samples that did and did not achieve pregnancy were unable to be separated by global lipidomic profiles. Nevertheless, univariate analysis showed a series of individual lipids that were increased in NP spermatozoa, highlighting the fact that infertile samples may have higher levels of some glycerophospholipids and sphingolipids.

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Compliance with ethical standards

Ethical approval Written informed consent was obtained from all patients and the study was approved by the Ethical Committee of IVI Valencia (#1209-C-108-RR).

Conflict of interest The authors declare that they have no conflict of interests.

References

1. Reproductive Health Outlook. Infertility: overview and lessons learned. available: http://www.rho.org. 2002.
2. Sakkas D, Ramalingam M, Garrido N, Barratt CL. Sperm selection in natural conception: what can we learn from Mother Nature to improve assisted reproduction outcomes? Hum Reprod Update. 2015;21:711–26.
3. WHO. Laboratory manual for the examination and processing of human semen. 5th ed; 2010. ISBN: 978 92 4 154778 9
4. Esteves SC. Clinical relevance of routine semen analysis and controversies surrounding the 2010 World Health Organization criteria for semen examination. Int Braz J Urol. 2014;40:443–53.
5. Esteves SC, Zini A, Aziz N, Alvarez JG, Sabanegh ES Jr, Agarwal A. Critical appraisal of World Health Organization's new reference values for human semen characteristics and effect on diagnosis and treatment of subfertile men. Urology. 2012;79:16–22.
6. Garrido N, Remohi J, Martinez-Conejero JA, Garcia-Herrero S, Pellicer A, Meseguer M. Contribution of sperm molecular features to embryo quality and assisted reproduction success. Reprod BioMed Online. 2008;17:855–65.
7. Egea RR, Puchalt NG, Escriva MM, Varghese AC. OMICS: current and future perspectives in reproductive medicine and technology. J Hum Reprod Sci. 2014;7:73–92.
8. Khoshvaght A, Towhidi A, Zare-shahneh A, Noruozi M, Zhandi M, Davachi ND, et al. Dietary n-3 PUFAs improve fresh and post-thaw semen quality in Holstein bulls via alteration of sperm fatty acid composition. Theriogenology. 2016;85:807–12.

9. Quehenberger O, Armando AM, Brown AH, Milne SB, Myers DS, Merrill AH, et al. Lipidomics reveals a remarkable diversity of lipids in human plasma. J Lipid Res. 2010;51:3299–305.
10. Henkel R. Sperm preparation: state-of-the-art–physiological aspects and application of advanced sperm preparation methods. Asian J Androl. 2012;14:260–9.
11. Paiva C, Amaral A, Rodriguez M, Canyellas N, Correig X, Ballesca JL, et al. Identification of endogenous metabolites in human sperm cells using proton nuclear magnetic resonance ((1) H-NMR) spectroscopy and gas chromatography-mass spectrometry (GC-MS). Andrology. 2015;3:496–505.
12. Gupta A, Mahdi AA, Ahmad MK, Shukla KK, Jaiswer SP, Shankhwar SN. 1H NMR spectroscopic studies on human seminal plasma: a probative discriminant function analysis classification model. J Pharm Biomed Anal. 2011;54:106–13.
13. Schroter F, Jakop U, Teichmann A, Haralampiev I, Tannert A, Wiesner B, et al. Lipid dynamics in boar sperm studied by advanced fluorescence imaging techniques. Eur Biophys J. 2016;45:149–63.
14. van Gestel RA, Brouwers JF, Ultee A, Helms JB, Gadella BM. Ultrastructure and lipid composition of detergent-resistant membranes derived from mammalian sperm and two types of epithelial cells. Cell Tissue Res. 2016;363:129–45.
15. Oresti GM, Penalva DA, Luquez JM, Antollini SS, Aveldano MI. Lipid biochemical and biophysical changes in rat spermatozoa during isolation and functional activation in vitro. Biol Reprod. 2015;93:140.
16. Bernabo N, Greco L, Ordinelli A, Mattioli M, Barboni B. Capacitation-related lipid remodeling of mammalian spermatozoa membrane determines the final fate of male gametes: a computational biology study. OMICS. 2015;19:712–21.
17. Deepinder F, Chowdary HT, Agarwal A. Role of metabolomic analysis of biomarkers in the management of male infertility. Expert Rev Mol Diagn. 2007;7:351–8.
18. Garcia-Herrero S, Garrido N, Martinez-Conejero JA, Remohi J, Pellicer A, Meseguer M. Differential transcriptomic profile in spermatozoa achieving pregnancy or not via ICSI. Reprod BioMed Online. 2011;22:25–36.
19. Garrido N, Garcia-Herrero S, Meseguer M. Assessment of sperm using mRNA microarray technology. Fertil Steril. 2013;99:1008–22.
20. Garcia-Herrero S, Garrido N, Martinez-Conejero JA, Remohi J, Pellicer A, Meseguer M. Ontological evaluation of transcriptional differences between sperm of infertile males and fertile donors using microarray analysis. J Assist Reprod Genet. 2010;27:111–20.
21. Garcia-Herrero S, Meseguer M, Martinez-Conejero JA, Remohi J, Pellicer A, Garrido N. The transcriptome of spermatozoa used in homologous intrauterine insemination varies considerably between samples that achieve pregnancy and those that do not. Fertil Steril. 2010;94:1360–73.
22. Munoz M, Cruz M, Humaidan P, Garrido N, Perez-Cano I, Meseguer M. The type of GnRH analogue used during controlled ovarian stimulation influences early embryo developmental kinetics: a time-lapse study. Eur J Obstet Gynecol Reprod Biol. 2013;168:167–72.
23. Meseguer M, Martinez-Conejero JA, O'Connor JE, Pellicer A, Remohi J, Garrido N. The significance of sperm DNA oxidation in embryo development and reproductive outcome in an oocyte donation program: a new model to study a male infertility prognostic factor. Fertil Steril. 2008;89:1191–9.
24. Alikani M, Calderon G, Tomkin G, Garrisi J, Kokot M, Cohen J. Cleavage anomalies in early human embryos and survival after prolonged culture in-vitro. Hum Reprod. 2000;15:2634–43.
25. van der Kloet FM, Bobeldijk I, Verheij ER, Jellema RH. Analytical error reduction using single point calibration for accurate and precise metabolomic phenotyping. J Proteome Res. 2009;8:5132–41.
26. Barr J, Caballeria J, Martinez-Arranz I, Dominguez-Diez A, Alonso C, Muntane J, et al. Obesity-dependent metabolic signatures associated with nonalcoholic fatty liver disease progression. J Proteome Res. 2012;11:2521–32.
27. Martinez-Arranz I, Mayo R, Perez-Cormenzana M, Minchole I, Salazar L, Alonso C, et al. Enhancing metabolomics research through data mining. J Proteome. 2015;127:275–88.
28. Jolliffe I. Principal component analysis. 2nd ed. New York: Springer; 2002.
29. Bylesjö M. OPLS discriminant analysis: combining the strengths of PLS-DA and SIMCA classification. J Chemometr. 2006;20:341.
30. Wiklund S, Johansson E, Sjostrom L, Mellerowicz EJ, Edlund U, Shockcor JP, et al. Visualization of GC/TOF-MS-based metabolomics data for identification of biochemically interesting compounds using OPLS class models. Anal Chem. 2008;80:115–22.
31. Garrido N, Bellver J, Remohi J, Simon C, Pellicer A. Cumulative live-birth rates per total number of embryos needed to reach newborn in consecutive in vitro fertilization (IVF) cycles: a new approach to measuring the likelihood of IVF success. Fertil Steril. 2011;96:40–6.
32. Garrido N, Bellver J, Remohi J, Alama P, Pellicer A. Cumulative newborn rates increase with the total number of transferred embryos according to an analysis of 15,792 ovum donation cycles. Fertil Steril. 2012;98:341,6.e1–2.
33. Muratori M, Luconi M, Marchiani S, Forti G, Baldi E. Molecular markers of human sperm functions. Int J Androl. 2009;32:25–45.
34. Flesch FM, Gadella BM. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochim Biophys Acta. 2000;1469:197–235.
35. Mann T, Lutwak-Mann C. Male reproductive function and semen. Springer, 1981. ISBN 978-1-4471-1300-3
36. Nissen HP, Kreysel HW. Analysis of phospholipids in human semen by high-performance liquid chromatography. J Chromatogr. 1983;276:29–35.
37. Lenzi A, Picardo M, Gandini L, Dondero F. Lipids of the sperm plasma membrane: from polyunsaturated fatty acids considered as markers of sperm function to possible scavenger therapy. Hum Reprod Update. 1996;2:246–56.
38. Force A, Grizard G, Giraud MN, Motta C, Sion B, Boucher D. Membrane fluidity and lipid content of human spermatozoa selected by swim-up method. Int J Androl. 2001;24:327–34.
39. Zhou X, Xia XY, Huang YF. Updated detection of the function of sperm plasma membrane. Zhonghua Nan Ke Xue. 2010;16:745–8.
40. Wathes DC, Abayasekara DR, Aitken RJ. Polyunsaturated fatty acids in male and female reproduction. Biol Reprod. 2007;77:190–201.
41. Aitken RJ, Muscio L, Whiting S, Connaughton HS, Fraser BA, Nixon B, et al. Analysis of the effects of polyphenols on human spermatozoa reveals unexpected impacts on mitochondrial membrane potential, oxidative stress and DNA integrity; implications for assisted reproductive technology. Biochem Pharmacol. 2016;121:78–96.
42. Watson PF. The roles of lipid and protein in the protection of ram spermatozoa at 5 degrees C by egg-yolk lipoprotein. J Reprod Fertil. 1981;62:483–92.
43. Holt WV, North RD. The role of membrane-active lipids in the protection of ram spermatozoa during cooling and storage. Gamete Res. 1988;19:77–89.
44. Moore AI, Squires EL, Graham JK. Adding cholesterol to the stallion sperm plasma membrane improves cryosurvival. Cryobiology. 2005;51:241–9.
45. Shadan S, James PS, Howes EA, Jones R. Cholesterol efflux alters lipid raft stability and distribution during capacitation of boar spermatozoa. Biol Reprod. 2004;71:253–65.

46. Serin I, Aksoy M, Ceylan A. Cholesterol-loaded cyclodextrin inhibits premature acrosomal reactions in liquid-stored rabbit spermatozoa. Anim Reprod Sci. 2011;123:106–11.
47. Moce E, Blanch E, Tomas C, Graham JK. Use of cholesterol in sperm cryopreservation: present moment and perspectives to future. Reprod Domest Anim. 2010;45(Suppl 2):57–66.
48. Purdy PH, Graham JK. Effect of adding cholesterol to bull sperm membranes on sperm capacitation, the acrosome reaction, and fertility. Biol Reprod. 2004;71:522–7.
49. Spizziri BE, Fox MH, Bruemmer JE, Squires EL, Graham JK. Cholesterol-loaded-cyclodextrins and fertility potential of stallions spermatozoa. Anim Reprod Sci. 2010;118:255–64.