A shift in follicular fluid from triacylglycerols to membrane lipids is associated with positive pregnancy outcome

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ABSTRACT: Follicular fluid (FF) is a liquid that surrounds the ovum. Its metabolite and, specifically, its lipid content have been associated with oocyte development. To characterize possible association between the lipid composition of FF and the outcome of pregnancy, we carried out a lipidomics study and compared the abundance of lipids from FF of patients with positive and negative outcomes. We found a differential lipid network wiring in positive-outcome FF, with a significant decrease (~2 fold; $P < 0.001$) in triacylglycerol levels and higher accumulation (10–50%; $P < 0.001$) of membrane lipids groups (phospholipids and sphingolipids). In addition to this major metabolic alteration, other lipid groups such as cholesteryl esters showed lower levels in positive-outcome patients, whereas derivatives of vitamin D were highly accumulated in positive-outcome FF, supporting previous studies that associate vitamin D levels in FF to pregnancy outcome. Our data also point to specific lipid species with a differential accumulation pattern in positive-outcome FF that predicted pregnancy in a receiver operating characteristic analysis. Altogether, our results suggest that FF lipid network is associated with the oocyte development, with possible implications in diagnostics and treatment.—Shehadeh, A., Bruck-Haimson, R., Saidemberg, D., Zacharia. A., Herzberg, S., Ben-Meir, A., Moussaieff, A. A shift in follicular fluid from triacylglycerols to membrane lipids is associated with positive pregnancy outcome. FASEB J. 33, 10291–10299 (2019). www.fasebj.org

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The metabolome (the sum of all small molecules found in a biologic sample) has been associated by many independent studies with the functional state of the cell or tissue examined (1, 2), including the follicular fluid (FF), a liquid composed of blood plasma constituents that cross the blood follicular barrier, and of secretions of follicle cells. It fills the follicular antrum and surrounds the oocyte, hence constructing its microenvironment. FF plays a key role in the nutritional and developmental support of the oocyte (3, 4), promoting oocyte meiosis (5, 6) and development (1, 7). FF metabolic profiling is therefore expected to uncover the metabolic network that supports oocyte development (1, 4, 7, 8). The lipidome (the composition of lipids) is a segment of the metabolome that raises special interest and is suggested to influence oocyte developmental potential (9).

Despite increasing awareness of the importance of the lipid content of the oocyte microenvironment, a characterization of lipid composition of the FF and its relation to pregnancy outcome is still lacking. A few pioneering studies on the accumulation of specific lipids in the FF and their association with the developmental potential of the oocyte indeed suggest such association; triacylglycerides [triglycerides or triacylglycerols (TAGs)] are the most abundant lipids in oocytes, constituting over 50% of all lipid material (8), and provide a large potential energy reserve. TAG levels inversely correlated to follicle size (10) and positively correlated to maternal body mass index (BMI) in several independent studies (11, 12). TAG accumulation in the FF was also correlated to the levels of adipokines and proinflammatory cytokines in FF (13).

Phospholipids (PLs) are the major component of biologic membranes and are involved in the modulation of

ABBREVIATIONS: BMI, body mass index; DAG, diacylglycerol; ES+, positive mode; FC, fold change; FDR, false discovery rate; FF, follicular fluid; IVF, *in vitro* fertilization; LC-MS, liquid chromatography mass spectrometry; MSE, mass spectrometry exploration; PL, phospholipid; PLS-DA, partial least-squares discriminant analysis; PPM, parts per million; ROC, receiver operating characteristic; SL, sphingolipid; TAG, triacylglycerol

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multiple cell functions and cellular interactions (14–17). Scarce information is available on the PL content of FF and its relation to embryo development. Several phosphocholines were found to show lower accumulation in poor ovarian responder patients (18). Choline and phosphocholine, precursors of phosphatidylcholines, also showed a differential accumulation in the FF of oocytes that developed into early cleavage-stage embryos (4). The total levels of PLs were implied to be inversely correlated with higher percentages of fertilized oocytes (3).

Cholesterol derivatives have also been implicated to be involved in female fertility; however, previous work on this group of lipids was mostly focused on gonadal hormones and lipoproteins (9). Recent studies have suggested a relationship between the abundance of molecular species of a vitamin D subgroup of cholesterol derivatives and *in vitro* fertilization (IVF) outcomes. However, current literature offers conflicting evidence for the levels and the roles of these lipids (19). In a few recent examples, vitamin D abundance was positively correlated to success of an IVF cycle (20), whereas others found that the FF level of 25-hydroxyvitamin D correlates negatively with the oocytes' ability to undergo fertilization and subsequent preimplantation embryo development (21) or that lower follicular 25-hydroxyvitamin D concentrations predicted a better response to ovarian stimulation (22).

Sphingolipids (SLs) form another notable group of bioactive lipids, some of them known to be mediating or regulating proliferative responses, growth inhibition, apoptosis, differentiation and senescence, and cell motility (23). The FF levels of 4 SL species were previously positively correlated with oocyte cleavage rate (24).

Given previous work that indicated roles played by specific lipid species in the development of the oocyte, we hypothesized that shifts in the lipid network of FF are associated with pregnancy outcome. We performed a lipidomics analysis of FF from IVF patients and found an association of FF lipid composition with pregnancy rate. The lipid signature of FF that corresponded with a positive outcome of pregnancy included lower accumulation of TAGs, diacylglycerols (DAGs), and cholesteryl esters but high accumulation of PLs, SLs, and vitamin D derivatives.

MATERIALS AND METHODS

Study population

Patients undergoing IVF were recruited at the Assisted Reproductive Technology center of the Hebrew University Hadassah Medical Center. The Institutional Review Board of Hadassah Medical Organization approved the study (decision number 0207-15-HMO), and each patient signed a consent form before oocyte retrieval. Exclusion criteria included male infertility or no embryo transfer. Patients underwent controlled ovarian hyper-stimulation by short GnRH agonist protocol or GnRH antagonist protocol as previously described in ref. 25. The ovarian response was assessed by ultrasound and E_2 levels every 2–3 d. Human chorionic gonadotropin, GnRH agonist, or both were administered to induce final oocyte maturation 36 h before oocyte retrieval. Oocyte retrieval was performed under general anesthesia using transvaginal aspiration with 16–17 gauge needles under ultrasonography guidance. After the oocytes were extracted by an embryologist, the residual FF was pooled and transferred to AM laboratory for sample preparation (shown herein). Fertilization was accomplished by IVF or intracytoplasmic sperm injection. The embryos were cultured in individual wells on a plate in a time-lapse incubator (EmbryoScope; VItrolife, Gothenburg, Sweden). Embryo transfer was done after 3–6 d according to the embryo morphologic grading. A pregnancy test was done 2 wk after embryo transfer.

Materials and reagents

All solvents but chloroform (HPLC grade) were liquid chromatography mass spectrometry (LC-MS) grade. Solvents were purchased from JT Baker (Phillipsburg, NJ, USA) except for isopropanol (Chemsolute; Th. Geyer, Renningen, Germany). Formic acid was purchased from Tokyo Chemical Industry (Tokyo, Japan). Leucine enkephaline-TOF-G2 XS Standard Kit was purchased from Waters (Milford, MA, USA), and sodium formate (98%) and ammonium fluoride (LC-MS) were purchased from Fluka (Buchs, Switzerland).

Sample preparation for LC-MS analysis

Following sample collection, FF samples were immediately centrifuged at 770 *g* for 10 min at 4°C to spin down cells, and the supernatant was collected. Samples were snap-frozen in liquid nitrogen and transferred to −80°C until analysis. For lipid extraction, we used a modified Bligh and Dyer biphasic extraction (26, 27). We optimized the extraction of lipids from FF in preliminary studies and found that the addition of 2% formic acid increased the total amount of lipids extracted as well as the number of lipid species detected. We therefore modified the Bligh and Dyer protocol by slightly acidifying the aqueous phase to improve PL yields and protein precipitation (27, 28). Extraction was performed on ice using ice-cold solvents. A total of 300 μl FF was thawed and transferred to clean glass tubes. A total of 375 μl chloroform and 750 μl methanol were added. Following 30 s of vortex, 375 μl of high-purity water with 2% formic acid was added. The mixture was vortexed for 30 s and then ultrasonicated for 30 s at 4°C × 5 cycles for extraction of lipids. Phase separation was carried out by centrifugation at 770 *g* for 10 min at 4°C. The lower phase containing lipids was transferred to clean glass tubes. Solvents were evaporated in a SC210A SpeedVac concentrator (Thermo Fisher Scientific, Waltham, MA, USA) at 30°C with a refrigerated solvent trap, and dry samples were kept at −80°C until analysis. For LC-MS run, samples were resuspended in 200 μl acetonitrile 95% 0.1% formic acid and then filtered through a 0.22 μm PTFE membrane for subsequent LC-MS analysis.

Ultra-HPLC quadrupole time-of-flight MS

Lipid analysis was performed using a Waters Acquity UPLC H-Class equipped with a photodiode array detector and a high-resolution, high-mass accuracy Xevo X2-XS Q-ToF (Waters) equipped with an electrospray ionization source. Electrospray ionization was used in positive (ES+) mode. A UPLC CSH C18 column (100 × 2.1 mm, 1.7 μm; Waters) was used for the separation of metabolites. The mobile phase consisted of 0.1% formic acid in water (phase A) and 0.1% formic acid (vol/vol) in acetonitrile (phase B). The linear gradient program was as follows: 60% mobile phase A (0.1% formic acid in water) and 40% mobile phase B for 1 min. The mobile phase B proportion was increased to 70% (v/v) in 5 min. From 5 to 8 min, mobile phase consisted of

24% A, 40% B, and 36% C (isopropanol); from 8 to 9 min, 20% A, 35% B, and 45% C; from 9 to 12 min, 18.4% A, 33% B, and 48.6% C; from 12 to 17 min, 12% A, 25% B, and 63% C; and up to 25 min, 0.4% A, 10.5% B, and 89.1% C. From 25.51 to 35 min, the system was allowed to re-equilibrate to the initial conditions. Following preliminary experiments, the retention time of 1.0–25 min was used for analysis. The flow rate was 0.4 ml/min, and the column temperature was kept at 60°C. Capillary spray was maintained at 3.0 kV, cone voltage at 40 eV, and collision energy at 15 eV. All masses went through MS^E analyses; collision energy was 40–65 eV for positive mode and 30–60 eV for negative mode. Full-scan and MS^E mass spectra were acquired from 30 to 2000 Da. Argon was used as the collision gas for collision-induced dissociation. The mass spectrometer was calibrated using sodium formate, and leucine enkephalin was used as the lock mass (*m/z* 556.2771, 200 pg/ml) and continuously infused at 6 μl/min; data was acquired from positive (ES+) mode. MassLynx software v.4.1 (Waters) was used to control the instrument and calculate accurate masses. Postcolumn derivatization was employed with ammonium fluoride to improve the yields of the neutral charged lipids in the ES+ mode as $[M+NH_4]^+$. One millimolar ammonium fluoride in 50:50 methanol:water was automatically continuously injected into the MS together for post-column derivatization in positive-mode runs to improve the yields of neutral-charged lipids.

Two quality control sets were used to assure data quality; a pool of all samples from the current analysis was injected after every 10 samples, and a second sample containing 9 lipid standards [arachidonic acid 5 μM; N-hexanoyl-D-sphingosine 10 μM; stearoyl-SN-glycero-3-phosphocholine 10 μM; 24:0 C24 ceramide-1-phosphate (d18:1/24:0) 10 μM; and triglyceride mix (a mix of 5 standards): triacetin (C2:0), tributyrin (C4:0), tricaproin (C6:0), tricaprylin (C8:0), and tricaprin (C10:0); MilliporeSigma, Burlington, MA, USA)] was injected every other 10 samples.

A standard was used to validate the identification of 25-hydroxyvitamin D3 monohydrate (MilliporeSigma).

MassLynx 4.1 (Waters) was used for mass spectra visualization, and Progenesis QI (Nonlinear Dynamics, Newcastle upon Tyne, United Kingdom) was used for spectra deconvolution, alignment, normalization, and identification. To exclude masses that were not originated in FF samples, blank samples (solvents that went through sample preparation but contained no FF) were injected. Masses with minimum intensity cutoff of 100 *m/z*, lowest mean abundance in blank, and fold change (FC) over 100 from blank were used for analysis. The generation of partial least-squares discriminant analysis (PLS-DA) and a heat map and the corresponding analyses (permutation test and volcano plot) were carried out using MetaboAnalyst 4.0 (29). For multivariate tests, range scaling was used to eliminate the dependence of the rank of the lipids on their measured abundance (30). MS^E was used for acquisition of exact mass precursor and fragment ion spectra from every detectable component of the samples. Lipid identification was then carried out according to exact mass (mass accuracy < 5 PPMs), retention time (the different lipid groups have typical retention times; see **Fig. 3*A*, *B***), isotope pattern, and fragmentation pattern. Data from all lipids was compared against 18 metabolite libraries compatible with Progenesis QI. The exact mass, isotope pattern, and fragmentation pattern of lipids were then further validated against theoretical data. Tandem MS experiments were carried out for further validation identities of 32 differential lipids [FC > 3 and false discovery rate (FDR)-adjusted $P \leq 0.05$ from the ES+ data set]. A receiver operating characteristic (ROC) curve was generated according to the data of 6 highly differential lipids suggested in **Fig. 4** using Matlab (v.R2017a; MathWorks, Natick, MA, USA). Prediction accuracy, prediction sensitivity, and specificity were calculated.

RESULTS

We sought to define the metabolic alterations in the microenvironment of the oocyte that are associated with pregnancy outcome and may influence the oocyte development. To optimize the sample preparation for the highest yield of metabolites from the FF, we tested different extraction systems. Surprisingly, we found that chloroform extraction of the aqueous FF yielded the highest number of metabolites and the highest abundant metabolites (Supplemental Fig. S1), suggesting that lipids constitute a major component of the FF metabolome. We further optimized the lipid extraction from the FF by a mild acidification that resulted in increase in the yield of lipids (Supplemental Fig. S1*B*, *C*). We therefore utilized this extraction system for the preparation of samples of the main cohort of IVF patients.

A total of 109 women underwent fresh embryo transfer with U.S. guidance. After the exclusion of patients with male factor background or unknown pregnancy outcome, the lipid composition of FF of 71 patients (**Fig. 1**) was taken for lipidomics analysis. Demographic and gynecologic features as well as IVF treatment–related data are presented in **Table 1**. As expected, the clinical data point to differences between the positive- and the negative-outcome groups in the age and BMI of the patients.

Our lipidomics study resulted in 9953 features detected (ES+). Following stringent exclusion of possible artifactual features (masses that match those found in blank samples; see Methods section), 1571 features were assigned as FF

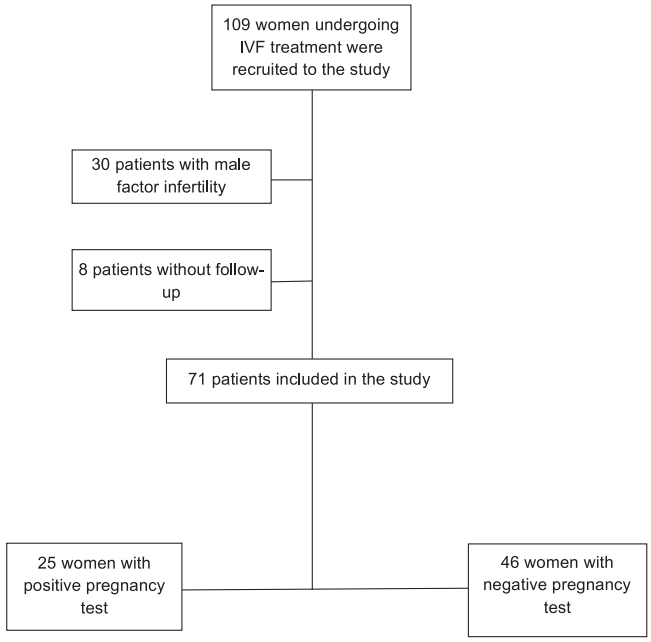

Figure 1. A flow chart of participants in the study.

TABLE 1. *Patient characteristics, IVF protocol, and pregnancy outcome*

Characteristic	Pregnancy + (n = 25)	Pregnancy − (n = 46)	P value
Age (yr)	34.8 ± 7.1	38.2 ± 5.1	0.02
BMI (kg\m^2)	23.1 ± 6.6	27.4 ± 6.8	0.01
Gestation	1.2 ± 1.2	0.8 ± 1.2	0.26
Deliveries	0.6 ± 0.9	0.2 ± 0.5	0.05
Miscarriages	0.5 ± 0.8	0.5 ± 0.9	0.93
Infertility diagnosis			
Ovulation dysfunction	4	7	0.93
Mechanical factor	4	5	0.53
Unexplained infertility	12	25	0.61
PGD	3	4	0.66
No. of cycles	1.8 ± 1.4	2.0 ± 1.3	0.54
Protocol			
Antagonist	17	27	0.44
Short agonist	8	17	0.68
Natural		1	-
Long protocol		1	-
No. of follicles	11.2 ± 5.7	8.5 ± 6.4	0.09
E_2 max (pM)	6356 ± 2633	5795 ± 3188	0.46
Oocytes no.	10.2 ± 6.0	8.7 ± 8.0	0.40

PGD, preimplantation genetic diagnosis.

lipids. A total of 1032 of these were putatively identified. The identification of 32 lipids that showed the most discriminative accumulation (>3 FC and $P \leq 0.05$ after the adjustment for FDR) was further validated by tandem MS experiments.

A of all FF-originated features (1571) showed a separation between the FF lipid composition of the positive- and negative-outcome patients (**Fig. 2*A***; $R^2 = 0.83$ and $Q^2 = 0.47$). To address a possible overfit, a permutation test was performed with 1000 permutations, suggesting prediction accuracy during training of empirical P value ($P < 0.001$). The alterations in the lipid composition of positive-outcome patients is underscored by a heat map of the top 100 discriminative lipids (based on Student's t test) between the positive- and negative-outcome FF samples (Fig. 2*B*).

To better understand the nature of the lipid signature and the metabolic processes that are responsible for the separation of the FF lipidome of positive- and negative-outcome patients, we grouped the lipids in the list of variable importance in projection. This list demonstrated a differential wiring of the lipid network throughout different biosynthetic lipid groups, suggesting shifts in the metabolism of TAGs, DAGs, PLs, lysophospholipids, SLs, sphingomyelins, cholesteryl esters, and vitamin D derivatives. Based on the characteristic retention time frames, we could see differences between positive- and negative-outcome FF even in the total ion current chromatograms (Fig. 3*A, B*). We measured the relative abundance of lipids from each of these biosynthetic groups in the FF of patients. We compared the accumulation of total lipid species detected in all samples (after the exclusion of possible

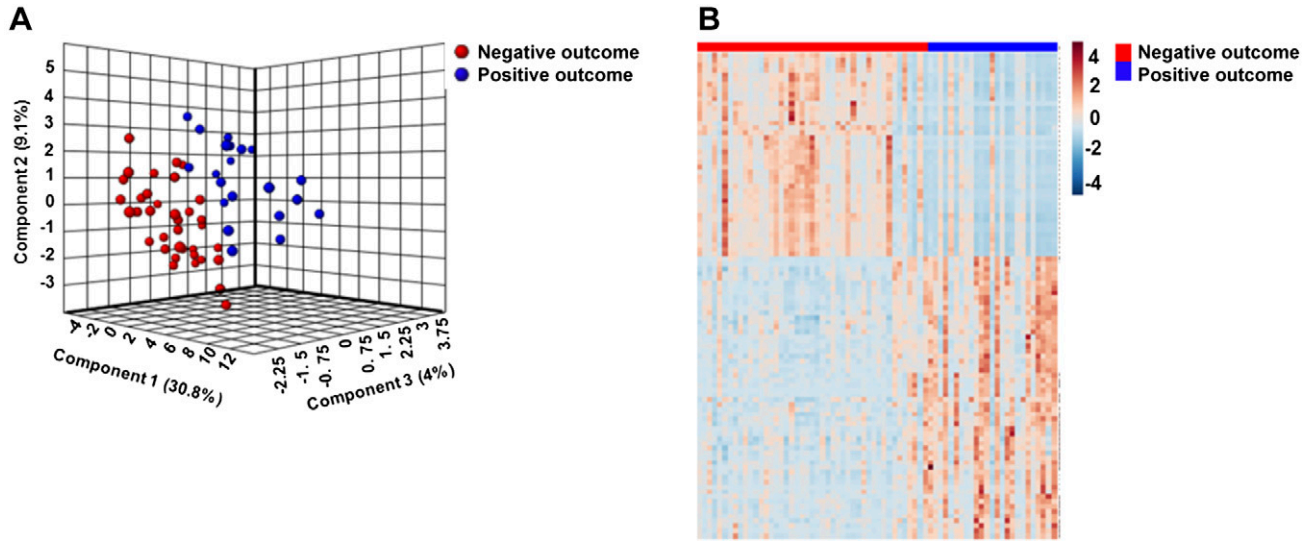

Figure 2. Positive-outcome FF lipid composition separates from that of negative-outcome FF. *A*) A PLS-DA of patients divided by the pregnancy outcome. *B*) A heat map of the top 100 lipids based on Student's t test.

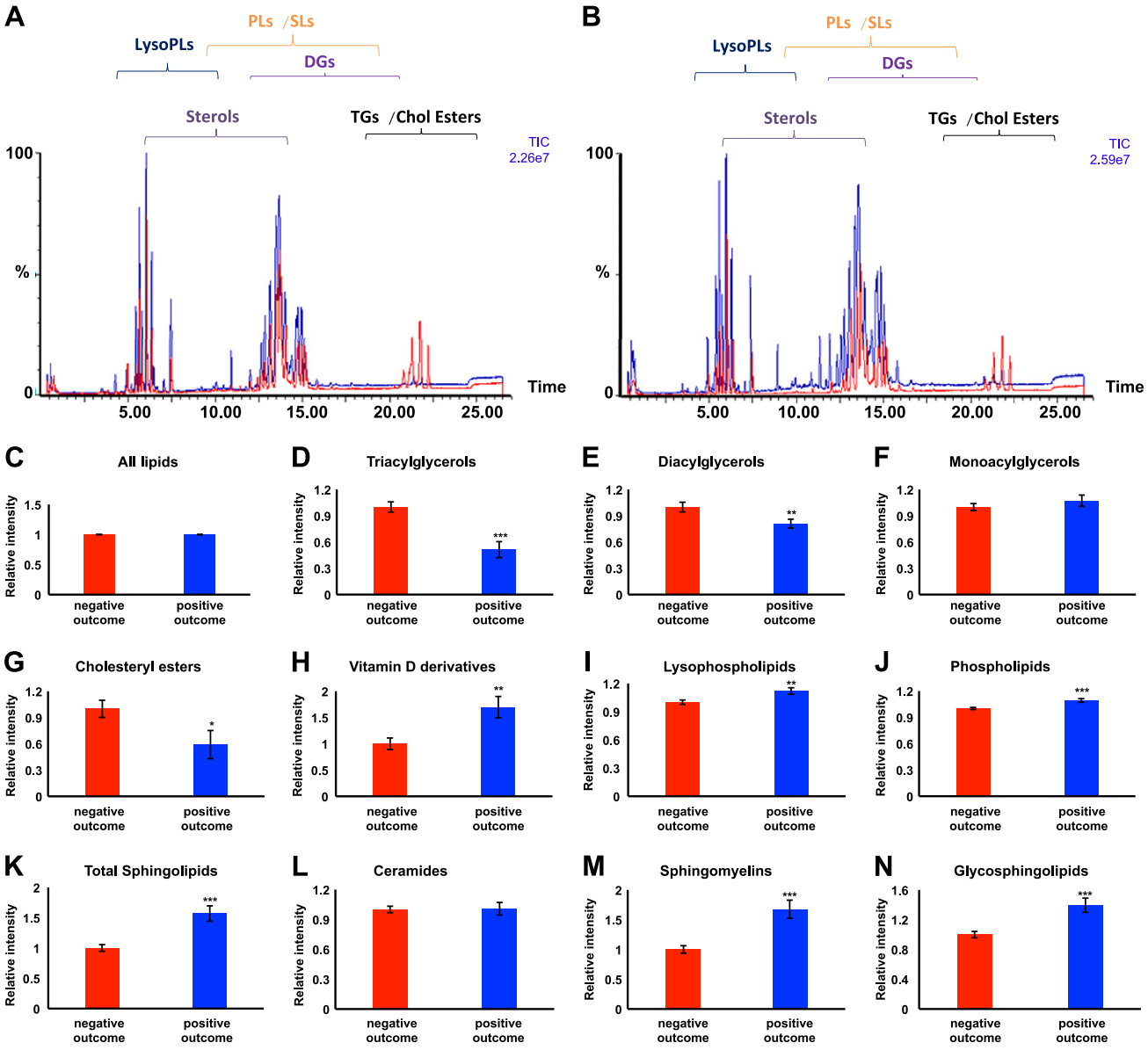

Figure 3. The accumulation of plasma lipids is lower in the FF of positive-outcome patients. *A*, *B*) Total ion chromatograms of FF from representative positive (blue) and negative (red) outcome patients. Two representative aged (*A*) and younger (*B*) patients are shown. *C–N*) The cumulative abundance is shown for all lipids (*C*), TAGs (*D*), DAGs (*E*), Monoacylglycerols (*F*), cholesteryl esters (*G*), vitamin D derivatives [25-hydroxyvitamin D3; trihydroxyvitamin D3; 1α,25-dihydroxy-23-oxavitamin D3; 2β-methoxy-1α,25-dihydroxyvitamin D3; 1α,25-dihydroxy-2β-(5-hydroxypentoxy)vitamin D3/1α,25-dihydroxy-2β-(5-hydroxypentoxy)-cholecalciferol; 24,25-dihydroxyvitamin D3; 1α-hydroxy-26,27-dimethylvitamin D3/1α-hydroxy-26,27-dimethylcholecalciferol] (*H*), lysophospholipids (*I*), PLs (*J*), total sphingolipids (*K*), and SL subgroups ceramides (*L*), sphingomyelins (*M*), and glycosphingolipids (*N*). Error bars represent SEM. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

artifactual features) and found no difference between the positive- and negative-outcome FF (Fig. 3*C*). However, we found changes across the different lipid biosynthetic groups. Importantly, while the accumulation of several lipid groups was lower in the positive-outcome FF, the accumulation of others was higher (Fig. 3*D–N*). The most striking difference in the lipid composition of positive- and negative-outcome FF was the lower accumulation of TAGs in positive-outcome patients (Fig. 3*D*). The levels of DAGs in the FF also showed an inverse correlation to pregnancy outcome, with milder but still highly significant differences (Fig. 3*E*), whereas no difference was noted in the accumulation of monoacylglygerols (Fig. 3*F*). We found changes in the accumulation of the cholesterol derivatives cholesteryl esters and vitamin D derivatives [25-hydroxyvitamin D3; trihydroxyvitamin D3; 1α,25-dihydroxy-23-oxavitamin D3; 2β-methoxy-1α,25-dihydroxyvitamin D3; 1α,25-dihydroxy-2β-(5-hydroxypentoxy)vitamin D3/1α,25-dihydroxy-2β-(5-hydroxypentoxy)cholecalciferol; 24,25-dihydroxyvitamin D3; 1α-hydroxy-26,27-dimethylvitamin D3/1α-hydroxy-26,27-dimethylcholecalciferol]. Interestingly, though the accumulation of cholesteryl esters is lower in the positive-outcome FF (Fig. 3*G*), the levels of vitamin D derivatives are higher (Fig. 3*H*), suggesting metabolic shift or shifts that take place downstream to the cholesterol synthesis. Given conflicting reports regarding the association

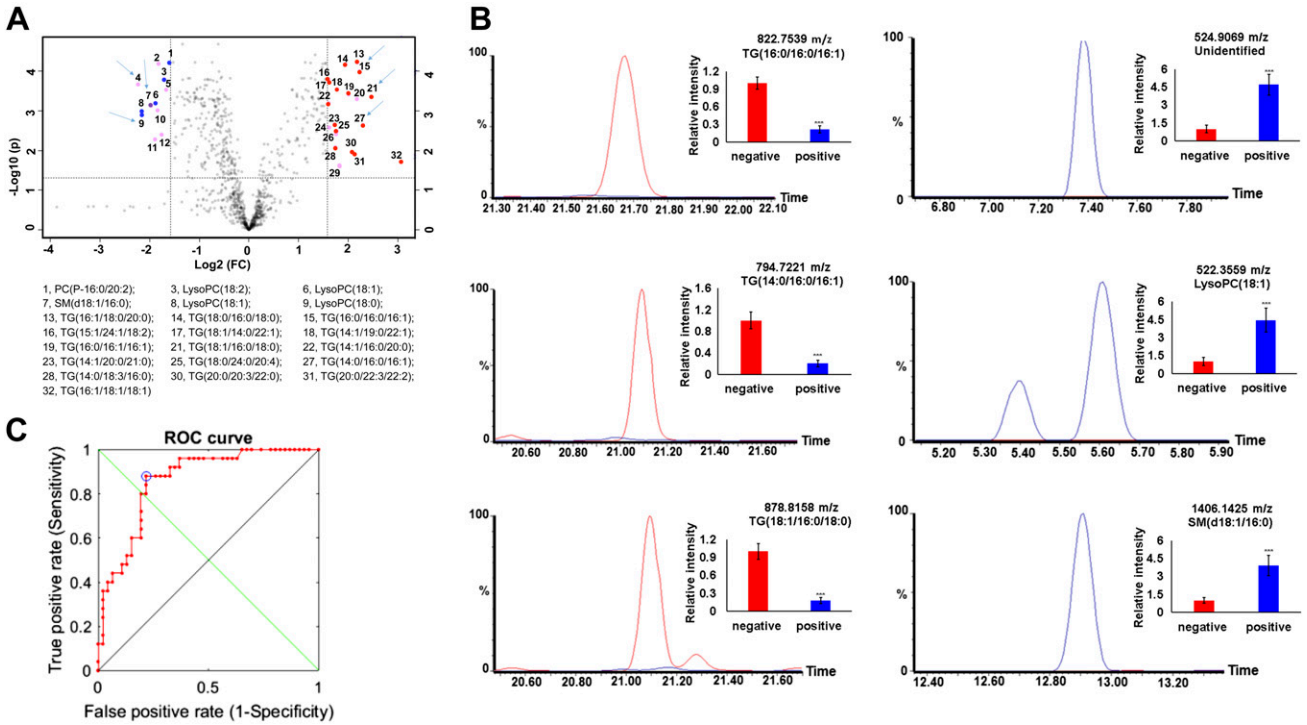

Figure 4. Highly differential FF lipids may be used for predicting successful pregnancies. *A*) A volcano plot representing lipids with a significant (FC > 3 and FDR-adjusted $P \leq 0.05$) accumulation in the FF of positive- and negative-outcome patients. Blue, lipids with high accumulation in FF of positive-outcome patients; red, lipids with low accumulation in FF of positive-outcome patients; pink, unidentified lipids. Lipids that are highly abundant in positive-outcome FF are in blue; lipids with low levels in the positive-outcome FF in red. Arrows point to the 6 most discriminant lipids. 1, PC(P-16:0/20:2); 3, LysoPC(18:2); 6, LysoPC(18:1); 7, SM(d18:1/16:0); 8, LysoPC(18:1); 9, LysoPC(18:0); 13, TG(16:1/18:0/20:0); 14, TG(18:0/16:0/18:0); 15, TG(16:0/16:0/16:1); 16, TG(15:1/24:1/18:2); 17, TG(18:1/14:0/22:1); 18, TG(14:1/19:0/22:1); 19, TG(16:0/16:1/16:1); 21, TG(18:1/16:0/18:0); 22, TG(14:1/16:0/20:0); 23, TG(14:1/20:0/21:0); 25, TG(18:0/24:0/20:4); 27, TG(14:0/16:0/16:1); 28, TG(14:0/18:3/16:0); 30, TG(20:0/20:3/22:0); 31, TG(20:0/22:3/22:2); 32, TG(16:1/18:1/18:1). *B*) The chromatograms of 6 selected differential lipids in positive-outcome FF (data from all patients are included in the graphs). The graph for 522.3559 represents the larger peak on the right side of the chromatogram. ***$P \leq 0.001$ by Student's *t* test. *C*) An ROC curve describing the predictive ability of 6 of the differential lipids. The blue circle represents the cutoff point. The area under the curve was 0.85 with an SE of 0.05.

between the accumulation of vitamin D in the FF and pregnancy rates, we further validated the identification of 25-hydroxy vitamin D, the major vitamin D metabolite, by use of a standard. Though the higher accumulation of 25-hydroxy vitamin D in positive-outcome FF did not reach significance, the accumulation of the total vitamin D derivatives was significantly higher in the positive-outcome FF.

Lysophospholipids and PLs (Fig. 3*I, J*) showed higher accumulation in positive-outcome patients. The change in the levels of PLs is especially notable given the tight regulation of their biosynthesis and abundance necessary for maintaining homeostasis (31). The total abundance of another group of membrane lipids, SL species, was also higher in positive-outcome FF (Fig. 3*K*). As SLs constitute an extremely versatile group of lipids with great structural and functional diversity, we studied the accumulation of notable subgroups of SLs. No difference was noted in the levels of ceramides, the simplest SL species (composed of sphingosine and a fatty acid; Fig. 3*L*). In contrast, sphingomyelins (sphingophospholipids; Fig. 4*M*) and glycosphingolipids (SLs with attached carbohydrate chains; Fig. 3*N*) followed the accumulation pattern of total SLs, with higher abundance in the positive-outcome FF. To exclude a possible age-related effect on lipid composition that may be irrespective of pregnancy outcome, we compared the accumulation of the lipid groups in the FF of positive- and negative-outcome patients within age groups, with similar results to the ones obtained for the data set that includes all age groups (Supplemental Figs. S2 and S3).

Finally, we wished to point to specific lipids with the most distinguishable accumulation in positive-outcome FF. We determined both the FC and the statistical evaluation (given as FDR-adjusted *P* values) of the abundance of specific lipid species in positive- and negative-outcome FF. These are presented as a volcano plot, which suggested the 32 most discriminant lipids [FC > 3 and an FDR-adjusted $P \leq 0.05$; Fig. 4*A*). The list of the lipids that demonstrated the highest FC together with significant *P* values consisted of TAGs, PLs, lysophospholipids, and 1 sphingomyelin (Fig. 4*A*). A table of the exact masses, retention time, mass fragments, and putative identification of these differential lipids is provided as Supplemental Table S1 in Supplemental Information. We further pursued our analyses to provide a possible straightforward and immediate MS-based assessment of the pregnancy

potential. By examining the accumulation of several selected lipids, the distinction of positive-outcome FF became apparent by their chromatograms, even with no biostatistical processing (Fig. 4*B*). A ROC curve analysis of the predictive ability of 6 highly differential lipids resulted in a diagnostic performance of 86% area under curve.

Altogether, our data provide an FF lipid signature of the outcome of pregnancy that may be easily and immediately determined.

DISCUSSION

Lipids serve a variety of functions as a source of energy, a major structural component of plasma and organelle membranes, signaling molecules, and protein regulators. To determine the alterations in FF lipid network in positive-outcome patients, we performed a lipidomics analysis that revealed an association between FF lipid composition and the outcome of pregnancy. Our analysis points to a profound alteration in lipid composition of the FF that is associated with positive outcome throughout the following major lipid groups: TAGs, PLs, SLs, and cholesterol derivatives. This implies the importance of the lipid metabolic network in the oocyte microenvironment. Interestingly, as a general trend, high levels of major plasma lipids such as TAGs, DAGs, and cholesteryl esters are associated with negative pregnancy outcome, whereas the accumulation of major membrane lipids such as PLs and SLs is associated with positive outcome.

The link between high FF TAG levels and decreased oocyte development may be causal because TAGs were reported to inflict endoplasmic reticulum stress and impaired mouse oocyte maturation (12). Importantly, though our lipidomics analysis is well aligned with findings of previous studies, pointing to an inverse association between the total levels of TAGs and pregnancy outcome, it does not support the notion of a global high lipid content of the microenvironment of the oocytes as has been previously suggested (12, 32, 33). In fact, our data point to high accumulation of specific groups of lipids (PLs, lysophospholipids, SLs, vitamin D derivatives) in the positive-outcome FF, whereas the levels of other lipid groups (TAGs, DAGs, and cholesteryl esters) are lower, thus providing a detailed characterization of the lipid composition of the FF. It has been demonstrated that the total lipid content in the FF is increased in women with obesity, leading to lipotoxicity and to impaired oocyte maturation and early embryonic loss (12, 32, 34). Though female fertility is sensitive to age and body weight (35), the mechanism by which maternal fat-related signals are transduced to affect the developing oocyte and embryo is not clear (8, 36–38). It is likely, however, that these signals are mediated by the FF, which is mediating maternal signals to the oocyte. Given that TAG levels were inversely correlated to follicle size (10) but positively correlated to maternal BMI (11, 12) and to the levels of adipokines and proinflammatory cytokines in FF (13), it would be of interest to study the possible role played by FF TAG levels in the effect of overweight on female fertility, potentially *via* increased inflammatory response.

The higher abundance of PLs in positive outcome FF demonstrated by our analyses is in seeming discrepancy with the data of Fayezi *et al.* (3) that suggest that PL total abundance is inversely correlated with higher percentages of fertilized oocytes. This seeming discrepancy may be the result of the different end points in the two studies. Fayezi *et al.* (3) measured the percentage of fertile oocytes, whereas our end point was pregnancy outcome. However, the differences in the conclusions of the two studies may also be the result of the different technologies used in the former study, in which the total PL content was separated by 1-dimensional thin-layer chromatography, and phosphorus content of PL was estimated after hydrolysis with concentrated perchloric acid. The researchers do not mention the use of PL standards for the thin-layer chromatography, the indicator for phosphorous content, or whether the phosphorous estimation included phosphorous from degraded lipoproteins.

Cholesteryl esters, formed by the esterification of cholesterol with long-chain fatty acids, constitute the means by which cholesterol is transported through the blood by lipoproteins. The levels of cholesteryl esters are lower, whereas that of vitamin D derivatives is higher in positive-outcome FF, suggesting a shift in the positive-outcome FF downstream to cholesterol. The finding of the differentially total accumulation of vitamin D derivatives sheds new light on an ongoing debate on the association between the abundance of vitamin D in blood plasma or FF and pregnancy outcome. Our results corroborate the notion that vitamin D metabolism is indeed associated with embryo quality (39) and successful pregnancy (20, 40, 41). Our results also point to the patient's age as one of the possible reasons to the discrepancies in the literature because significant differences were only demonstrated in the younger patient group.

It would be important to note that the similar pattern of lipid accumulation in 2 age groups (with the exception of vitamin D derivatives) supports the differences found in specific lipid groups in the positive-outcome FF in the analysis of the main data set and overrules possible age-related bias in the results. Taken together with previous literature (*e.g.*, the work of Yang *et al.*) (12), our analyses point to a link between FF lipid profile and pregnancy outcome and imply that a supportive FF lipid composition may be essential for the development of the embryo.

To the best of our knowledge, the changes in the lipid network demonstrated in our results provide the first detailed characterization of lipid composition in the FF and thus sheds new light on many previous studies that associate the lipid content to oocyte development. In addition to the contribution to basic understanding of the metabolic microenvironment of the oocyte, these results may have implications in terms of our ability to predict pregnancy. Such prediction may be straightforward and immediate by using potential markers of

positive and negative outcome, such as the ones suggested by our analyses, and by establishing the correlation between their ratios and pregnancy outcome. However, further work is needed to establish that the outcome of pregnancy may be predicted by the lipid composition of FF. The current study may also provide new targets for potential interventions that would improve the metabolic support of the developing oocyte. FJ

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AUTHOR CONTRIBUTIONS

A. Shehadeh collected and prepared samples, performed research, and analyzed data; R. Bruck-Haimson collected and prepared samples, and performed research; S. Herzberg collected FF samples and analyzed clinical data; D. Saidemberg analyzed data; A. Zacharia analyzed data; A. Ben-Meir provided FF samples and analyzed clinical data; and A. Moussaieff designed research, analyzed data, interpreted data, and drafted the manuscript.

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