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A prospective analysis of circulating plasma metabolites associated with ovarian cancer risk

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Abstract

Ovarian cancer has few known risk factors, hampering identification of high-risk women. We assessed the association of pre-diagnostic plasma metabolites (N=420) with risk of epithelial ovarian cancer, including both borderline and invasive tumors. 252 cases and 252 matched controls from the Nurses' Health Studies were included. Multivariable logistic regression was used to estimate odds ratios (OR) and 95% confidence intervals (CI) comparing the 90th-10th percentile in metabolite levels, using the permutation-based Westfall and Young approach to account for testing multiple correlated hypotheses. Weighted gene co-expression network analysis (WGCNA) modules (n=10 metabolite modules) and metabolite set enrichment analysis (MSEA; n=23 metabolite classes) were also evaluated. An increase in pseudouridine levels from the 10th to the 90th percentile was associated with a 2.5-fold increased risk of overall ovarian cancer (OR=2.56, 95%CI=1.48-4.45; p=0.001/adjusted-p=0.15); a similar risk estimate was observed for serous/poorly-differentiated tumors (n=176 cases; comparable OR=2.38, 95%CI=1.33-4.32, p=0.004/adjusted-p=0.55. For non-serous tumors (n=34 cases), pseudouridine and C36:2 phosphatidylcholine (PC) plasmalogen had the strongest statistical associations (comparable OR=9.84, 95%CI=2.89-37.82; p<0.001/adjusted-p=0.07; and OR=0.11, 95%CI=0.03-0.35; p<0.001/adjusted-p=0.06, respectively). Five WGCNA modules and 9 classes were associated with risk overall at FDR≤0.20. Triacylglycerols (TAGs) showed heterogeneity by tumor aggressiveness (case-only heterogeneity-p<0.0001). The TAG association with risk overall and serous tumors differed by acyl carbon content and saturation. In summary, this study suggests that pseudouridine may be a novel risk factor for ovarian cancer and that TAGs may also be important, particularly for rapidly fatal tumors, with associations differing by structural features.

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Keywords

ovarian cancer risk; circulating metabolomics; serous ovarian cancer risk; prospective analysis

Introduction

Ovarian cancer is the fifth leading cause of female cancer death in the U.S. (1). However, there are few known risk factors, such that current risk prediction models have a modest predictive capability, necessitating the identification of new risk factors to identify women at high risk.

Advances in technology have led to precise measures of small molecule metabolites that are critical for growth and maintenance of cells in biologic fluids (2). Several studies have identified metabolites as biomarkers of cancer risk. For example, branched chain amino acids were strongly associated with risk of pancreatic cancer (3) and lipid metabolites were inversely associated with risk of aggressive prostate cancer (4). Further, prediagnostic serum concentrations of metabolites related to alcohol, vitamin E, and animal fats were modestly associated with ER^+ breast cancer risk (5), while BMI-related metabolites were strongly related to increased risk (6). These findings support metabolomics profiling as a valuable strategy for identifying new cancer risk biomarkers.

Therefore, we used metabolomics assays to quantify several classes of circulating metabolites in plasma samples collected three to twenty-three years prior to ovarian cancer diagnosis within a nested case-control study, and, in an agnostic analysis, assessed their potential as biomarkers of ovarian cancer risk.

Materials and Methods

Study population

We conducted nested case-control studies within the Nurses Health Studies (NHS (7), NHSII (8)). The NHS was established in 1976 among 121,700 US female nurses aged 30–55 years, and NHSII was established in 1989 among 116,429 female nurses aged 25–42 years. Participants have been followed biennially by questionnaire to update information on exposure status and disease diagnoses. Details are provided in the supplementary file.

Incident cases of epithelial ovarian cancer were identified through biennial questionnaires or linkage with the National Death Index, for whom we obtained related medical records and pathology reports or linked to the relevant cancer registry when medical records were unattainable. A gynecologic pathologist reviewed the records to confirm the diagnosis and abstract date of diagnosis, invasiveness, stage, and histotype (serous, poorly differentiated [PD], endometrioid, clear cell [CC], mucinous, other/unknown), which is highly concordant with centralized pathology review (9). Date of death was extracted from the death certificate.

Confirmed cases were diagnosed with ovarian cancer three years after blood collection until June 1, 2012 (NHS), or June 1, 2013 (NHSII): two hundred fifty-two cases of invasive and borderline epithelial ovarian cancer (212 in NHS and 40 in NHSII). We excluded cases diagnosed within three years of blood collection (N=46) as most ovarian cancer cases are diagnosed at a late stage, with evidence suggesting preclinical disease up to 3 years before diagnosis (10). Cases were matched to one control on: cohort (NHS, NHSII); menopausal status and hormone therapy use at blood draw (premenopausal, postmenopausal/ hormone therapy use, postmenopausal/ no hormone therapy use, missing/ unknown); menopausal status at diagnosis (premenopausal, postmenopausal, or unknown); age (±1 year), date of blood collection (±1 month); time of day of blood draw (±2 hours); and fasting status (>8 hours or ≤8 hours); women in NHSII who gave a luteal sample were matched on the luteal date (date of the next period minus date of blood draw, ±1 day).

Completion of the questionnaire was considered to imply informed consent when the study protocol was approved in 1976 (NHS) and 1989 (NHSII) by the institutional review boards of the Brigham and Women's Hospital and Harvard T.H. Chan School of Public Health, and those of participating registries as required. The studies were conducted in accordance with recognized ethical guidelines (Declaration of Helsinki).

Metabolite profiling

Plasma metabolites were profiled at the Broad Institute of MIT and Harvard (Cambridge, MA) using three complimentary liquid chromatography tandem mass spectrometry (LC-MS/MS) methods designed to measure polar metabolites and lipids as well as free fatty acids. Details are provided in the supplementary file.

In total, 608 known metabolites were measured. Metabolites with a coefficient of variation (CV) >25% or an intraclass correlation coefficient (ICC) <0.4 among blinded QC samples were excluded (N=132, Supplementary Table 1). Furthermore, metabolites with poor stability due to delayed processing (11) were excluded (N=56, Supplementary Table 1). Included metabolites (e.g., amino acids, amino acids derivatives, amines, lipids, fatty acids, bile acids; N=420 [69%]; Supplementary Table 1) exhibited good reproducibility within person over one year (11) and over 10 years. 197 metabolites had no missing values among participant samples.

Statistical analysis

Identification of individual metabolites associated with risk—Missing values for metabolites (N=211) with <10% missingness were imputed with 1/2 of the minimum value measured for that metabolite. We included a missing value indicator for metabolites (N=12) with more than 10% missingness. Continuous metabolite values were transformed to probit scores to reduce the influence of skewed distributions and heavy tails on the results and to scale the measured metabolite values to the same range. Conditional logistic regression was used to evaluate metabolite associations, modeled continuously (with an additional indicator if >10% missingness), with risk of overall ovarian cancer. We present the odds ratios (OR) and 95% confidence intervals (95% CI) for an increase from the 10^{th} to 90^{th} percentile in metabolite levels or the indicator variable.

We compared conditional logistic regression to unconditional logistic regression adjusting for the matching factors and found similar results (Supplementary Table 1.1). Thus, subsequent analyses by histotype, rapidly fatal status, time between blood collection and diagnosis, and sensitivity analyses were conducted using the latter approach, allowing the use of all controls.

We conducted stratified analyses restricting to serous/poorly differentiated (PD) tumors (cases=176/controls=252), endometrioid/clear cell (CC) tumors (cases=34/controls=252), rapidly fatal invasive cases (death occurring <3 years after diagnosis; cases=86/controls=252), and less aggressive invasive tumors (all other cases; cases=138/controls=252), as well as premenopausal (cases=82/controls=82) and postmenopausal women (cases=137/controls=137) at blood collection and those diagnosed 3–11 years (cases=121/controls=252) and 12–23 years after blood collection (cases=131/controls=252). Models were adjusted for matching factors, duration of oral contraceptive use (none or <3 months, 3 months to 3 years, 3 to 5 years, >5 years), tubal ligation (yes/no) and parity (none, 1, 2, 3, 4+ children). We calculated heterogeneity by histotype, time to diagnosis and tumor aggressiveness using case-only analyses and by menopausal status at blood collection by introducing an interaction term between the metabolite and menopausal status.

We conducted sensitivity analyses excluding borderline tumors (N=25), known low-grade serous cases (N=4), samples processed >24 hours after collection (N=13 cases, N=6 controls), cases with a diagnosis of a prior cancer (N=17), or women with a diagnosis of another cancer after their matched case's diagnosis (N=35 controls).

A permutation test (N=5000) was used to control the family-wise error rate (i.e. account for multiple testing) while accounting for the correlation structure of metabolites using the stepdown min P approach by Westfall and Young (12). Details are in the supplemental file. We report unadjusted and multiple comparison adjusted p-values and discuss individual metabolites associated with ovarian cancer risk at unadjusted p-values≤0.01 given the hypothesis generating nature of the study.

Identification of groups of metabolites associated with risk—Metabolite Set Enrichment Analysis (MSEA) (13) was used to identify groups of molecularly or biologically similar metabolites that were enriched among the metabolites associated with risk of overall ovarian cancer and histotypes and Weighted Gene Co-expression Network Analysis (WGCNA) (14) was used to identify metabolite modules and their association with ovarian cancer risk; details are in the supplemental file. We report nominal p-values and false discovery rates (FDR) (15) for all metabolite groups and modules, discussing those at FDR≤0.2. All analyses were performed using the statistical computing language R, version 3.5.0 (16).

Results

Study population

Of the 252 cases, 176 cases were diagnosed with serous/PD tumors, while 34 were classified as endometrioid/CC; 86 represented rapidly fatal tumors with death within 3 years of diagnosis (Table 1). Mean follow-up was 12.3 years. Distributions of ovarian cancer risk factors were generally in the expected directions.

Measured metabolites and their association with ovarian cancer risk

Of the 420 metabolites passing our QC filtering criteria, there were 159 lipids; 158 amino acids, amino acids derivatives amines and cationic metabolites; and 103 free fatty acids, bile acids and lipid mediators. Eight metabolites were associated with risk of overall ovarian cancer at a nominal p-value ≤0.01 (Table 2A, Figure 1 and Supplementary Table 1). Odds ratios for an increase from the 10th to the 90th percentile of levels ranged between 0.49 and 2.56. The top three metabolites associated with risk were pseudouridine (OR=2.56, 95% CI=1.48–4.45; p-value=0.001), C18:0 sphingomyelin (SM) (OR=2.10, 95% CI=1.26–3.49; p-value=0.004) and 4-acetamidobutanoate (OR=2.10, 95% CI=1.24–3.56; p-value=0.006). Pseudouridine had an adjusted-p=0.15 (accounting for all tested metabolites and their correlation structure); all other metabolites had adjusted-p>0.5. The test of the global null hypothesis that no metabolite was associated with risk had p=0.15. Results did not change in sensitivity analyses excluding specific case and control populations (Supplementary Table 1.1-1.5).

Five metabolites were associated with risk of serous/PD tumors at a nominal p-value ≤0.01 (Table 2B, Figure 1 and Supplementary Table 2). Odds ratios for an increase from the 10th to the 90th percentile of metabolites levels for these metabolites ranged between 1.99 and 2.38. The top three metabolites were pseudouridine (OR=2.38, 95% CI=1.33–4.32; p-value=0.004), C52:5 triacylglycerol (TAG) (OR=2.09, 95% CI=1.23–3.59; p-value=0.007) and C52:4 TAG (OR=2.03, 95% CI=1.21–3.47; p=0.008). However, none of the metabolites remained significant after accounting for multiple comparisons via permutation (adjusted p-value >0.55). The test of the global null hypothesis that no metabolite was associated with risk had p=0.55. Results did not change in sensitivity analyses in which we excluded low-grade serous cases (Supplementary Table 2.1).

Thirty metabolites were associated with risk of endometrioid/CC tumors at a nominal p-value ≤0.01 (Table 2C, Figure 1 and Supplementary Table 2). Odds ratios for an increase from 10th to the 90th percentile of metabolites levels for these metabolites ranged between 0.11 and 0.24 for inverse associations, and between 3.85 and 9.84 for positive associations. The top three metabolites positively associated with risk were pseudouridine (OR=9.84, 95% CI=2.89–37.82; p=0.0003), C2 carnitine (OR=7.4, 95% CI=2.37–25.35; p=0.001) and C56:7 TAG (OR=5.85, 95% CI=2.04–18.02; p=0.001). The top three metabolites inversely associated with risk were C36:2 phosphatidylcholines (PC) plasmalogen (OR=0.11, 95% CI=0.03–0.35), p=0.0003), C34:1 PC plasmalogen-A (OR=0.18, 95% CI=0.05–0.54, p=0.003), C22:0 lysophosphatidylethanolamine (LPE) (OR=0.21, 95% CI=0.07–0.59; p=0.004). C36:2 PC plasmalogen and pseudouridine had an adjusted-p=0.06 and 0.07, respectively (accounting for all tested metabolites and their correlation structure). All other metabolites had adjusted-p≥0.14. The test of the global null hypothesis that no metabolite was associated with risk had p=0.06.

On the individual metabolite level, histograms and QQ-plots of the nominal p-values (Supplementary Figure 1) together with the results of the permutation-based approach to account for testing multiple correlated metabolites (Westfall and Young's stepdown min p approach) suggest the existence of a metabolomic signal for overall ovarian cancer and non-serous tumors.

Metabolite groups associated with risk of ovarian cancer

In the MSEA analysis, nine metabolite groups were enriched among metabolites associated with risk of ovarian cancer overall at an FDR ≤0.2 (Figure 2 and Supplementary Table 3). The top five groups were organic acids and derivatives; PE plasmalogens; TAGs; cholesteryl esters; and PC plasmalogens. Nine metabolite groups were associated with risk of serous/PD tumors with FDR ≤0.20 (Figure 2 and Supplementary Table 3). The top five were: nucleosides, nucleotides and analogues; TAGs; carnitines; sphingomyelins; and alkaloids and derivatives. Finally, eleven metabolite groups were associated with risk of endometrioid/CC tumors at FDR ≤0.20 (Figure 2 and Supplementary Table 3). The top five associated metabolite groups were TAGs, DAGs, fatty acyls, lysophosphatidylserines (LPS), and carnitines. TAGs were enriched in the above at FDR≤0.05. Notably, we observed differential associations by acyl carbon number and double bond content with risk of ovarian cancer overall (Supplementary Figure 2) and serous/PD tumors (Supplementary Figure 3, but not with endometrioid/CC tumors (Supplementary Figure 4). Specifically, TAGs with higher number of acyl carbon atoms and double bonds were associated with increased risk, while TAGs with lower number of acyl carbon atoms and double bonds were associated with decreased risk. We did not observe similar patterns for other lipid classes (Supplementary Figures 2-4).

Metabolite modules associated with risk of ovarian cancer

WGCNA identified seven metabolite modules associated with risk of ovarian cancer with FDR ≤0.20 (Table 3 and Figure 3, panels A-D). Module 1 (M1, characterized by steroids and steroid derivatives, organic acids and derivatives, and organonitrogen compounds [Supplementary Figures 5-7, Supplementary Table 4]), M2 (characterized by TAGs, PCs, PE, LPCs, and LPEs), M6 (characterized by TAGs, LPEs and CEs), and M7 (characterized by TAGs, DAGs, ceramides and CEs) were associated with increased risk of ovarian cancer overall, OR, increase from 10^{th} to 90^{th} percentile=1.99 (p=0.013/FDR=0.072), 1.62 (p=0.093/FDR=0.186), 1.56 (p=0.081/FDR=0.186) and 1.8 (p=0.015/FDR=0.072), respectively. M4 (characterized by carnitines, pseudouridine [inversely weighted], and organic acids and derivatives) was associated with decreased risk (OR=0.5, p=0.022/FDR=0.072). M7 was associated with increased risk of serous/PD tumors (OR=1.97; p=0.012/FDR=0.117). Finally, four modules were associated with risk of endometrioid/CC tumors: M2 (OR=6.14; p=0.002/FDR=0.011), M4 (OR=0.17; p=0.003/FDR=0.011), M5 (PC and PE plasmalogens; OR=0.35; p=0.041/FDR=0.072), and M8 (fatty acyls, OR=0.22; p=0.007/FDR=0.017).

Metabolites associated with ovarian cancer risk by menopausal status at blood collection

C22:0 LPS isomer was suggestively associated with increased risk among postmenopausal women (OR=1.83, 95%CI=0.92–3.63; p=0.085) and decreased risk among premenopausal women (OR=0.44, 95%CI=0.17–1.08; p=0.074), with a heterogeneity p=0.004 (Supplementary Table 5). C38:4 PC plasmalogen was suggestively associated with increased risk among postmenopausal women (OR=1.92, 95%CI=0.96–3.85; p=0.066) and decreased risk among premenopausal women (OR=0.16, 95%CI=0.05–0.51; p=0.002), with a heterogeneity p=0.005. Among premenopausal women, 14/22 (63%) metabolites associated with risk at p≤0.1 were inversely related, but among premenopausal women only 15/98 (15%) metabolites showed inverse associations. Pseudouridine did not show heterogeneity by menopausal status (heterogeneity p=0.32).

Metabolites associated with ovarian cancer risk by time between blood collection and diagnosis

Hydroxyvitamin D3 was associated with increased risk among participants with blood collection 12–23 years before diagnosis (OR=1.84, 95%CI=1.02–3.37; p=0.044) but not among participants with blood collection 3–11 years before diagnosis (OR=0.64, 95%CI=0.36–1.15; p=0.141), with a heterogeneity p=0.002 (Supplementary Table 6). C40:6 phosphatidylserine (PS) was associated with decreased risk among participants with blood collection 12–23 years before diagnosis (OR=0.55, 95%CI=0.3–0.99; p=0.049) but not among participants with blood collection 3–11 years before diagnosis (OR=1.41, 95%CI=0.79–2.51; p=0.245), with a heterogeneity p=0.008. Pseudouridine showed suggestively stronger associations (heterogeneity p=0.066) among women for whom sample collection was 3–11 years before diagnosis (OR=4.48, 95%CI=2.25–9.24; p≤0.001) compared to participants with samples collection 12–23 years before diagnosis (OR=2.00, 95%CI=1.06–3.85; p=0.035).

Metabolites associated with ovarian cancer risk by tumor aggressiveness

Fifty-three lipid-related metabolites (26 TAGs, 7 PCs, 6 LPEs, 3 PEs, 3 LPC, 4DAGs, 2 LPSs, and 2 PSs) showed differences by tumor aggressiveness at heterogeneity p≤0.01 (Supplementary Table 7). Seven metabolites (6 TAGs and 1 PS) were associated with increased risk of rapidly fatal disease with ORs ranging between 2.56 and 3.07 at p≤0.008, but not with less aggressive tumors (p>0.62), with heterogeneity p≤0.001. Several lipid-related metabolite classes (DAGs, LPCs, LPEs, PCs, PEs, PSs, and TAGs with high acyl carbon content and saturation) were overrepresented in rapidly fatal tumors versus controls, while carnitines were overrepresented in less aggressive tumors (Supplementary Figure 8, panels A and B). TAGs with lower acyl carbon content and saturation were inversely associated with less aggressive tumors. Pseudouridine did not show heterogeneity by tumor aggressiveness (heterogeneity p=0.13).

Discussion

We conducted the first large-scale agnostic analysis of metabolomics and risk of ovarian cancer. We identified a potential novel risk factor, plasma pseudouridine, which was associated with an increased risk of ovarian cancer overall and non-serous tumors and suggestively for serous/PD disease. Stronger associations for pseudouridine were observed among cases diagnosed within 3–11 years after blood collection. We identified several metabolite groups and metabolite modules associated with risk of ovarian cancer risk, as well as multiple subtype-specific associations, that open up new opportunities for assessing novel metabolite pathways involved in ovarian cancer development.

Pseudouridine

Pseudouridine is the most abundant post-transcriptionally modified nucleoside, and is an isomer of uridine. It is produced by pseudouridine synthase by isomerizing uridines from transfer RNA, which is involved in in protein translation, or spliceosomal snRNA, which plays a role in pre-mRNA splicing. Pseudouridine was nominally associated with risk overall and for both histotypes, with no significant heterogeneity (p=0.16) by histotype. This suggests that pseudouridine may represent a common etiologic mechanism underlying different histotypes of ovarian cancer, which has been observed for other risk factors, such as aspirin and CRP. In retrospective studies, pseudouridine was elevated in urine (17) and plasma (18) from epithelial ovarian cancer patients versus healthy controls. This, in combination with our finding that pseudouridine had a stronger association when assessed 3–11 years before diagnosis, suggests that this modified nucleotide may be important in progression of preclinical lesions to fully overt invasive disease, which for high-grade serous ovarian cancer appears to be about 7–9 years (19). Increasing evidence suggests that pseudouridylation plays a role in cancer-associated splicing distributions, which are more variable than in normal tissues. Notably, tissue-specific alternative splicing reverts to a default cancer pattern that directly contributes to cellular transformation and cancer progression (20). This has been observed in serous carcinomas, which have highly dysregulated splicing compared to normal tissue (21). Further, aberrant pseudouridylation may lead to altered and reduced translational fidelity of p53 (22), which is mutated in nearly all high-grade serous tumors (23). Another potential mechanism is via circular RNA activity, which is altered due to isomerization of uridine to pseudouridine, and has been shown to be dysregulated in ovarian cancer (24). Interestingly, pseudouridine is associated with the estimated glomerular filtration rate, a marker of kidney function (25). While kidney function alters the immune response, potentially contributing to the development of cancer (26), associations with cancer incidence are mixed, although a recent study observed that thiazide diuretics, which can affect kidney function, are associated with a higher risk of ovarian cancer (27). Additional research should explore the potential role of pseudouridine in precursor lesions to ovarian cancer, the relation between circulating pseudouridine to ovarian and fallopian tube tissue levels, and if kidney function plays a role in the initiation or development of ovarian cancer.

Triacylglycerides

Notably, several individual TAGs were nominally related to risk and showed significantly stronger associations with rapidly fatal tumors. Evidence suggests that established ovarian cancer risk factors vary by tumor aggressiveness (28). As high grade serous ovarian cancer was the predominant histotype among rapidly fatal as well as less aggressive tumors, our data suggest that there are potential differences between the metabolic profiles of these two groups of tumors independent of histotype. TAGs as a group were enriched in the MSEA analysis, and 3 of 7 WCGNA modules related to risk were characterized by TAGs. Long chain fatty acids, a main source of energy in the human body, are stored and transported from the small intestine and liver to peripheral cells as TAGs (29). Lipid synthesis and metabolism that releases free fatty acids from TAGs are dysregulated in ovarian tumors, increasing cell migration and invasive potential (30). Further, several human studies reported suggestive associations of ovarian cancer risk with total cholesterol (31) (positive) or HDL (inverse) (32). Additionally, ovarian cancer metastasizes preferentially to the adipose-rich omentum (33). Omental fat possesses a distinct lipidomic signature with several lipid groups, including TAGs, DAGs, and SMs, showing differences when compared to subcutaneous fat (34). Finally, plasma TAGs represent known risk factors for cardiovascular disease and coronary heart disease. A recent study identified that TAGs at the extremes of carbon atoms and saturation had differential associations with diabetes risk (35). We also observed differential associations by TAG fatty acids length and saturation, with higher number of carbon atoms and double bonds related to an increased risk and lower number of carbon atoms and double bonds related to decreased risk, particularly for serous/PD tumors. A similar pattern was observed in a retrospective study of serum samples from high-grade serous ovarian cancer cases and controls (36). Together with our results, these findings suggest that circulating TAG levels may be a risk biomarker for ovarian cancer, particularly for rapidly fatal tumors. Additional prospective studies are needed to validate these associations in different populations and assess the potential differential role of various TAG species in ovarian carcinogenesis.

Other metabolite groups

A number of metabolite groups and classes were associated with ovarian cancer risk, including organic acids and derivatives, and SMs, the latter of which was hypothesized a priori as a potential risk biomarker and is discussed elsewhere (37). A metabolite module driven by carnitines, organic acids and derivatives, carboxylic acids and derivatives, which included pseudouridine (highly negatively weighted), was associated with decreased risk of overall ovarian cancer and non-serous tumors. This module includes asymmetric dimethylarginine (ADMA), which has been related to risk of cardiovascular disease (38), and inhibits nitric oxide synthesis and may have antiproliferative properties (39) including in ovarian tumors (40). LPEs were also represented in WCGNA modules associated with increased risk of overall and endometrioid/CC ovarian cancers. LPEs have been shown to increase migration in response to chemotherapy as well as have invasive potential in ovarian cancer cell lines (41). In MSEA analyses, several metabolite classes had a significant negative enrichment score, including PE plasmalogens, PC plasmalogens and cholesteryl esters, independent of subtype.

Little work has examined these markers in ovarian cancer development or etiology. Sphingolipids ([SL]; including SMs, PCs, PEs, LPCs, LPEs, cholesteryl esters, acylcarnitines) are associated with a series of conditions that may be related to ovarian cancer, including thrombosis in a mouse study (42), myocardial infraction among symptomatic coronary artery disease patients (43), type 1 and type 2 diabetes in human studies (44), diabetic kidney disease in mice (45, 46) and airway inflammation and asthma in mouse and human studies (47-49). Notably, patients with ovarian cancer have the highest incidence of venous thromboembolism (VTE) of all solid tumor types (50), which is significantly related to higher mortality (51). Coagulation activation by tumors promotes development of VTE which in turn favors cancer progression through tumor growth, angiogenesis, invasion, immune evasion, and metastasis (52). Further, cholesterol-lowering statins have anti-inflammatory, anti-proliferative, apoptotic and anti-invasive qualities (53-57), and can lower SMs (58). Data on ovarian cancer risk reduction by statins have been mixed. A meta-analysis of existing studies suggested a lower risk for ovarian cancer associated with statin use (59) while post-diagnostic statin use was inversely associated with overall survival and ovarian cancer specific mortality (60-62). Additional work should evaluate whether these conditions and medications associated with the identified metabolites represent novel risk factors for ovarian cancer, preferably using large consortia to ensure power.

Our study has several strengths and limitations. Importantly, this is a prospective study of ovarian cancer risk with coverage of multiple different metabolite classes. Additional strengths include the long follow-up time and detailed covariate information. Our cohort consisted of registered nurses, a group that are not representative of the general population (e.g. social economic status), although established risk factor associations in these cohorts are similar to those in other more representative studies (63). While we had over 250 ovarian cancer cases and controls, we had more limited sample sizes for specific histotypes, which have been shown to have different associations for known risk factors (64). We used medical records and pathology reports to confirm diagnosis and extract histotype and cannot rule out the possibility of histotype misclassification, although we previously showed high concordance to centralized slide review (9). To maximize power, borderline and tumors of unknown morphology were included. We did not include information on family history of ovarian cancer. However, only 2 cases were diagnosed before age 45 suggesting that early onset disease, likely due to high risk mutations, does not play a role in this study. We also applied stringent QC criteria to limit identification of spurious associations. Another limitation is that we only analyzed blood samples collected at one point in time; however, we demonstrate that the majority of the measured metabolites have a high within person stability over time (11). Further, we do not have an independent validation dataset. As this type of data becomes more common, further population studies are needed to validate the results discussed here, while experimental studies are required to understand the biological mechanisms underlying these associations.

In summary, circulating levels of plasma pseudouridine were associated with higher risk of ovarian cancer 3–23 years before diagnosis, with stronger associations among participants with samples collected closer to diagnosis. Additionally, several metabolite groups and metabolite modules were associated with risk of disease overall and by subtype. While independent prospective studies are needed for validation, our results highlight some potentially important novel metabolites that may play a role in the etiology of ovarian cancer. Potential experimental studies to understand the biological mechanisms of these risk biomarkers could examine their role in carcinogenic tendencies in ovarian and fallopian tube cancer cell lines (with and without p53 mutations), mouse models of early and late ovarian lesions leading to ovarian cancer as well as xenograft mouse models in which pseudouridine production has been impaired or enhanced. Adding these new risk biomarkers to current risk prediction models may help the identification of high-risk women.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of significance: Pseudouridine represents a potential novel risk factor for ovarian cancer and triglycerides may be important particularly in rapidly fatal ovarian tumors.

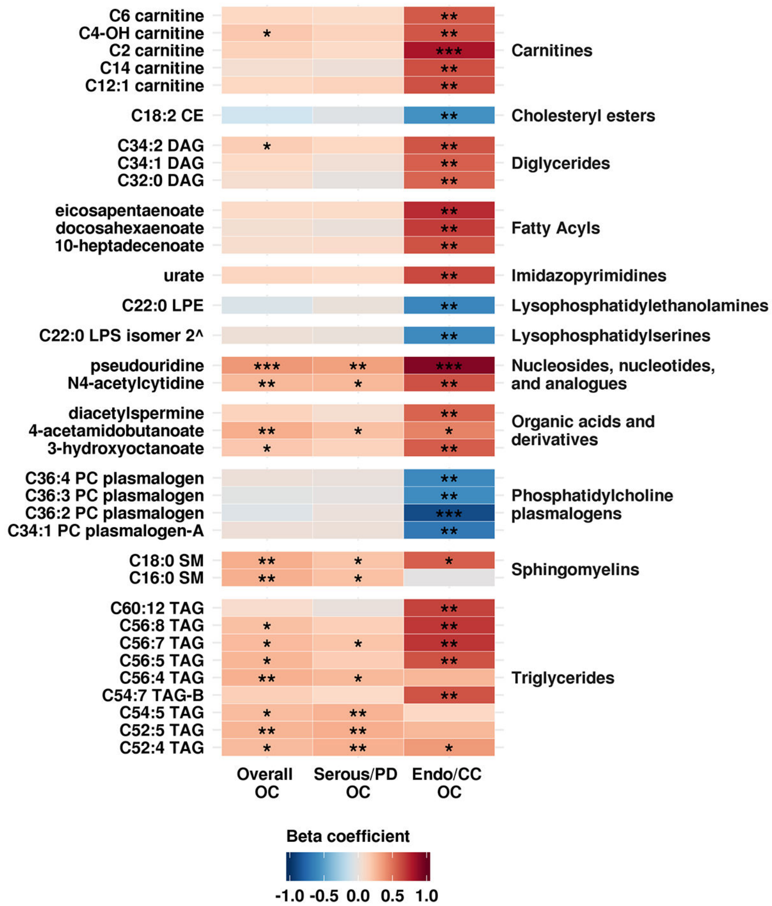

Figure 1: Beta coefficients of the association between metabolites and overall OC, serous/poorly differentiated OC (Serous/PD OC) and endometrioid/clear cell OC (Endo/CC OC).

Coefficients with a p-value ≤0.01 in any of the analyses are shown. Shades of red represent positive coefficients while shades of blue indicate negative coefficients. Significance of the association (using unadjusted p-values) is overlaid on the heat map and marked as follows: * p-values≤0.1, ** p-values≤0.01, *** p-values≤0.001; all other p-values are >0.1. Based on the Westfall and Young stepdown min p approach for multiple comparisons, only pseudouridine for overall OC and endo/CC OC as well as C36:2 PC plasmalogen and C2 carnitine had an adjusted p-value<0.2. ^ preliminary ID

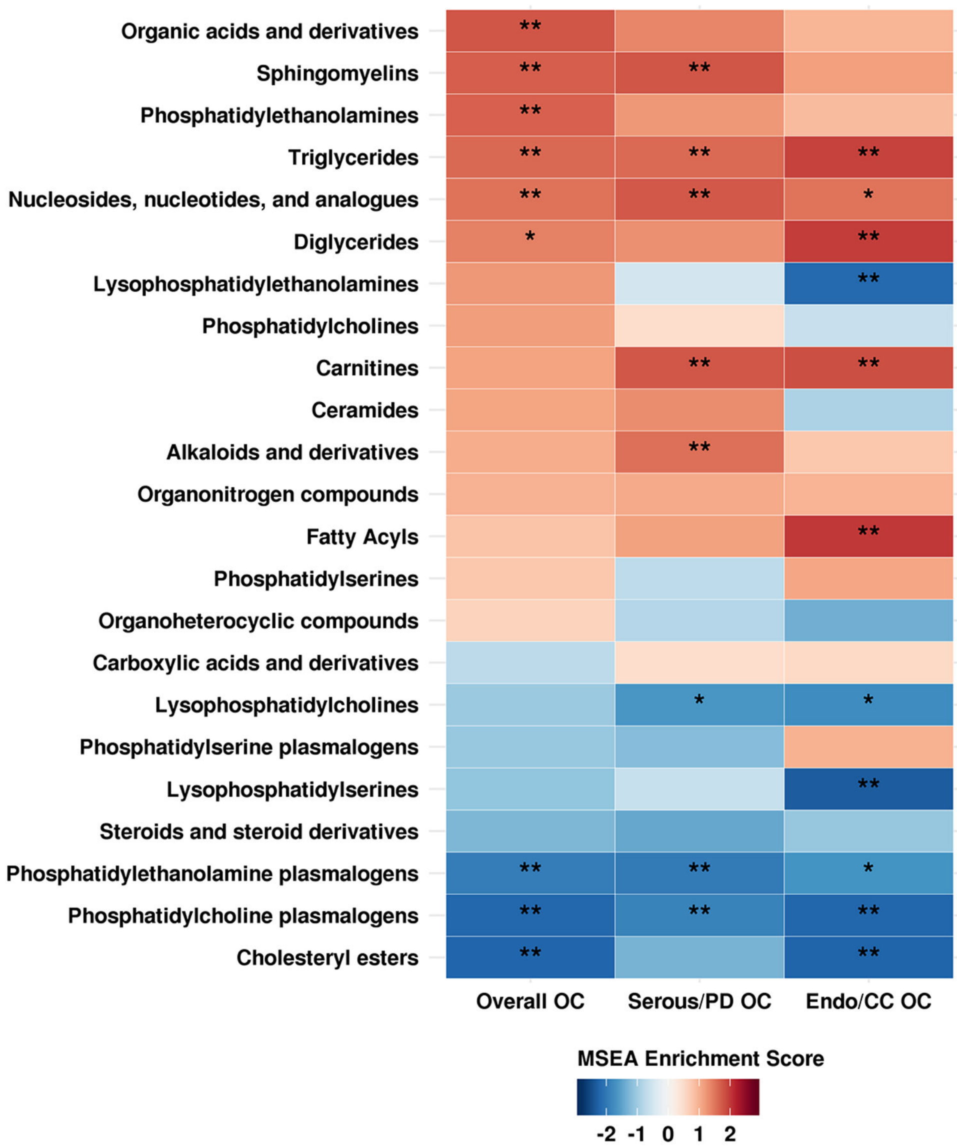

Figure 2: MSEA results. Enriched metabolite groups associated with risk of overall OC, serous/ poorly differentiated OC (Serous/PD OC) and endometrioid/clear cell OC (Endo/CC OC).
Significance of the association is overlaid on the heat map and marked as follows: * FDR ≤0.2, ** FDR ≤0.05; all other FDR >0.2.

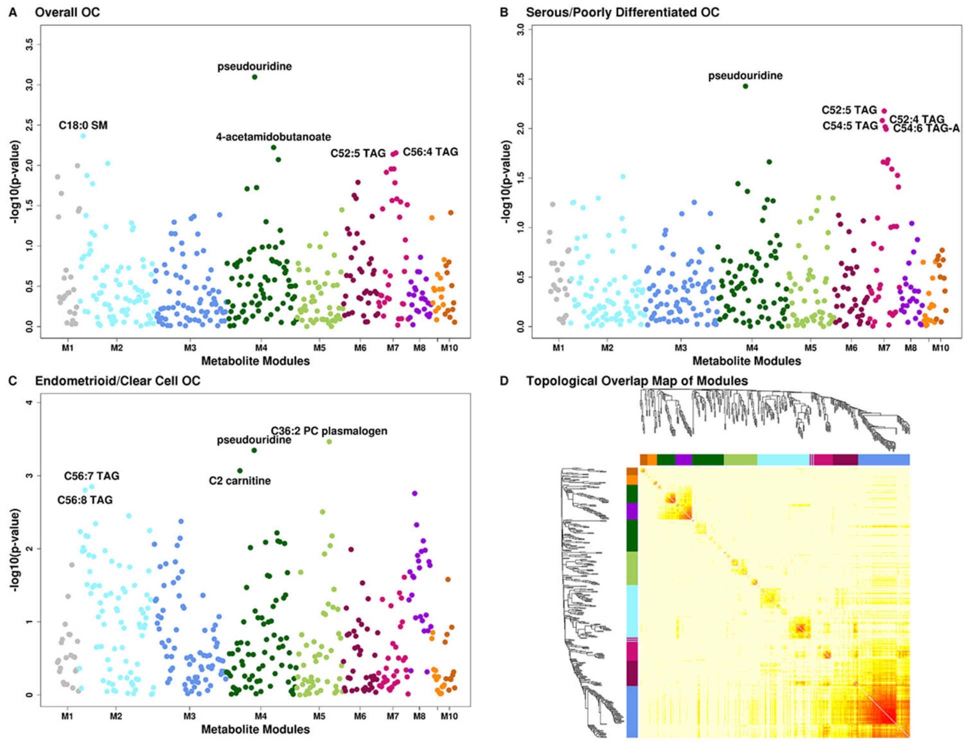

Figure 3: METhattan plots.

Man*hattan* plots of *met*abolites by metabolite groups, with each group being shown in a different color. **A** Overall ovarian cancer. **B** Serous/poorly differentiated ovarian cancer. **C** Endometrioid/clear cell ovarian cancer**. D** Topological Overlap Matrix (TOM). Metabolites in the rows and columns are sorted by the clustering tree. Light yellow shades represent low topological overlap (low similarity). Darker red shades represent higher overlap and similarity. Metabolite modules correspond to the squares along the diagonal.

Table 1:

Characteristics of overall, serous/poorly differentiated (PD) and endometrioid/clear cell (CC) ovarian cancer (OC) cases, rapidly fatal tumors, and all controls at time of blood collection.

	All Controls (N = 252)	Overall OC (N = 252)	Serous/PD OC (N = 176)	Endometrioid/CC OC (N = 34)	Other histotypes (N = 42)	Rapidly fatal tumors (N=86)
Mean (SD)						
Age at blood draw*	55.6 (7.8)	55.5 (7.9)	55.3 (7.9)	54.0 (8.1)	57.8 (7.5)	58.5 (6.8)
BMI at blood draw	24.7 (4.1)	25.0 (4.7)	24.5 (4.2)	26.9 (5.8)	25.5 (5.4)	25.3 (5.3)
Time to diagnosis (years)	-	12.3 (5.2)	12.8 (5.3)	12.1 (5.1)	10.9 (4.7)	12.7 (5.2)
Age at diagnosis (years)	-	69.7 (9.7)	68.1 (9.8)	66.0 (9.8)	68.7 (9.2)	71.2 (8.7)
N (Percent)						
Tumor morphology						
Invasive	-	227 (90)	163 (93)	33 (97)	31 (74)	86 (100)
Borderline	-	22 (9)	13 (7)	1 (3)	8 (19)	0 (0)
Unknown	-	3 (1)	0 (0)	0 (0)	3 (7)	0 (0)
Menopausal status blood draw*						
Premenopausal	82 (33)	82 (33)	56 (32)	16 (47)	10 (24)	14 (16)
Postmenopausal, No HT use	71 (28)	68 (27)	47 (27)	5 (15)	16 (38)	29 (34)
Postmenopausal, HT use	66 (26)	69 (27)	48 (27)	8 (24)	13 (31)	34 (40)
Unknown	33 (13)	33 (13)	25 (14)	5 (15)	3 (7)	9 (10)
Cohort*						
NHS	212 (84)	212 (84)	147 (84)	27 (79)	38 (90)	79 (92)
Race						
White	251 (100)	251 (100)	175 (100)	34 (100)	42 (100)	86 (100)
Oral contraceptive use duration						
None or <3 months	123 (49)	118 (47)	81 (46)	18 (53)	19 (45)	48 (56)
3 months to 3 years	33 (13)	32 (13)	22 (12)	3 (9)	7 (17)	10 (12)
3 to 5 years	45 (18)	63 (25)	46 (26)	8 (24)	9 (21)	16 (19)
5+ years	51 (20)	39 (15)	27 (15)	5 (15)	7 (17)	12 (14)
Parity						
No children	12 (5)	24 (10)	16 (9)	5 (15)	3 (7)	7 (8)

	All Controls (N = 252)	Overall OC (N = 252)	Serous/PD OC (N = 176)	Endometrioid/CC OC (N = 34)	Other histotypes (N = 42)	Rapidly fatal tumors (N=86)
1 child	11 (4)	13 (5)	8 (5)	1 (3)	4 (10)	2 (2)
2 children	72 (29)	89 (35)	60 (34)	15 (44)	14 (33)	23 (27)
3 children	77 (31)	65 (26)	45 (26)	9 (26)	11 (26)	25 (29)
4+ children	80 (32)	61 (24)	47 (27)	4 (12)	10 (24)	29 (34)
Tubal ligation						
Yes	43 (17)	39 (15)	30 (17)	5 (15)	34 (10)	17 (20)

[*] matching factors; HT any type of hormone therapy

Table 2:

Odds ratio (OR) for an increase from the 10th to the 90th percentile of metabolite levels and 95% confidence intervals (CI) of associations with risk of overall, serous/poorly differentiated and endometrioid/clear cell ovarian cancer.

Results with p-values ≤0.01 are shown for overall and serous/poorly differentiated ovarian cancer. The top 10 (out of 30) metabolites with p-values ≤0.01 are shown for endometrioid/clear cell tumors. Complete results are available in Supplementary Tables 1-2.

A Overall Ovarian Cancer (N = 252 cases and 252 controls)				
HMDB ID	**Metabolite**	**OR (95% CI)**	**P-value**	**Adjusted P-value**
HMDB00767	pseudouridine	2.56 (1.48-4.45)	0.001	0.150
HMDB01348	C18:0 SM	2.10 (1.26-3.49)	0.004	0.570
HMDB03681	4-acetamidobutanoate	2.10 (1.24-3.56)	0.006	0.672
HMDB05398 *	C56:4 TAG	2.03 (1.21-3.39)	0.007	0.722
HMDB05380 *	C52:5 TAG	1.96 (1.20-3.20)	0.007	0.733
HMDB05923	N4-acetylcytidine	1.88 (1.18-3.02)	0.008	0.772
HMDB10169	C16:0 SM [1]	2.06 (1.19-3.56)	0.009	0.807
--	armillane [2]	0.49 (0.28-0.85)	0.010	0.824
B Serous/Poorly differentiated ovarian cancer (N = 176 cases and 252 controls)				
HMDB ID	**Metabolite**	**OR (95% CI)**	**P-value**	**Adjusted P-value**
HMDB00767	pseudouridine	2.38 (1.33-4.32)	0.004	0.552
HMDB05380 *	C52:5 TAG	2.09 (1.23-3.59)	0.007	0.745
HMDB05363 *	C52:4 TAG	2.03 (1.21-3.47)	0.008	0.809
HMDB05391 *	C54:6 TAG-A	1.99 (1.18-3.38)	0.010	0.862
HMDB05385 *	C54:5 TAG	1.99 (1.19-3.39)	0.010	0.851
C Endometrioid/Clear cell ovarian cancer (N = 34 cases and 252 controls)				
HMDB ID	**Metabolite**	**OR (95% CI)**	**P-value**	**Adjusted P-value**
HMDB11243 *	C36:2 PC plasmalogen	0.11 (0.03-0.35)	0.0003	0.056
HMDB00767	pseudouridine	9.84 (2.89-37.82)	0.0004	0.072
HMDB05462 *	C56:7 TAG	5.85 (2.04-18.02)	0.001	0.236
HMDB00201	C2 carnitine	7.4 (2.37-25.35)	0.001	0.143
HMDB05392 *	C56:8 TAG	5.75 (2.00-17.72)	0.002	0.260
HMDB01999	eicosapentaenoate	6.25 (2.05-20.65)	0.002	0.286
HMDB11208 *	C34:1 PC plasmalogen-A	0.18 (0.05-0.54)	0.003	0.468
HMDB07103 *	C34:2 DAG	4.46 (1.64-12.85)	0.004	0.577
HMDB11520	C22:0 LPE	0.21 (0.07-0.59)	0.004	0.517
HMDB02183	docosahexaenoate	5.49 (1.74-18.72)	0.005	0.617

* representative ID

-- HMBD ID not available

[1] significantly associated with risk in our analysis of lipid-related metabolites and risk of ovarian cancer (manuscript in revision)

[2]preliminary ID

Table 3:

P-values, FDR, odds ratio (OR) for an increase from the 10^{th} to the 90^{th} percentile of metabolite levels and 95% confidence intervals (CI) of WGCNA metabolite modules associated with risk of ovarian cancer overall and by histotype.

	Overall OC				Serous/PD OC				Endometrioid/CC OC			
Module / number of metabolites	**OR (95% CI)**	**P-value**	**FDR**	**explained variance [%]**	**OR (95% CI)**	**P-value**	**FDR**	**explained variance [%]**	**OR (95% CI)**	**P-value**	**FDR**	**explained variance [%]**
M1 / 24	1.99 (1.15-3.42)	0.013	0.072	14.51	1.64 (0.89-3.05)	0.114	0.46	14.87	1.09 (0.38-3.11)	0.875	0.613	14.62
M2 / 79	1.62 (0.92-2.85)	0.093	0.186	23.63	1.16 (0.65-2.07)	0.624	0.701	23.35	6.14 (1.97-20.67)	0.002	0.011	24.54
M3 / 76	0.9 (0.56-1.46)	0.678	0.682	65.22	1.06 (0.62-1.8)	0.839	0.839	65.18	0.48 (0.17-1.28)	0.151	0.212	66.23
M4 / 74	0.5 (0.28-0.9)	0.022	0.072	17.86	0.64 (0.35-1.15)	0.138	0.46	18.13	0.17 (0.05-0.54)	0.003	0.011	17.63
M5 / 49	0.91 (0.56-1.46)	0.682	0.682	24.23	1.2 (0.71-2.03)	0.49	0.701	23.94	0.35 (0.12-0.94)	0.041	0.072	21.61
M6 / 37	1.56 (0.95-2.58)	0.081	0.186	40.38	1.17 (0.68-2.01)	0.575	0.701	40.32	1.4 (0.5-3.99)	0.522	0.456	40.16
M7 / 32	1.8 (1.12-2.88)	0.015	0.072	32.45	1.97 (1.17-3.36)	0.012	0.117	32	1.94 (0.71-5.5)	0.203	0.237	32.69
M8 / 24	0.82 (0.49-1.37)	0.441	0.552	65.98	0.81 (0.47-1.4)	0.451	0.701	65.34	0.22 (0.07-0.65)	0.007	0.017	64.15
M9 / 14	0.73 (0.45-1.19)	0.205	0.341	37.33	0.88 (0.51-1.5)	0.631	0.701	38.1	0.79 (0.29-2.21)	0.651	0.506	36.26
M10 / 11	0.75 (0.43-1.29)	0.293	0.419	46.53	0.7 (0.38-1.29)	0.257	0.644	45.92	1.88 (0.57-6.46)	0.305	0.305	47.51