

Urine Metabolite Analysis Offers Potential Early Diagnosis of Ovarian and Breast Cancers

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Abstract

Purpose: Metabolomics is a new, rapidly expanding field dedicated to the global study of metabolites in biological systems. In this article metabolomics is applied to find urinary biomarkers for breast and ovarian cancer.

Experimental Design: Urine samples were collected from early- and late-stage breast and ovarian cancer patients during presurgical examinations and randomly from females with no known cancer. After quantitatively measuring a set of metabolites using nuclear magnetic resonance spectroscopy, both univariate and multivariate statistical analyses were employed to determine significant differences.

Results: Metabolic phenotypes of breast and ovarian cancers in comparison with normal urine and with each other revealed significance at Bonferroni-corrected significance levels resulting in unique metabolite patterns for breast and ovarian cancer. Intermediates of the tricarboxylic acid cycle and metabolites relating to energy metabolism, amino acids, and gut microbial metabolism were perturbed.

Conclusions: The results presented here illustrate that urinary metabolomics may be useful for detecting early-stage breast and ovarian cancer. *Clin Cancer Res*; 16(23): 5835–41. ©2010 AACR.

Currently, there are no available tests for the general screening of epithelial ovarian cancer (EOC). Although EOC accounts for 3% of cancers in women, it is the leading cause of death from gynecologic cancer and the fifth leading cause of all cancer-related deaths among women (National Cancer Institute, <http://www.cancer.gov>). Because early-stage EOC is usually asymptomatic, more than two thirds of cases are diagnosed with advanced (FIGO stage III/IV) disease, where the tumor has spread within and outside the abdomen. Until we have reliable and appropriate methods to screen and diagnose early EOC, the majority of patients will continue to present with advanced disease. Despite improvements in multimodality treatment, the 5-year overall survival (OS) for advanced ovarian cancer is only 20% to 30% (National Cancer Institute, <http://www.cancer.gov>). However, for stage I ovarian cancer, 5-year survival rates are more than 90% (National Cancer Institute, <http://www.cancer.gov>). Thus, a screening method that

facilitates early diagnosis would be advantageous. It used to be recommended that individuals at high risk of having ovarian cancer obtain an endovaginal ultrasound together with a CA-125 test every 6 months. However, because of the low sensitivity of these tests (1), they are no longer recommended.

For breast cancer, which has a higher incidence (1 in 8 versus 1 in 71 for EOC; National Cancer Institute, <http://www.cancer.gov>), screening mammography is considered the gold standard for early detection; however, the sensitivity of this test is between 54% and 77% depending on the type of mammography (2). Furthermore, mammography is uncomfortable for many patients and exposes them to radiation. As a result, many women do not obtain yearly mammograms. There is a need to find a general screening test for all cancers that would ideally be noninvasive and have high sensitivity and specificity.

Measuring small-molecule metabolites has essentially been the cornerstone of clinical chemistry, and indeed clinical practice, for more than 1,000 years. Monitoring of blood or urine for glucose and creatinine continues to be an integral part of diagnostic tests run today. Although these 1- or 2- component chemical tests provide a quick and inexpensive way to monitor health, what distinguishes metabolomics from clinical chemistry is that metabolomics measures tens to hundreds and potentially thousands of metabolites at once rather than just 1 or 2.

The field of metabolomics is an emerging science dedicated to the global study of metabolites, their composition, interactions, dynamics, and responses to disease or

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Translational Relevance

Breast and ovarian cancer are 2 common cancers that represent a significant disease burden. If detected at an early stage, 5-year survival approaches 100% for breast and 90% for ovarian cancer. Late-stage disease survival is low. Although breast mammography has been used for decades for detecting breast cancer, its sensitivity remains low, particularly in women with dense breast tissue. Furthermore, there is no screen for ovarian cancer. There is a need for novel markers that can help diagnose breast and ovarian cancer at an early stage and allow treatment response to be monitored. This work represents a significant step forward in defining a novel noninvasive cancer screen for ovarian and breast cancer. Once validated in a prospective fashion in a larger sample set, the biomarker profiles described here will represent a breakthrough in clinical screening and rapid detection of early-stage breast and ovarian cancers.

environmental changes in cells, tissues, and biofluids. We have shown that NMR spectroscopy coupled with targeted profiling techniques offers a powerful approach to generate high-density metabolic data on biofluids (3–8). Multivariate statistical analysis, including principal component analysis (PCA), partial least-squares-discriminant analysis (PLS-DA), or orthogonal partial least-squares-discriminant analysis (OPLS-DA) can be applied to these data or complex spectral data to aid in the characterization of changes related to a biological perturbation or disease. Metabolomics has been successfully applied to studies of cancer (9–15) and, through urinary measurement, has the potential to become a general screening test because it is convenient, easy to obtain, and noninvasive. In this paper we apply metabolomics to study urine from women with either breast or ovarian cancer.

Materials and Methods

Populations

Written informed consent was obtained from each subject before entering this study, and the institutional ethics committees approved the protocols outlined in the following text. Patients were recruited at presurgical examination at the Royal Alexandra or Misericordia hospitals in Edmonton.

Patients with breast cancer

Forty-eight females with either ductal carcinoma ($n = 37$), ductal carcinoma *in situ* (DCIS; $n = 7$), or lobular carcinoma ($n = 4$) were recruited. Tumor sizes ranged from less than 1 cm to 9 cm in diameter, with the majority between 1 and 2 cm. A total of 10 patients had at least 1 positive lymph node. They ranged in age from 30 to 86, with a median age of 56. Ten samples were randomly selected and set aside as a test set.

Patients with ovarian cancer

Fifty females with EOC were recruited. EOC patients were diagnosed with histopathologic features and stages, for a total of 2 with stage IV, 32 with stage III, 2 with stage II, 10 with stage I, and 4 with undocumented stage. They ranged in age from 21 to 83, with a median age of 56. Ten samples were randomly selected and set aside as a test set.

Healthy volunteers

Seventy-two female volunteers with no known history of either breast or ovarian cancer ages 19 to 83 years (median age 56) were recruited. Ten samples were randomly selected and set aside as a test set.

Data collection

Urine samples were obtained from volunteers, transferred into urine cups, and subsequently frozen within 1 hour at -20°C , followed by long-term storage at -80°C . Prior to NMR data collection, samples were thawed, and 585 μL of sample supernatant was mixed with 65 μL of internal standard containing approximately 5 mmol/L of DSS- d_6 [3-(trimethylsilyl)-1-propanesulfonic acid- d_6], 0.2% NaN_3 , in 99.8% D_2O . For each sample, the pH was adjusted to 6.8 ± 0.1 by adding small amounts of NaOH or HCl. Sample (600 μL) was subsequently transferred into 5 mm 535 pp NMR tubes (Wilmad LabGlass), and samples were stored at 4°C until NMR acquisition (within 24 hours of sample preparation). NMR spectra were acquired as previously described (7).

Data analysis

Metabolite identification and quantitation was accomplished through the technique of targeted profiling using Chenomx NMR Suite 4.6 (Chenomx Inc.; ref. 8). Metabolites were selected from a library of approximately 300 compounds. Of these 300 compounds, 67 metabolites could be identified in all spectra, 6 of which were tentative assignments and are indicated in the article as "unknown singlet." These metabolites accounted for more than 80% of the total spectral area. To account for variations in metabolite concentration due to dilute or concentrated urine, probabilistic quotient normalization of the metabolite variables using a median calculated spectrum (16) was performed prior to chemometric and statistical analysis. Multivariate statistical data analysis (PCA, PLS-DA, and OPLS-DA) was performed on \log_{10} -transformed normalized metabolite concentrations to account for the non-normal distribution of the concentration data and reduce the chance of skewed variables, using SIMCA-P (version 11; Umetrics) with mean centering and unit variance scaling applied. Significance tests using Wilcoxon's rank-sum test was performed using GraphPad Prism version 4.0c for Macintosh (GraphPad Software). Significance was determined after Bonferroni correction and set at $\alpha = 0.0082$.

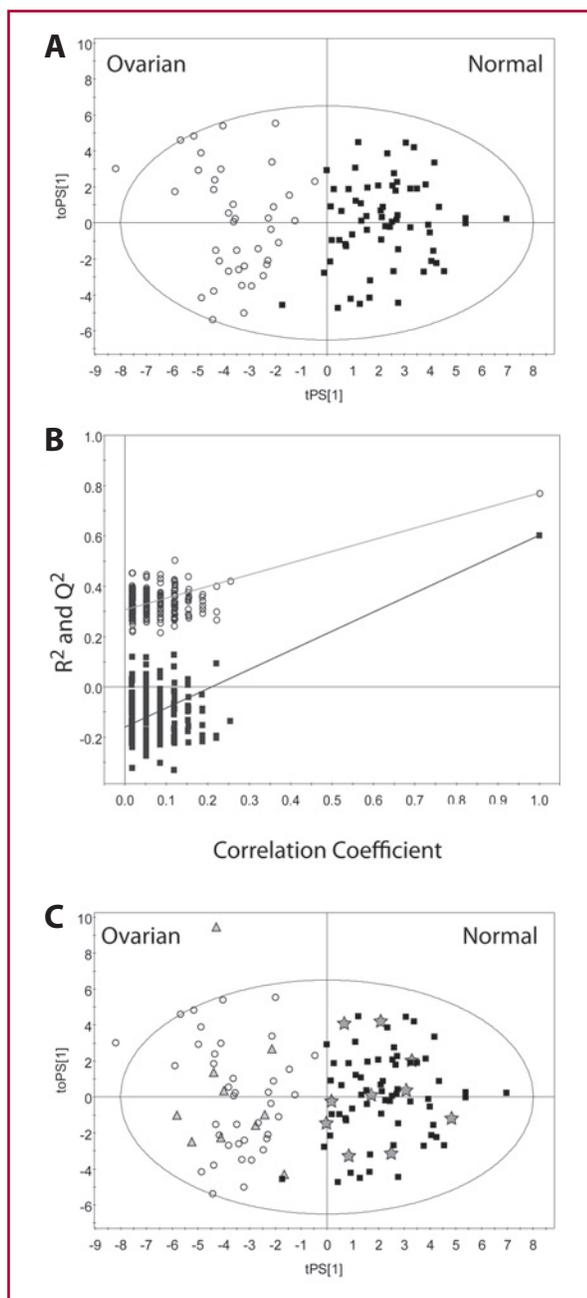


Fig. 1. Urinary metabolite profiles derived from ovarian cancer patients are different from healthy subjects. A, OPLS-DA model (based on 67 measured metabolites) with 40 ovarian cancer subjects (○) and 62 healthy female subjects (■; $R^2 = 0.77$; $Q^2 = 0.60$). B, statistical validation of the corresponding PLS-DA model by permutation analysis. R^2 is the explained variance, and Q^2 is the predictive ability of the model. C, OPLS-DA prediction of 20 additional subjects (10 each of healthy and ovarian cancer subjects). For ease of presentation, after prediction healthy subjects were indicated as a star, and ovarian subjects were indicated as a triangle.

Results

Comparison of 67 metabolite concentrations measured in urine from a cohort of apparently healthy female sub-

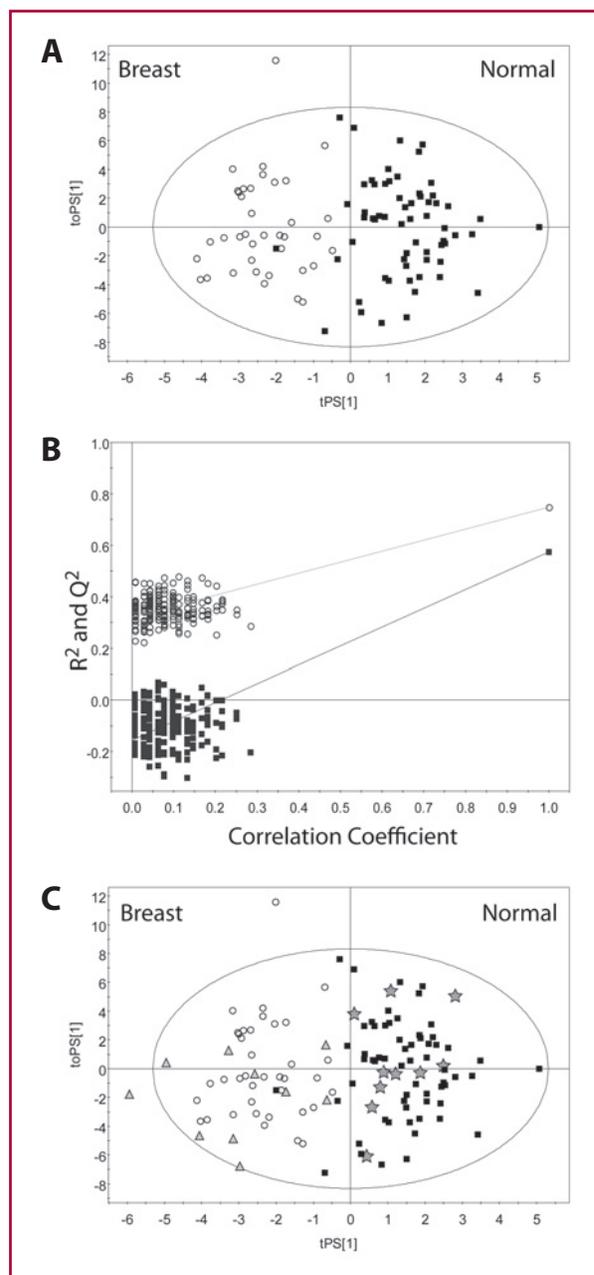


Fig. 2. Urinary metabolite profiles derived from breast cancer patients are different from healthy subjects. A, OPLS-DA model (based on 67 measured metabolites) with 38 breast cancer subjects (○) and 62 healthy female subjects (■) ($R^2 = 0.75$; $Q^2 = 0.57$). B, Statistical validation of the corresponding PLS-DA model by permutation analysis. C, OPLS-DA prediction of 20 additional subjects (10 each of healthy and breast cancer subjects). For ease of presentation, after prediction healthy subjects were indicated as a star, and ovarian subjects were indicated as a triangle.

jects ($n = 62$) and subjects with ovarian cancer ($n = 40$) revealed substantial differences. Application of OPLS-DA to the data set resulted in distinction between individuals with EOC and those that were healthy (Fig. 1A). One healthy individual in the learning set appeared in the

Table 1. Metabolite changes in human urine with breast and ovarian cancer when compared with a healthy group

Metabolite ^a	Healthy versus ovarian cancer			Healthy versus breast cancer		
	% Change ^b	P ^c	Rank ^d	% Change ^b	P ^c	Rank ^d
Unknown singlet @ 4.34 ppm	-80	< 0.0001	6	-67	0.0005	19
Creatine	-77	< 0.0001	3	-75	0.0010	23
Acetate	-74	< 0.0001	5	-68	< 0.0001	9
Succinate	-71	< 0.0001	4	-70	< 0.0001	2
Levogluconan	-65	< 0.0001	14	-	0.0141	39
Unknown singlet at 3.35 ppm	-65	< 0.0001	1	-46	< 0.0001	31
Lactate	-64	< 0.0001	7	-59	< 0.0001	36
Pyroglutamate	-63	< 0.0001	19	-48	0.0003	15
Formate	-62	< 0.0001	8	-43	< 0.0001	1
Isoleucine	-61	< 0.0001	9	-43	< 0.0001	11
Sucrose	-61	< 0.0001	12	-39	0.0016	24
Unknown singlet at 3.94 ppm	-60	< 0.0001	32	-51	0.0009	26
Trigonelline	-59	< 0.0001	28	-	0.0099	33
Leucine	-59	< 0.0001	10	-52	< 0.0001	6
Asparagine	-58	< 0.0001	15	-51	< 0.0001	7
Urea	-58	< 0.0001	2	-37	< 0.0001	13
Glucose	-58	< 0.0001	30	-42	0.0081	52
Ethanolamine	-56	< 0.0001	22	-48	0.0003	18
Dimethylamine	-55	0.0001	31	-41	0.0003	17
4-Hydroxyphenylacetate	-55	< 0.0001	11	-50	< 0.0001	14
Creatinine	-54	< 0.0001	26	-42	0.0001	12
Alanine	-54	< 0.0001	13	-42	0.0003	16
Unknown singlet at 2.36 ppm	-54	0.0004	42	-39	0.0012	37
Hippurate	-54	< 0.0001	23	-49	< 0.0001	5
1-Methylnicotinamide	-53	< 0.0001	18	-	0.0650	51
Unknown singlet at 3.79 ppm	-52	< 0.0001	24	-	0.1832	62
Uracil	-52	< 0.0001	28	-52	< 0.0001	4
Valine	-52	< 0.0001	20	-47	0.0008	22
Unknown singlet at 2.60 ppm	-50	< 0.0001	16	-44	< 0.0001	10
<i>trans</i> -Aconitate	-49	< 0.0001	21	-46	0.0003	20

^aMetabolites ranked according to % change for ovarian cancer patients.

^bChange calculated as difference in median concentration between cancer and healthy group. Values that are significant after Bonferroni correction are indicated.

^cP value calculated using Wilcoxon's rank-sum test.

^dVariable rank was determined from the OPLS-DA variable importance to projection for the two models.

cancer category, and 1 cancer individual appeared in the healthy category. Model parameters for the explained variation, R^2 , and the predictive capability, Q^2 , were significantly high ($R^2 = 0.77$; $Q^2 = 0.60$), and validation of the PLS-DA is suggestive of an excellent model (Fig. 1B). OPLS-DA class prediction was performed on a total of 20 subjects that were not used in the generation of the model, 10 each of ovarian cancer and healthy subjects (Fig. 1C). For ease of presentation, those subjects with ovarian cancer were later indicated as grey triangles, and those that were "healthy" were later indicated as grey stars. As may be observed, all test subjects were correctly predicted as either ovarian cancer or normal.

Comparison of 67 metabolite concentrations from healthy subjects ($n = 62$) and subjects with breast cancer ($n = 38$) revealed significant differences. Application of OPLS-DA to this dataset resulted in distinction between individuals with breast cancer and those without (Fig. 2A). Five of the healthy individuals overlapped with the breast cancer category. The model parameters and validation of the PLS-DA suggested a good model ($R^2 = 0.75$; $Q^2 = 0.57$; Fig. 2B). OPLS-DA class prediction was performed as for the EOC subjects, on a total of 20 subjects, 10 each of breast cancer and healthy (Fig. 2C). As may be observed, all breast cancer and healthy test subjects were correctly classified.

Table 2. Metabolite changes in human urine of ovarian cancer when compared with a breast cancer group

Metabolite ^a	% Change ^b	P ^c	Rank ^d
Acetone	84	0.0002	36
Allantoin	80	0.0006	2
Unknown singlet at 3.79 ppm	70	0.0021	5
Carnitine	57	0.0005	1
Methanol	55	0.0015	7
Urea	49	0.0007	3
1-Methylnicotinamide	49	0.0034	4
Levogluconan	39	0.0060	8
Unknown singlet at 2.82 ppm	-63	0.0022	6

^aMetabolites ranked according to % change for ovarian cancer patients.

^bChange calculated as difference in median concentration between cancer and healthy group.

^cP value calculated using Wilcoxon's rank-sum test.

^dVariable rank was determined from the OPLS-DA variable importance to projection for the model.

Analysis of urinary metabolite changes revealed that many metabolites decreased in relative concentration with a cancer (both EOC and breast) phenotype when compared with healthy (Table 1). However, the extent of the change

was different for ovarian and breast cancers. For example, the singlet at 3.35 ppm, tentatively assigned as methanol, was ranked as the most important metabolite responsible for separating EOC patients, with a 65% decrease in concentration relative to normal subjects. For breast cancer patients, this metabolite was ranked as the 31st important metabolite, with a 46% decrease in concentration. In fact, there are several metabolites that are significantly different between breast and ovarian cancers (Table 2), and comparison of breast and ovarian cancer metabolite profiles revealed good separation (Fig. 3). Certain metabolites, such as propylene glycol and mannitol, which strictly come from ingestion, were unchanged in concentration between healthy, ovarian, or breast cancer (data not shown).

Discussion

This study demonstrates for the first time that urinary metabolic profiling shows changes in metabolite concentrations that can be specifically correlated with breast or ovarian cancer and that at least 2 types of cancer can be subtyped using urine metabolomics. Remarkably, we discovered that nearly all metabolites that were significantly different between the cancers and normal were lower in concentration in both the EOC and breast cancer groups as compared with normal. As the data was normalized to account for dilution, the explanation was not one of excess fluid intake by the cancer patients.

In these datasets, there were few misclassifications. In the ovarian cancer model, the "healthy" individual who overlapped with the ovarian cancer patients was a 61-year-old with arthritis and gastroesophageal reflux disease. The misclassified EOC patient was 79-year-old with stage IC papillary serous and a CA-125 level more than 35. At this time, it is not known why her profile appeared on the edge of the healthy cohort. Interestingly, 10 of the ovarian cancer patients had CA-125 levels less than 35, and the metabolomics test was able to detect these cancers. In the

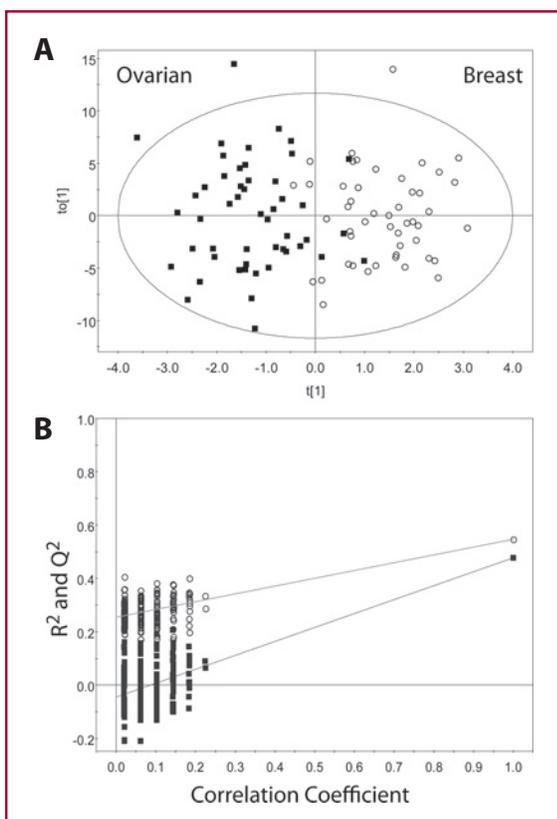


Fig. 3. Urinary metabolite profiles derived from subjects with breast and ovarian cancer are different. A, OPLS-DA model (based on 67 measured metabolites) comparing 48 breast cancer (○) and 50 ovarian cancer (■) subjects ($R^2 = 0.55$; $Q^2 = 0.48$). B, Statistical validation of the OPLS-DA model by permutation analysis.

breast cancer model, there was 1 “healthy” individual that was clearly classified as breast cancer, and another 4 appeared on the edge of the breast cancer category. None of the breast cancer patients overlapped with the “healthy” cohort. Also of interest, all 5 of these individuals were 60 years and older, and 1 (the square marker on the lower left of Fig. 2A just inside the breast cancer cohort) is the same individual that appeared in the ovarian cancer category on the ovarian cancer model plot (Fig. 1A).

That the majority of urinary metabolites appeared to decrease in concentration in cancer patients is a similar result to what has been seen in colon cancer tissue metabolomics (17). Interestingly, some metabolites that were shown to increase in cancer tissue (such as some of the amino acids) were lower in the urine of cancer patients. Our results are in agreement with other publications involving measurements of metabolites in blood (18), where concentrations of many amino acids decrease in cancer patients relative to healthy. Decreases in tricarboxylic acid (TCA) cycle intermediates are suggestive of a suppressed TCA cycle. In a study of urinary markers of colorectal cancer, it was observed that several TCA-cycle intermediates decrease in those with colorectal cancer as compared with those without (19). The biological reason behind the metabolite changes is largely speculative at this point but likely involves a shift in energy production, as tumors rely primarily on glycolysis as their main source of energy. This phenomenon is known as the Warburg effect (20), and decreases in TCA cycle intermediates and glucose in the urine could be indicative of this phenomenon. Clearly, lower glucose concentrations were observed in women with ovarian cancer as compared with breast cancer. This could be because of the fact that more of the women with ovarian cancer were in advanced stage disease. Furthermore, the use of amino acids by tumors requires the upregulation of amino acid transporters (21), pulling these metabolites from the blood. Decreases in circulating glucose and amino acids could subsequently result in an overall decrease in energy metabolism elsewhere in the body, diminishing other metabolic pathways such as the urea cycle, resulting in lower concentrations of urea and creatine, and potentially affecting gut microbial population and/or metabolism. These observations will undoubtedly be the subject of future studies.

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The fact that we found almost no false negatives (98% and 100% sensitivity for ovarian and breast cancer, respectively) and few false positives (99% and 93% specificity for ovarian and breast cancer, respectively) suggests that our test would be an effective screening tool with no harmful side effects. Indeed, breast mammography, where the number of false positives and false negatives are many times what we have demonstrated, has resulted in a significant decrease in mortality (22). We suggest that our novel urine test is faster, easier to administer, less costly, and noninvasive, and could be used as a prescreen to other forms of more invasive or uncomfortable screening. The majority of the breast cancers in this study were small ductal carcinomas and even DCIS, that is, very small cancers that were confined to the breast tissue, and they were easily detected by our methods. We have shown that metabolomics is proving useful as a potential screening tool. In the future, we will undertake a study of a larger prospective cohort to further validate the accuracy of this test.

In summary, patients with either breast or ovarian cancer show distinct changes in their urinary metabolite signature. Urinary metabolite measurements have the capacity to revolutionize cancer detection, and potentially cancer treatment, if the early stage can be identified and treated.

Disclosure of Potential Conflicts of Interest

C.M. Slupsky currently holds equity interest in Metabolistics Inc., a biotechnology company in the metabolomics domain, and holds IP interest in this field. H. Steed and M.B. Sawyer hold advisory interests in Metabolistics Inc.

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