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Identification of novel candidate plasma metabolite biomarkers for distinguishing serous ovarian carcinoma and benign serous ovarian tumors

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Abstract

OBJECTIVE—Serous ovarian carcinoma (OC) represents a leading cause of cancer-related death among U.S. women. Non-invasive tools have recently emerged for discriminating benign from malignant ovarian masses, but evaluation remains ongoing, without widespread implementation. In the last decade, metabolomics has matured into a new avenue for cancer biomarker development. Here, we sought to identify novel plasma metabolite biomarkers to distinguish serous ovarian carcinoma and benign ovarian tumor.

METHODS—Using liquid chromatography-mass spectrometry, we conducted global and targeted metabolite profiling of plasma isolated at the time of surgery from 50 serous OC cases and 50 serous benign controls.

RESULTS—Global lipidomics analysis identified 34 metabolites (of 372 assessed) differing significantly ($P < 0.05$) between cases and controls in both training and testing sets, with 17 candidates satisfying FDR $q < 0.05$, and two reaching Bonferroni significance. Targeted profiling of ~150 aqueous metabolites identified a single amino acid, alanine, as differentially abundant ($P < 0.05$). A multivariate classification model built using the top four lipid metabolites achieved an estimated AUC of 0.85 (SD=0.07) based on Monte Carlo cross validation. Evaluation of a hybrid model incorporating both CA125 and lipid metabolites was suggestive of increased classification

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Author contributions

Conception and design: M.F.B., D.R., C.I.L. Subject recruitment: N.U., C.D. Data acquisition: H.G., J.Z., D.D. Analysis and interpretation of data: M.F.B. Drafting of the manuscript: M.F.B., H.G., D.D., J.Z. Study supervision: D.R., C.I.L. All authors critically revised the manuscript for intellectual content.

Conflicts of Interest

D.R. holds equity and an executive role in Matrix-Bio, Inc. (IN, USA). D.R., H.G., and C.I.L. have filed a provisional patent application based on the use of plasma lipid metabolite biomarkers for clinical triage of ovarian masses. The authors have no other potential conflicts of interest to disclose.

accuracy (AUC=0.91, SD=0.05) relative to CA125 alone (AUC=0.87, SD=0.07), particularly at high fixed levels of sensitivity, without reaching significance.

CONCLUSIONS—Our results provide insight into metabolic changes potentially correlated with the presence of serous OC versus benign ovarian tumor and suggest that plasma metabolites may help differentiate these two conditions.

Introduction

Serous ovarian carcinoma (OC) represents a leading cause of cancer-related death among U.S. women [1]. Among women who present with an ovarian mass and are referred for surgical excision, an important clinical challenge involves identifying, prior to surgery, those with malignancy versus benign disease. Prognosis and survival are significantly improved when surgical interventions are performed by tertiary care specialists [2]. Recently-developed methods for assessing the likelihood of cancer, such as the Risk of Ovarian Malignancy Algorithm (ROMA) and the “OVA1” test, are based on blood levels of CA125 and other proteins—HE4, prealbumin, transferrin, β 2 microglobulin, and apolipoprotein AI [3–7]. These approaches have shown promise but continue to be evaluated, and will likely require further validation in multi-center trials prior to widespread clinical adoption [8–12].

Metabolomics has emerged as a promising domain for identifying molecular signatures associated with cancer [13–15]. In contrast to genomics, transcriptomics, or proteomics, metabolomics describes the study of concentrations and fluxes of low molecular weight metabolites in biofluids or tissue [16, 17]. As metabolic fluctuations lie downstream of alterations at the DNA, RNA, and protein levels, metabolomics offers a sensitive and comprehensive functional read-out of biological systems. Metabolite alterations in tissue or biofluids have been linked to a broad spectrum of human cancers [18–21]. Adaptive changes such as increased rates of aerobic glycolysis (the Warburg effect), amino acid metabolism, and lipid turnover are thought to be important features of the neoplastic process that facilitate sustained cellular proliferation and tumor expansion [13].

Several past metabolomics studies have focused on OC, and used liquid chromatography (LC)-mass spectrometry (MS), gas chromatography (GC)-MS, or nuclear magnetic resonance (NMR) to evaluate tissue [22, 23], blood [24–27], or urine [28, 29]. While these reports have described changes in the levels of multiple classes of metabolites (purines/pyrimidines, lipids, tricarboxylic acid (TCA) intermediates, and amino acids) in women with versus without ovarian cancer, minimal consensus has emerged with respect to robust, consistent metabolic signatures, either within or across biospecimen types (tissue, blood, urine). Nevertheless, three independent studies have implicated alterations in lipids and/or phospholipids in either plasma or tissue from OC cases [22, 26, 27]. Comprehensive lipidomics profiling on OC specimens, however, has not been conducted previously.

We used two independent LC-MS-based metabolomics platforms, including a recently-optimized in-house global lipidomics approach, to screen for differentially abundant plasma metabolites between cases with serous ovarian carcinoma and controls with benign serous ovarian tumor. Plasma samples isolated at the time of surgery from 50 cases and 50 controls were selected from a high-quality local biorepository and subjected to i) global lipidomics

profiling of >600 lipid metabolites, and ii) targeted aqueous profiling [21] of ~160 selected metabolites encompassing several major metabolic pathways. The potential utility of derived metabolite profiles to discriminate between cases and controls was investigated through construction and evaluation of multivariate classification models.

Subjects and Methods

Study population

Participants were recruited between May 2006 and August 2011 to support protocols of the Pacific Ovarian Cancer Research Consortium (POCRC) by physicians at Pacific Gynecology Specialists, Swedish Medical Center (Seattle, WA). Specimen collection was funded by the National Cancer Institute's Specialized Programs of Research Excellence (SPORE) program. All subjects were postmenopausal women who had been referred for surgical excision of an ovarian mass, and had no prior history of cancer. 50 cases with serous ovarian carcinoma and 50 controls with benign serous ovarian tumor were selected for analysis. Controls were frequency-matched to cases by age, ethnicity, body-mass index (BMI), parity, use of oral contraceptives and year of blood draw. All diagnoses were made in accordance with uniform histological and pathological guidelines. Demographic and lifestyle characteristics were obtained through questionnaires filled out by patients prior to surgery. Cases and controls were randomly and equally distributed into either a training set (n=50) or testing set (n=50). This study was approved by the Institutional Review Board at the Fred Hutchinson Cancer Research Center.

Plasma specimens

Twelve-hour fasted blood samples from included study participants were collected at the time of surgery and processed according to standardized protocols, as described previously [30]. All blood specimens were processed within four hours post-draw. Plasma samples selected for analysis were distributed into three batches, such that case and control specimens were equally represented and randomly ordered within-batch. Each sample was assigned a unique laboratory identification number, which specified the order of processing and blinded laboratory personnel to sample identities. Specimens were delivered on dry ice to the Northwest Metabolomics Research Center and stored at -80 °C until use.

Sample preparation

Detailed protocols for the preparation of samples analyzed via targeted aqueous metabolite profiling have been published previously [21]. For global lipidomics, frozen plasma samples were thawed at room temperature for 30 min. 20 µL of each sample was mixed with 200 µL chloroform/methanol (2:1, v:v). The chloroform/methanol solution contains the following five lipids standards: PC(17:0/0:0) at 8.75 µM, PC(17:0/17:0) at 5.85 µM, PE (17:0/17:0) at 6.19 µM, TG(17:0/17:0/17:0) at 4.20 µM, and Cer(d18:1/17:0) at 6.46 µM. The lipid standards were used to monitor sample analysis performance, and were purchased from Avanti Polar Lipids, Inc (Alabaster, Alabama). The mixture was then vortexed for 2 minutes and incubated for 30 minutes at room temperature (25°C), followed by centrifugation at 14,000 × g for 5 min. 120 µL of the lower (organic) phase were then collected and loaded into a glass vial for lipidomics analysis.

LC-MS/MS Analysis

Targeted LC-MS/MS experiments were performed on an Agilent 1260 LC (Agilent Technologies, Santa Clara, CA) AB Sciex QTrap 5500 MS (AB Sciex, Toronto, Canada) system, controlled by Analyst 1.5 software (AB Sciex, Toronto, Canada), as described previously [21]. Two sample injections (2 μ L and 10 μ L) were used for LC-MS/MS analysis in positive and negative ion modes, respectively. To assess instrument performance and process reproducibility, replicates of two independent quality control (QC) serum samples were injected after every 10 patient samples. Chromatographic separations were performed in hydrophilic interaction chromatography (HILIC) mode (SeQuant ZIC-cHILIC, 150 $\times$ 2.1 mm, 3.0 μ m, Merck KGaA, Darmstadt, Germany). The flow rate and column temperature were set to 300 μ L/min and 45 $^{\circ}$ C, respectively. Multiple reaction-monitoring (MRM) mode was used to detect metabolites of interest. The 154 metabolites selected for targeted analysis represent numerous major metabolic pathways (eg. glycolysis/TCA cycle, amino acid, nucleotide, and lipid metabolism) [21].

Global Lipidomics Analysis

LC-QTOF-MS experiments were performed using an Agilent 1200 SL LC system coupled online with an Agilent 6520 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA). Each prepared sample (4 μ L for positive ESI ionization, 8 μ L for negative ESI ionization) was injected onto an Agilent Zorbax 300 SB-C8 column (2.1 $\times$ 50 mm, 1.8- μ m), which was heated to 50 $^{\circ}$ C. The flow rate was 0.4 mL/min. Mobile phase A was 5 mM ammonium acetate and 0.1% formic acid in water, and mobile phase B was 5% water in ACN containing 5 mM ammonium acetate and 0.1% formic acid. The mobile phase composition was kept isocratic at 35% B for 1 min, and was increased to 95% B in 19 min; after another 10 min at 95% B, the mobile phase composition was returned to 35% B. The ESI voltage was 3.8 kV. The mass accuracy of our LC-MS system is generally less than 5 ppm; the Q-TOF MS spectrometer was calibrated prior to each batch run, and a mass accuracy of less than 1 ppm was often achieved using the standard tuning mixture (G1969-85000, Agilent Technologies, Santa Clara, CA). The mass scan range is 100–1600, and the acquisition rate was 1.5 spectra/s. The absolute intensity threshold for MS data collection was set to 100, and the relative threshold was 0.001%.

Measurement of CA125

A customized in-house bead-based ELISA assay was used to assess plasma CA125 levels, as described previously [30, 31]. All measurements were conducted in the laboratory of Dr. Nicole Urban at the Fred Hutchinson Cancer Research Center.

Data processing and regression analyses

For global lipidomics profiling, raw spectra were processed using MassHunter Qualitative Analysis Software and Mass Profiler Professional Software (Agilent). For targeted aqueous profiling, peak intensities for detected metabolites were integrated using Analyst 1.5 software (AB SCIEX). Peak integrals were used for further analyses. Profiled analytes were excluded if i) detectable signal was absent in >33% of all global lipidomics study samples, or >4% of all targeted aqueous study samples, or ii) the coefficient of variation (CV) across

all QC samples exceeded 20% (global) or 15% (targeted). 618 of 1706 lipid metabolite features had detectable signal intensities in 67% of samples in both training and testing sets, and 372 of 618 were retained for analysis (CV 20%). 98 of ~160 metabolites in the targeted analysis had detectable signal intensity in 96% of all samples, and 90 of 98 were retained (CV 15%). A total of four study samples were excluded entirely from either the global (n=2) or targeted (n=2) analysis due to loss of material during sample preparation. Targeted profiling measurements for an individual metabolite were normalized to the within-batch mean signal among QC samples for that metabolite. Global lipidomics measurements were not normalized, as all samples were run in one continuous batch.

Linear regression analyses were conducted in which $\log_2$ -transformed metabolite values were regressed on case status (X defined as 0/1), with adjustment for covariates (C_i): $M \sim \alpha_0 + [\alpha_1 C_1 + \dots + \alpha_n C_n] + \beta X$. Metabolites for which β differed significantly from zero (at $P < 0.05$) in the training set were advanced to the testing set and similarly assessed, with those satisfying $P < 0.05$ selected as preliminary candidates. Correction for multiple comparisons in the testing set was conducted using the Benjamini-Hochberg false discovery rate (FDR) approach, with Bonferroni-level significance also evaluated. Inclusion of an individual covariate (C_i) in a given regression model was determined by assessment of the association between values of the metabolite and the covariate in control samples: $M \sim \alpha + \gamma_i C_i \mid X=0$. Covariates for which γ_i differed significantly from zero (at $P < 0.05$) were included in the primary regression model for the selected metabolite. Adjustment was considered for several OC risk factors—age (grouped-linear: 54–64, 65–74, 75+), BMI (grouped-linear: 18.5–24.9, 25–29.9, 30+), parity (grouped-linear: NP, 1, 2), history of oral contraceptive use (yes/no)—and duration of specimen storage (grouped-linear: 2–3, 4–5, or 6–8 y). Fold changes in the geometric means of metabolite signals between cases and controls were calculated using covariate-adjusted metabolite values. For a given metabolite, if covariates C_1 and C_2 were included in the main regression model, adjusted metabolite values were obtained as $M_{\text{adj}} = M_{\text{unadj}} - \delta_1 C_1 - \delta_2 C_2$, where δ_1 and δ_2 are coefficients derived from the regression $M \sim \alpha + \delta_1 C_1 + \delta_2 C_2 \mid X=0$.

Classification models

Multivariate classifiers for discriminating cases from controls were constructed using regularized logistic regression with an elastic net penalty (R package: glmnet) [32]. After exclusion of 14 subjects with missing CA125 values and two subjects missing all global lipidomics profiles, 84 subjects (44 cancers and 40 benign controls) were retained for analysis. Missing values in the lipidomics dataset were imputed using the k-nearest neighbor method (R package: impute), after exclusion of analytes with non-detectable signal in >33% of study samples. Cases and controls were randomly allocated to a training set (75%: n=63), used for variable selection and model selection, or a testing set (25%: n=21), used for (preliminary) model validation. With the mixing parameter (α) set to 0.5, five-fold cross-validation was conducted within the training set only to select the optimal value of the penalty parameter (λ). Using this value of λ , a model was generated using the complete training set data and used to predict class values for subjects in the testing set. For a hybrid model based on multiple metabolites and CA125, the coefficient penalty factor for CA125 was set to zero (versus one) to force inclusion of this predictor. Monte Carlo cross validation

(MCCV) was conducted, such that the entire procedure described was repeated using 100 different training (and associated testing) sets randomly selected from the study sample. Mean estimated area under the receiver-operating curve (AUC) was calculated across the 100 testing sets. Performance of CA125 alone was estimated using the same MCCV framework. Composite average ROC curves were constructed to summarize overall classification accuracy (R package: ROCR) [33]. All univariate statistical analyses were conducted using Stata v13.1 (College Station, TX), while multivariate modeling was conducted using R v3.03, as indicated.

Results

Demographic and reproductive characteristics for serous ovarian cancer cases and benign serous controls are shown in Table 1. Cases and controls are well-balanced with respect to frequency-matching covariates: age, race, BMI, parity, and oral contraceptive use. None of the observed differences in the distributions of these variables between cases and controls, in either the training or testing set, reached statistical significance when assessed by χ^2 tests (data not shown). Similarly, covariate distributions were highly similar between controls (or cases) in the training versus testing set. The majority of included cases (~80%) were Stage III disease, with only 6% of cancers classified as Stage IV.

Metabolite profiling

Global lipidomics profiling conducted using the LC-Q-TOF-MS system provided high sensitivity, resolution, and mass accuracy for a large number of lipids. 86 of 372 metabolites assessed exhibited a nominally significant ($P < 0.05$) signal when comparing cases to controls in the training set, and 34 of these 86 reached significance ($P < 0.05$) in the testing set. After correction for multiple comparisons, 17 metabolites remained significant ($FDR\ q < 0.05$) (Table 2A), with two satisfying the highly stringent Bonferroni threshold ($P < 0.05/86 = 5.81 \times 10^{-4}$) (Figure 1). All 17 of these metabolites, which included a number of glycerolipids and glycerophospholipids, were found to be decreased in abundance in cases relative to controls, in both the training and testing sets. Fold changes in the testing set ranged from 0.43 to 0.67, with similar ratios observed in the training set.

Targeted aqueous profiling of the same 100 specimens was also conducted using an optimized LC-MS platform. Nine of 90 metabolites assessed exhibited differential abundance in cases versus controls in the training set ($P < 0.05$), while only a single metabolite of these nine, the amino acid alanine, reached significance ($P < 0.05$, $q = 0.22$) in the testing set (Table 2B). Alanine showed reduced signal in cases relative to controls in both training and testing sets (fold change 0.83–0.86).

Multivariate classification models

To explore the utility of combining multiple metabolites into a composite panel for discriminating between benign and malignant serous ovarian tumors, we selected the top four lipid metabolites listed in Table 2A, and conducted multivariate modeling, after imputing missing values via the k-nearest neighbor method. The mean AUC of models built using these four lipid markers, and assessed across 100 rounds of MCCV, was 0.85

(SD=0.07), with estimated specificity of ~35% at 95% sensitivity (Figure 2). Given the availability of CA125 plasma measurements on the majority of study participants, we also assessed the classification accuracy of a multi-marker model based on *both* CA125 *and* these four lipid metabolites, relative to CA125 *alone*. Performance assessments via MCCV were suggestive of a modest gain in overall classification accuracy for the hybrid model relative to CA125 alone (mean AUC 0.91 versus 0.87, respectively), although the observed AUC differences did not reach statistical significance (Table S1 and Figure S1). At high fixed levels of sensitivity (eg. 95%), we observed substantially higher levels of estimated specificity for the joint model (~43%) versus CA125 alone (<10%). Expanded models built using all 17 lipid metabolites listed in Table 2A (FDR $q < 0.05$) achieved very similar accuracy (Figures S2–S3).

Discussion

Ovarian cancer is a leading cause of cancer-related mortality among U.S. women [1]. As most of these cancers are detected at late and incurable stages, an overarching long-term research objective is the development of novel non-invasive methods for early detection that could be implemented in population-based screening programs. In parallel, a second important challenge, likely more tractable in the short-term, relates to the effective clinical triage of women presenting with a pelvic/ovarian mass, and specifically the ability to discriminate benign from malignant tumors [12]. New clinical tools based on blood-based protein markers have recently become available to address this second challenge [3, 4], but evaluation remains ongoing, and performance improvement remains a key goal. We employed two independent metabolomics profiling approaches and screened for plasma metabolite biomarkers that can differentiate benign from malignant serous ovarian tumors. Seventeen lipid metabolites were identified as differentially abundant (all reduced) in the plasma of cases versus controls. When combined into a multivariate classifier, four of these markers exhibited good discriminatory ability (AUC=0.85), and showed further potential in a hybrid model (AUC=0.91) to enhance the accuracy achieved by CA125 alone (AUC=0.87), especially at fixed high levels of sensitivity.

A number of metabolomics-based studies of ovarian cancer in the past decade have provided evidence of metabolite alterations in tissue, blood, and urine associated with the presence of OC; suggested the utility of metabolite-based classifiers for differentiating disease states; and offered initial insights into metabolic perturbations linked to this cancer [22–29, 34, 35]. This body of work has also highlighted a number of familiar challenges in the metabolomics field: small sample sizes; differences in study designs, inclusion criteria, case definitions, sample protocols, profiling platforms, and analysis approaches; and identification of many unknown “features”. These factors (among others) likely underlie the limited concordance observed among prior reports, with respect to specific metabolite markers and signatures. In the largest study to date [27], a total of 53 differential metabolites were identified in plasma from cases with epithelial OC versus benign ovarian tumor (BOT), including phospholipids, acylcarnitines, amino acid metabolites, and other analytes. Four metabolites described in a previous independent study [26] (by the same authors) were also identified in this larger analysis, including two lysophospholipids (LPC(18:2) and LPC(14:0)). Plasma concentrations of several phospholipids were reduced in cases with Stage III/IV disease

relative to BOT, but increased in early-stage cancers. Disturbances in lipid metabolism have also been suggested by at least two other metabolomics reports [22, 36]. None of these studies deeply interrogated the tissue or plasma lipidome, however, as standard widely-used extraction procedures and platforms were employed.

Of the 35 differentially-abundant metabolites ($P < 0.05$) identified in our primary analyses, the vast majority (34/35) were identified via lipidomics, with only a single metabolite (alanine) found using our aqueous platform. The 34 lipid metabolites included 16 glycerophospholipids, eight glycerolipids (all triacylglycerols), one sphingolipid, one sterol lipid, and eight unknowns, while the top 17 candidates ($q < 0.05$) comprised seven glycerolipids, six glycerophospholipids, one sterol lipid, and three unknowns. Strikingly, the plasma levels of all 34 of these lipids were reduced in cases versus benign controls. While altered lipid metabolism has been linked to OC by past studies, specific findings have been inconsistent, with either increases or decreases in various phospholipids and lipid precursors reported in both tissue and plasma from OC cases [22, 23, 27, 36, 37]. Lipid alterations in the context of OC pathogenesis may reflect higher rates of cell division [22], enhanced activity of phospholipase A2 and inflammation [27], elevated PI3-kinase activity, and increased membrane degradation or changes in cellular morphology [36]. However, the extent to which circulating plasma lipid metabolite levels correlate with those found in ovarian tissue remains uncertain. Future parallel analyses of paired pre-operative plasma and resected tissue specimens isolated from the same subjects may help clarify this relationship.

Clinical discrimination of benign from malignant ovarian tumors remains an important challenge with significant implications for patient prognosis and survival. Women with OC who are referred to tertiary care specialists have increased survival relative to those treated by general gynecologists/surgeons, likely reflecting optimal surgical debulking and clinical management [2]. Treatment of women with benign tumors by specialists does not appear to confer medical benefit (or harm), but increases resource utilization and may elevate patient anxiety. A cost-benefit simulation analysis [38] has argued for *universal* referral of women with an ovarian mass to specialized tertiary care, and suggested that any triaging plan should prioritize *high sensitivity*, while secondarily maximizing specificity. Referral has typically been based on a combination of personal history, clinical examination, ultrasound, and CA125 blood levels, components of which were formalized into an algorithm in 1990 known as the Risk of Malignancy Index (RMI) [39]. Two additional algorithms have been developed: ROMA, based on serum CA125 and HE4 with menopausal status [3]; and OVA1, based on CA125 in combination with additional blood protein markers discovered via mass spectrometry [4–7]. The only available head-to-head evaluation of these two approaches was a recent small study (50 post-menopausal benign controls and 26 post-menopausal ovarian cancers), which reported 42% specificity at 96% sensitivity for OVA1, and 76% specificity at 88.5% sensitivity for ROMA [8]. This level of performance for OVA1 appears quite similar to that of our hybrid model incorporating CA125 and multiple lipid metabolites.

Our study represents the first global lipidomics profiling analysis of OC, and is atypical of past reports in that it integrates data from two independent metabolomics platforms. Together, the profiling approaches employed provided broad coverage of both small lipids and aqueous analytes. Global lipid profiling enabled far more comprehensive interrogation

of this important class of metabolites than has been reported in >10 past metabolomics studies of OC. We restricted our analysis to the subset of metabolites that exhibited the most reproducible profiles based on assessment of included QC samples (CV 20%), thereby focusing attention on the most robust signals. Our use of a training-testing set framework with adjustment for multiple comparisons allowed for rigorous statistical assessment and minimization of type I error, while inclusion of several covariates in our regression models helped control for the effects of potential confounders, and increased the probability of identifying metabolite markers independent of known risk factors. The use of penalized regression methods further allowed for the development of optimized classifiers based on inclusion of an expanded pool of predictors variables.

This work also has certain limitations. First, our overall sample size of 50 cases and 50 controls restricted our statistical power and likely resulted in a number of missed candidate markers among the profiled metabolites. The extent of missing data (~20%) for several covariates such as BMI, parity, and oral contraceptive use further reduced the number of available samples for our primary regression analyses, while missing values for CA125 (~14%) limited the pool of participants in which we could assess hybrid CA125-metabolite classification models. Second, a number of “unknown features” were identified in our global analysis, as the corresponding MS peaks could not be mapped to compounds in the Agilent and METLIN chemical libraries. Third, our study design was retrospective, and the extent to which selected participants with serous OC or serous BOT were representative of the larger populations of women with these conditions remains unknown. While serous OC and BOT comprise a large percentage of ovarian masses, real-world prospective triaging also requires discrimination between benign and malignant tumors of multiple disparate histologies. With respect to our multivariate modeling, it should be noted that our estimates of classification accuracy were obtained using the same samples used to identify the included metabolite predictors; thus external validation will be essential to obtain truly unbiased performance estimates. Lastly, the cases included in our study sample consisted primarily of women with Stage III tumors, likely reflecting the stage distribution most commonly seen among OC patients presenting with an ovarian mass. It remains to be determined whether the identified candidate markers also exhibit similar fluctuations at earlier phases of disease progression. Of interest, recent evidence from prospective multi-institutional trials supports the potential utility of the OVA1 test for identifying early-stage OC [40].

Our results provide evidence that alterations in circulating plasma lipid metabolites are associated with the presence of malignant ovarian carcinoma (versus benign ovarian tumor). Our use of a global profiling platform focused specifically on lipid molecules has significantly expanded the pool of candidate markers that may be implicated in, or reflective of, the OC disease process. Additional studies in larger independent populations are required for validation. It will be of significant interest to determine if combining plasma metabolites with established protein-based markers (eg. CA125, HE4, prealbumin, transferrin) may enable superior classification of benign versus malignant tumors, and ultimately lead to the more effective clinical triage of women presenting with an ovarian mass.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Ovarian cancer represents a leading cause of cancer-related mortality among U.S. women, with an urgent need for non-invasive diagnostic approaches.
- Using global lipidomics profiling, we identified novel plasma lipid metabolite candidate biomarkers to discriminate benign from malignant serous ovarian tumors.
- The combination of such markers with CA125 may help identify women in need of tertiary surgical care to optimize prognosis/survival.

Figure 1. Box plots for selected top differentiating metabolites between benign controls (white) and OC cases (gray)

Covariate-adjusted metabolite values were plotted by case status, stratified by training or testing set. Median metabolite signals are indicated by solid black lines. Values for the 25th percentile and 75th percentile are indicated by the lower and upper box boundaries, respectively, with dotted lines marking the distribution of observed values, and outliers indicated by gray dots.

Figure 2. Receiver operating characteristic (ROC) curves for multivariate classifiers based on the top four lipid metabolites with or without CA125, and for CA125 alone

Study participants (n=84) were randomly divided into a training (75%) or testing (25%) set. A multivariate classifier was derived in the training set using the top four lipid metabolites (Table 2A), and evaluated in the testing set. After 100 rounds of Monte Carlo cross validation (MCCV), a composite average ROC curve was generated. The procedure was repeated using these four lipid metabolites plus CA125, and performance of CA125 alone was similarly assessed via the MCCV framework. Mean area under the ROC curve (AUC) was calculated across all MCCV iterations. Estimated specificity at 95% sensitivity indicated by black circles.

Table 1

Subject characteristics

	Training set				Testing set			
	Controls (n=25)		Cases (n=25)		Controls (n=25)		Cases (n=25)	
	n	%	n	%	n	%	n	%
Age								
54-64	10	40	16	64	10	40	16	64
65-74	9	36	5	20	9	36	6	24
75+	6	24	4	16	6	24	3	12
Race/ethnicity ^a								
White	19	95	21	95.5	19	100	21	95.5
Non-white	1	5	1	4.5	0	0	1	4.5
BMI (kg/m ²) ^{a,b}								
18.5-24.9	7	36.8	7	33.3	5	29.4	7	33.3
25-29.9	4	21.1	5	23.8	6	35.3	10	47.6
30.0+	8	42.1	9	42.9	6	35.3	4	19
Parity ^a								
Npc	2	11.1	3	15.8	2	10.5	3	14.3
1	2	11.1	4	21.1	3	15.8	3	14.3
2-3+	14	77.8	12	63.2	14	73.7	15	71.4
OC use ^{a,d}								
No	8	40	4	19	6	33.3	6	27.3
Yes	12	60	17	81	12	66.7	16	72.7
Stage								
I			3	12			2	8
II			2	8			1	4
III			19	76			20	80
IV			1	4			2	8

^aNumbers may not add to total subjects due to missing data

Oral contraceptive use

d

Nulliparous,

N_0

Body mass index,

q

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Table 2

Top metabolite marker candidates identified in (A) global lipidomics or (B) targeted aqueous profiling.

A	Metabolite ^a	Training set (n=49)					Testing set (n=49)						
		Mas ^b	RT ^c	N ^d	FC ^e	P ^f	Co ^g	N ^d	FC ^e	P ^f	Co ^g	q ^h	
1	C52 H79 N O5 S	829.6	16.8	42		0.69	0.0056	39	0.56	0.0001 [#]		0.01	
2	PS(O-18:0/0:0)	493.3	4.50	47	▼	0.56	0.0006	46	▼	0.46	0.0004 [#]	0.02	
3	18:3 Cholesteryl ester + 22.7	819.7	22.7	49	▼	0.75	0.0038	49	▼	0.66	0.0010	0.02	
4	TG(16:0/16:1(9Z)/16:1(9Z))[iso3] + 23.5	705.5	23.5	38	▼	0.55	0.0103	2;	▼	0.43	0.0010	2;	0.02
5	PG(P-20:0/12:0)	817.7	17.4	37	▼	0.73	0.0378	35	▼	0.54	0.0010	0.02	
6	TG(16:1(9Z)/16:1(9Z)/16:1(9Z)) + 22.7	729.5	22.7	39	▼	0.49	0.0021	2;	▼	0.45	0.0012	2;	0.02
7	PS(O-18:0/16:1(9Z))	872.7	16.8	47	▼	0.56	0.0002	48	▼	0.59	0.0015	0.02	
8	TG(17:2(9Z,12Z)/17:2(9Z,12Z)/20:5(5Z,8Z, 11Z,14Z,17Z))[iso3] + 22.2	841.7	22.3	45	▼	0.80	0.0440	46	▼	0.67	0.0024	0.02	
9	TG(16:1(9Z)/17:2(9Z,12Z)/17:2(9Z,12Z))[iso3] + 22.2	843.7	22.2	39	▼	0.55	0.0021	2;	▼	0.52	0.0027	2;	0.02
10	TG(16:1(9Z)/17:1(9Z)/17:2(9Z,12Z))[iso6]	749.5	22.8	39	▼	0.58	0.0036	2;5	▼	0.52	0.0029	2;5	0.02
11	PE(18:1(9Z)/20:3(8Z,11Z,14Z))	749.5	16.2	44	▼	0.66	0.0054	48	▼	0.61	0.0035	0.03	
12	C57 H102 N2 O S	862.8	24.6	38	▼	0.51	0.0116	2;	▼	0.46	0.0040	2;	0.03
13	TG(16:0/16:0/16:1(9Z))[iso3]	821.8	24.7	37	▼	0.54	0.0345	2;	▼	0.46	0.0044	2;	0.03
14	C29 H47 N9 O2	553.4	5.90	48	▼	0.48	0.0001	49	▼	0.46	0.0044	0.03	
15	PS(O-20:0/0:0)	521.4	6.60	46	▼	0.64	0.0082	47	▼	0.60	0.0048	0.03	
16	PE(18:1(9Z)/20:3(8Z,11Z,14Z))	749.5	16.3	48	▼	0.73	0.0226	46	▼	0.65	0.0049	0.03	
17	TG(16:1(9Z)/17:0/17:2(9Z,12Z))[iso6]	845.8	23.7	39	▼	0.68	0.0222	2;	▼	0.55	0.0053	2;	0.03
B	Metabolite	Mas ^b	RT ^c	N ^d	FC ^e	P ^f	Co ^g	N ^d	FC ^e	P ^f	Co ^g	q ^h	
1	Alanine	90.0	3.8	48	▼	0.83	0.0416	50	▼	0.86	0.0246	0.22	

^a Assigned metabolite identity based on mapping to Agilent/METLIN chemical libraries,

^b Daltons (Da),

^c Retention time (min),

^d Number of subjects with detectable signal for indicated metabolite and non-missing covariate values where applicable,

^e Fold change of geometric mean signal, comparing cases to controls,

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f_i p value derived from linear regression of metabolite values on case status, with adjustment for indicated covariates,
 g Covariates included in linear regression model: age (1), BMI (2), parity (3), OC use (4), specimen storage duration (5),
 h False discovery rate (Benjamini and Hochberg),
Significant after Bonferroni correction