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Diagnostic Markers for Early Detection of Ovarian Cancer

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Abstract

Purpose: Early detection would significantly decrease the mortality rate of ovarian cancer. In this study, we characterize and validate the combination of six serum biomarkers that discriminate between disease-free and ovarian cancer patients with high efficiency.

Experimental Design: We analyzed 362 healthy controls and 156 newly diagnosed ovarian cancer patients. Concentrations of leptin, prolactin, osteopontin, insulin-like growth factor II, macrophage inhibitory factor, and CA-125 were determined using a multiplex, bead-based, immunoassay system. All six markers were evaluated in a training set (181 samples from the control group and 113 samples from OC patients) and a test set (181 sample control group and 43 ovarian cancer).

Results: Multiplex and ELISA exhibited the same pattern of expression for all the biomarkers. None of the biomarkers by themselves were good enough to differentiate healthy versus cancer cells. However, the combination of the six markers provided a better differentiation than CA-125. Four models with <2% classification error in training sets all had significant improvement (sensitivity 84%-98% at specificity 95%) over CA-125 (sensitivity 72% at specificity 95%) in the test set. The chosen model correctly classified 221 out of 224 specimens in the test set, with a classification accuracy of 98.7%.

Conclusions: We describe the first blood biomarker test with a sensitivity of 95.3% and a specificity of 99.4% for the detection of ovarian cancer. Six markers provided a significant improvement over CA-125 alone for ovarian cancer detection. Validation was performed with a blinded cohort. This novel multiplex platform has the potential for efficient screening in patients who are at high risk for ovarian cancer.

Ovarian cancer is the fifth leading cause of cancer-related death in women in the United States and is the leading cause of gynecologic cancer deaths. Despite being one-tenth as common as breast cancer, it is three times more lethal and carries a 1:70 lifetime risk. This year, approximately 20,180 women will be diagnosed with ovarian cancer, and 15,310 will die from the disease (1).

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The high mortality rate of ovarian cancer is due to the lack of a screening strategy to detect early-stage disease. Ovarian cancer presents with very few, if any, specific symptoms. Twenty percent of patients are diagnosed at stage I and II when the disease is still confined to the ovary. In patients diagnosed with advanced disease, the 5-year survival rate ranges from 20% to 25%, depending on the stage and grade of tumor differentiation (2, 3). Of these patients, 80% to 90% will initially respond to chemotherapy, but less than 10% to 15% will remain in permanent remission (2, 4, 5). Therefore, an adequate screening test for early detection of ovarian cancer should greatly improve patient survival.

Currently, in some institutions, the screening strategy for ovarian cancer is annual pelvic examinations. Transvaginal ultrasound and serial measurements of the biomarker CA-125, have been included for the high-risk population, but with little success. CA-125 exhibits a sensitivity of less than 60% in early stages of the disease (6, 7). Even the addition of ultrasound screening to CA-125 measurement improves the positive predictive value (PPV) to only about 20% (8 – 10).

Recently, we described the characterization of a panel of biomarkers that can recognize ovarian cancer, including stage I and II (11). The test consisted of four serum proteins, which when analyzed together gave a sensitivity of 95% and a specificity of 94%. Although the reported sensitivity and specificity for this assay is better than any other presently available test, due to the low prevalence of ovarian cancer in the

Table 1. Characteristics of the patient population

Ovarian cancer type	Stage I	Stage II	Stage III	Stage IV/X	Benign tumors
	Mean Age = 48	Mean Age = 62	Mean Age = 64	Mean Age = 65	Mean Age = 45
Serous carcinoma	2	10	47	19	—
Endometrioid carcinoma	1	2	5	5	—
Mucinous carcinoma	2	1	2	1	—
Mixed carcinoma	1	3	5	5	—
Borderline	3	—	—	—	—
Stromal cell	2	1	3	0	—
Germ cell	2	1	0	1	—
Undifferentiated carcinoma	—	3	6	3	—
Clear cell carcinoma	—	2	4	2	—
Squamous cell carcinoma	—	—	1	1	—
Small cell carcinoma	—	—	1	—	—
Unstaged	—	—	—	9	—
Cystadenoma	—	—	—	—	3
Cyst	—	—	—	—	9
Fibrosis	—	—	—	—	1
Ovary benign	—	—	—	—	4
Number	13	23	74	46	17

general population (40/100,000), it could be argued that it is not specific enough as a clinical test for ovarian cancer detection. In order to outweigh the benefit of early detection with the complication of surgery for false-positive screening results, an ovarian cancer screening strategy must achieve a minimum of 99.6% specificity (6).

The objective of this study is to evaluate whether the inclusion of additional biomarkers and the use of an alternative platform may improve the sensitivity and specificity of this test and will enable its use as a screening tool for the detection of ovarian cancer. We describe the development and characterization of a serum biomarker test with a sensitivity of 95.3% and a specificity of 99.4%.

Materials and Methods

Patient population

Ovarian cancer group. The disease group (n = 156) includes women with newly diagnosed ovarian cancer (pelvic mass). All samples were collected previous to diagnosis at the gynecologic oncology clinic. Of the 1,375 blood samples collected between August 2002 and November 2006, 156 samples were classified as newly diagnosed ovarian cancer and were used in this study (stages I-IV and ages ranging from 25-88 years). The diagnosis of ovarian cancer was determined by clinical, surgical, histologic, and pathologic diagnosis. Of the 156 patients with ovarian cancer, 36 women were diagnosed with stage I/II and 120 with stage III/IV disease. The histology types are summarized in Table 1 for each stage. Inclusion and exclusion criteria are described in the Supplementary Material.

Control group. The healthy control group (n = 362) included age-matched healthy individuals who came for a regular gynecologic examination. These individuals did not have a diagnosis of any type of cancer, were not genetically predisposed to develop ovarian cancer, and were disease free at least 6 months after sample collection. A total of 568 samples were collected between August 2002 and November 2006, and 362 were included in this study. Inclusion and exclusion criteria for this group are described in the Supplementary Material. In addition, samples from 17 women diagnosed with benign masses were also included.

Patients were enrolled in the "Discovery to Cure" early detection program in the Department of Obstetrics and Gynecology at Yale University under HIC protocol No. 10425/26784. Information about diagnosis, staging histology, and grade was provided by the Gynecologic Oncology Clinic at the Yale New Haven Hospital. No data allowing identification of the patients were provided.

Qualified personnel obtained informed consent from each individual participating in this study.

Sample collection

Ten mL of peripheral blood was drawn from subjects using standardized phlebotomy procedures (11). Within 2 to 4 hours of collection, samples were processed using guidelines set by the National Cancer Institute Inter-Group Specimen Banking Committee and stored at -80°C in the Tissue/Sera Bank of the Discovery to Cure program.

Multiplex analysis

Details of the assay are described in the Supplementary Material. In short, the Beadlyte 6-plex Ovarian Cancer Panel Kit includes two panels: one for leptin, prolactin, macrophage inhibitory factor (MIF), osteopontin, and CA-125 (Beadlyte 5-plex Ovarian Cancer Panel) and a separate panel for IGF-II (Beadlyte Anti-Human IGF-II Bead Set).

Multiplex study design

The characterization and validation of the multiplex assay was done using 535 individual serum samples divided in two study phases. In phase 1 (characterization), the training set consisted of 294 serum samples: 181 samples from healthy individuals (control group) and 113 samples from patients with newly diagnosed ovarian cancer (stage I-IV; case group).

In stage 2 (validation/test), 224 samples were evaluated in a blinded manner (the technical personnel running the test and the statistician had no prior information related to the samples). This test set consisted of 181 samples obtained from healthy individuals (43 samples from newly diagnosed ovarian cancer patients [Stage I-IV]). A smaller number of cases in the validation/test set was due to inclusion of only those samples that had never been used in the discovery process leading to the identification of the above six markers or combination rules, to avoid optimism in reporting performance. An additional 17 samples from patients with benign disease were evaluated with the final model.

Serum analysis using ELISA analysis. ELISA was used as previously described (11).

Statistical analysis of data

Three independent statisticians from two different institutions analyzed the data (ZF and GL from the Fred Hutchinson Cancer

Table 2. Statistical analysis of ovarian cancers (OC)

	Training set		Validation set				
	Cross-validation error (%)	**Cross-validation sensitivity at 95% specificity**	**Total (OC and controls) /misclassified**	**Misclassification error (%) (SD)**	**Sensitivity for OC at 95% specificity**	**Total (early OC and controls) /misclassified**	**Sensitivity for early-stage OC at 95% specificity**
Model I	2.1	0.95	224/3	1.3	0.98	191/2	0.90 (0.70, 1.0)
Model II	1.6	0.951	224/3	1.3	0.95	191/1	0.90 (0.70, 1.0)
Model III	0.79	0.987	224/14	6.3	0.84	191/11	0.60 (0.30, 0.90)
Model IV	0.80	0.988	224/16	7.1	0.88	191/15	0.70 (0.40, 1.0)
CA-125	0.72	0.72	224/17	7.6	0.72	191/9	0.47 (0.20, 0.85)

NOTE: Model I: logistic regression with six proteins; Model II: logistic regression with four proteins (leptin and MIF dropped); Model III: split point model by cutoff point at 95% specificity; Model IV: split point model with cutoff point maximizing classification accuracy.
Prediction model for Model I: logit (probability of OC) = -2.355801 - 0.0130787 * leptin + 0.0703821 * prolactin + 0.0002253 * OPN + 0.0112188 * IGF-II + 0.0031604 * MIF + 1.418684 * CA-125. Prediction model for Model II: logit (probability of OC) = -0.9963645 + 0.0704505 * prolactin + 0.0002704 * OPN + 0.0127398 * IGF-II + 1.274593 * CA-125.

Research Center, Seattle, Washington and YL from George Washington University, Washington DC).

Training data analysis. Each individual marker was evaluated by a receiving operational characteristic (ROC) curve, the area under the ROC curve (AUC), and sensitivity at 95% specificity (ROC [0.05]). Confidence intervals (CIs) were calculated by 1,000 bootstrap samples. Four models were used to combine markers. Model I used logistic regression on raw values of each marker. Model II was similar to model I, except it allowed to drop some markers from the model. Model III created an indicator for each marker at the cutoff point corresponding to 95% specificity (1 if above cutoff, 0 otherwise, invert marker sign if smaller value indicates cancer). A logistic regression model was then used to find a linear combination of the six indicators to minimize the total classification error. Model IV was similar to model III, except the

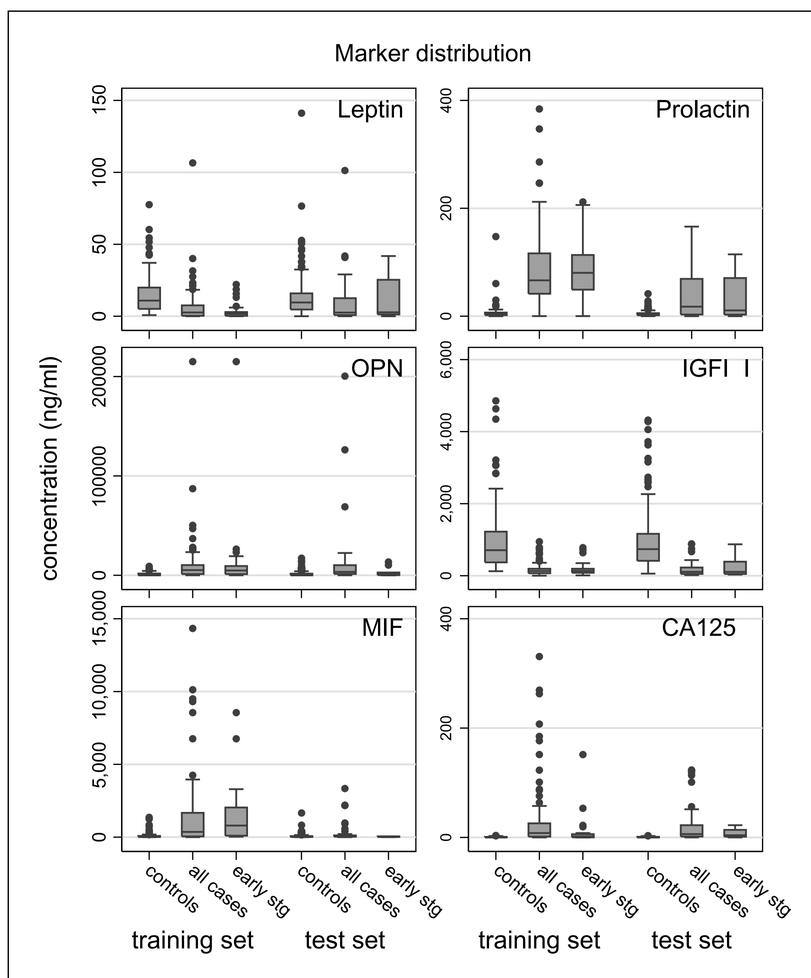

Fig. 1. Boxplot display of biomarker distributions for controls, ovarian cancer cases, and the subset of early stage (I and II) cases, by training and validation set assignment. Horizontal box boundaries and midline denote sample quartiles. Whiskers mark *adjacent values: upper adjacent value = largest marker value x such that x < = 75th percentile + 1.5 * interquartile distance. Similarly, lower adjacent value = lowest marker value such that x > = 25th percentile - 1.5 * interquartile distance. The interquartile distance = 75th-25th percentile (25).

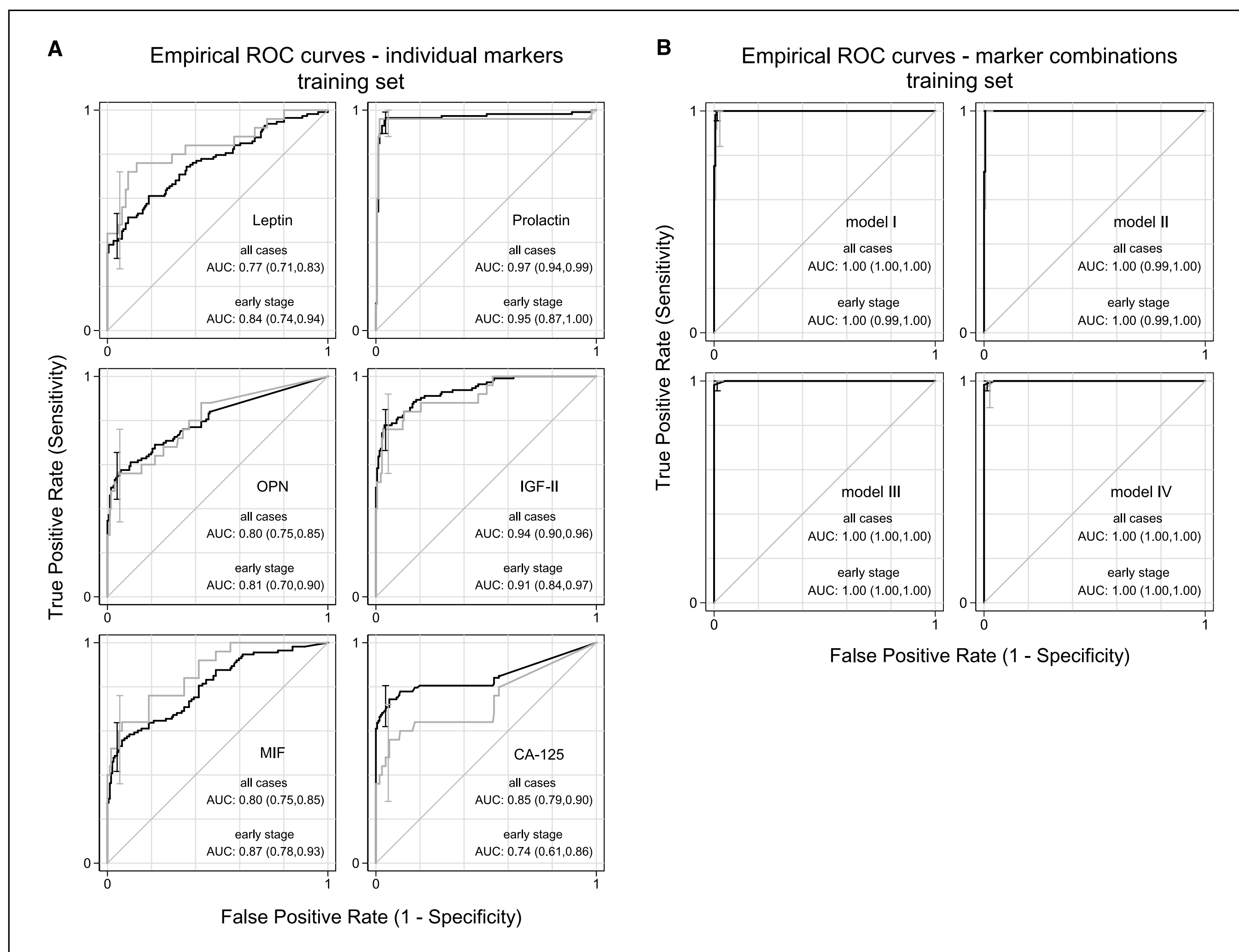

Fig. 2. Biomarker ROC curves for cases and controls assigned to the training set (*A*) and validation set (*C*). Lighter line types correspond to ROC curves for the subset of cases with early-stage disease. Ninety-five percent CIs for the TPR (sensitivity) at 95% specificity and for the AUC are based on bootstrap distribution percentiles. ROC curves for composite predictors corresponding to models I through IV as applied to subjects in the training (*B*) and validation sets (*D*). The composite predictors for both subject sets is based on a fit of the respective model in the training set only.

cutoff point was chosen to minimize the total classification error. Models III and IV aimed to produce a simple intuitive classification rule for clinicians with possible reduction in classification accuracy compared with models I and II. Ten-fold cross validations (CV) were used for estimating classification accuracies. To reduce the impact of randomness on 10-fold CV, 1000 random sample divisions were performed.

Validation set data analysis. Sensitivity, specificity, and total classification accuracy were calculated using the validation set data for each marker and for each of four models obtained from training data (Table 2).

Results

Evaluation of ELISA and multiplex platforms. First, we determined whether a multiplex assay could adequately replicate the results previously obtained with ELISA (11). Comparison of concentrations obtained for prolactin, leptin, OPN, and IGF II by both multiplex and ELISA in 50 serum samples from newly diagnosed ovarian cancer patients and 50 serum samples from age-matched healthy individuals exhibited the same pattern for the four markers (Supplementary Fig. S1). Statistical analysis of the values obtained from the multiplex assay showed similar results to those previously reported using ELISA (95% sensitivity and 94% specificity; data not shown).

Characterization of six biomarkers in a multiplex platform: Training set. To increase the sensitivity and specificity of the multiplex assay, we added two additional markers to the multiplex platform: MIF, which we found to be highly expressed in the sera of ovarian cancer patients (11) and CA-125. First, we evaluated the serum concentrations of six markers with a training set consisting of 113 newly diagnosed ovarian cancer patients and 181 age-matched healthy individuals as the control group. MIF levels were significantly higher in the sera from ovarian cancer patients compared with healthy individuals (1302.45 pg/mL ± (232) vs 85.56 ± 25.2 pg/mL, $P < 0.001$) confirming our previous results using ELISA (12). Similarly, serum from patients with ovarian cancer showed elevated levels of prolactin, OPN, and CA-125 as well as low

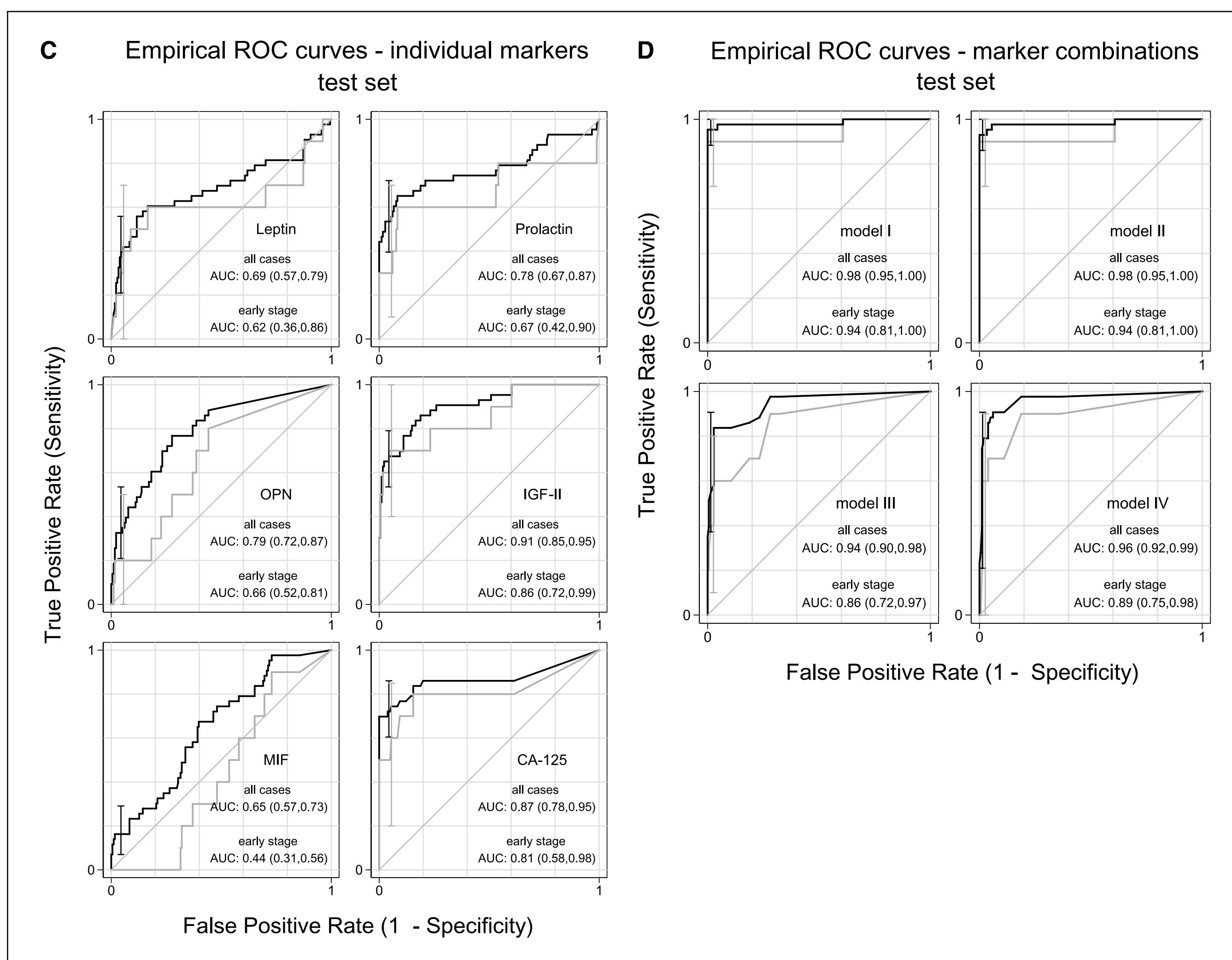

Fig. 2 *Continued*.

levels of leptin and IGF-II compared with the healthy group (Supplementary Table S1). The distributions of each marker in ovarian cancer (all ovarian cancers and early-stage ovarian cancers) and healthy control groups were compared with boxplots (Fig. 1). (The distribution summary for all six markers by case control/status is included in Table 1 of the Supplementary Material.).

The ROC curve for each individual marker on the training set (Fig. 2A) showed that classification performance for all six markers has significant discriminating ability to differentiate ovarian cancer patients from healthy controls, as AUCs are all significantly above 0.5. Among the six biomarkers, prolactin and IGF-II had significantly larger AUCs than that of CA-125; leptin, OPN, and MIF had compatible AUCs with CA-125. All four models have greater than 98% classification accuracy in training set (Fig. 2B), and misclassification by CV was between 0.8% and 2.1% (Table 2), as compared with a misclassification rate of 14% for CA-125. These results were not surprising because they were the same samples that were used to establish the model.

Performance of the six biomarkers in a multiplex platform: Blind study (test set). Our next objective was to determine whether the panel of six markers could differentiate normal versus ovarian cancer samples in a complete different cohort, which we identified it as the test set. AUCs in the test set decreased in the biomarkers, showing the importance of using a different cohort for the validation of the training model. Prolactin had the biggest decrease in AUC, while IGF-II and OPN had the smallest drop. However, all markers still had AUC significantly above 0.5 except for early stage cases in which only IGF-II, CA-125, and OPN have AUC significantly above 0.5, which meant that each biomarker is still indicative for ovarian cancer (Fig. 2C). The four models described in the statistical methods section were used to evaluate the performance of the markers. All four models continue to have better AUC (0.95-0.98) and ROC (0.05) (0.84-0.98) than CA-125 (AUC 0.87, ROC [0.05] = 0.72). For early-stage ovarian cancer, the four models have AUC 0.88-0.94 and ROC (0.05) 0.60-0.90, as compared with CA-125, AUC = 0.81, ROC (0.05) = 0.50, respectively (Fig. 2D). There is clear loss of diagnostic ability due to simplifying continuous markers into dichotomous values when comparing model I and II versus model III and IV (Table 2), although their 95%

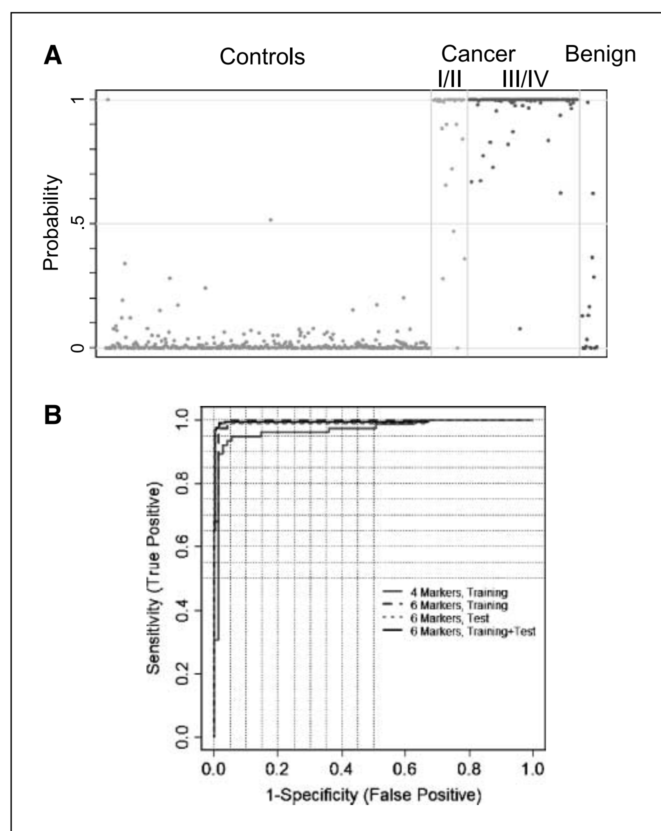

Fig. 3. *A,* scatterplot of final model predicted disease probabilities by disease group for subjects in the combined training and test sets. Predicted probabilities for controls and ovarian cancer cases correspond to the average of 1,000 10-fold CV fits of the model in the combined set (not including benign subjects). Subjects are randomly ordered on the x-axis within disease group. *B,* ROC curves for composite predictors corresponding to final model using four markers and the six markers with the different cohorts. Note the improvement with the six markers and the similarity of the results independent on the cohort analyzed.

CIs overlapped. The prediction model parameters are given in Table 2.

Design of the final model for the six biomarkers in a multiplex platform. We then evaluated the performance of model I for both sets of samples, the training and test set. No differences were observed when we applied model I to the test set or the combination of training and test (Fig. 3B and Supplementary Table 2), therefore, we fit a "final model" using observations from the combined training and test sets:

Logit [probability of ovarian cancer] = -3.13 + 0.0202*leptin + 0.0763*prolactin + 0.000277*OPN - 0.00834*IGF-II + 0.00292*MIF + 1.15*CA-125 <comp: set equation>.

The concentration for each biomarker in the healthy and cancer groups are summarized in Supplementary Table S3.

The performance of the final model was evaluated using 10-fold CV. From 362 healthy samples, two were misclassified, and from 156 ovarian cancer samples, five were misclassified (Fig. 3A). Evaluation of the results according to disease stages showed that from 36 stage I and II samples, 4 were misclassified (91.6%), whereas 119 of 120 stage III and IV samples were correctly classified (100%). The misclassified stage I and II samples were granulosa cell carcinoma, undifferentiated carcinoma, and a papillary serous carcinoma. From 17 samples obtained from patients diagnosed with a benign mass, two samples were misclassified as cancer, and the remaining 15 were correctly classified as normal (88.2%).

With this unique combination of six markers, we were also able to accurately recognize all of the different histologic subtypes of primary ovarian cancers: epithelial (papillary serous, clear cell, endometroid, and mixed), germ cell (yolk sac and dysgerminomas), and sex cord stromal (granulosa cell) tumors. The misclassified cancer samples were a stage I granulosa cell tumor, a stage II undifferentiated carcinoma, and a stage II papillary serous carcinoma.

ROC curves were used to examine the performance characteristic of the six markers in the training set, the test set, and these sets together, as well as comparing the results obtained from ELISA. As shown in Fig. 3B, the multiplex test using the six markers provided a significant improvement to the sensitivity and specificity compared with the 4-plex ELISA platform. As indicated above, no significant differences were found between the ROC curves for the training set, the test set, and the combination. Therefore, the statistical results for the final model are sensitivity 95.3% and specificity 99.4%. The PPV = TP/(TP+FP) for the test sample is 99.3% and the negative predictive value = TN/[FN+TN] is 99.2%. The final model provides more optimistic results compared with the test group, because it includes the training set; however, there are not major differences in terms of specificity and sensitivity for either model. Nevertheless, this final model will be validated in a multicenter validation study.

Discussion

In the present study, we describe the characterization of a novel blood test, based on the quantitative analysis of six biomarkers using a multiplex platform. This test can discriminate between disease-free women and ovarian cancer patients (stage I-IV) with high specificity (99.4%) and sensitivity (95.3%).

Ovarian cancer does not show specific symptoms that allow its identification during early stages. Diagnosis is frequently made only after progression to later stages, at which point the dissemination of the cancer limits effective treatment. Therefore, the development of sensitive and specific methods for early detection has been a priority as a means for improving the treatment of this disease.

The potential implication of early detection of ovarian cancer on patient outcome is shown by the differential survival rates of women diagnosed at different stages of disease progression. The 10-year survival rate is nearly 90% when the disease is localized to the ovaries at the time of diagnosis and drops to 20% when the disease has spread to distant sites at the time of diagnosis (13).

Serum markers are useful tools for the diagnosis and follow-up of patients with different forms of cancer (14). Numerous proteins have been identified as specific markers for ovarian cancer either in blood or urine (13, 15, 16). However, no single protein has provided adequate sensitivity and specificity. As shown in this study, each marker by itself poorly differentiates between cancer and healthy individuals, whereas the combination of these markers was able to significantly increase both the specificity and sensitivity of the assay. Lokshin's group reported a multiplex assay consisting of 24 cytokines in combination with CA-125 that when used in

different combinations could differentiate early-stage ovarian cancer (17).

Recently, Anderson suggested a road map for the creation of a viable diagnostic biomarker, which is composed of the following steps: discovery, verification/validation, and clinical implementation (14, 18). Our entire approach in the development of this test has followed the suggested "road map." For the discovery phase, we used protein arrays, which is a powerful technology for discovery but not for diagnostics. In addition, we limited the output to proteins associated with the control of cell growth. With this approach, we were able to avoid covering the whole proteome. The identified candidates were measured in a coherent technology platform, ELISA, which provided sufficient uniformity across samples and laboratories. In the validation stage, the samples were evaluated in a blind manner, the statistical analysis was confirmed by different approaches, and the results were confirmed by validation within the research community. For the third stage, the potential clinical implementation for a diagnostic test, we have included two additional markers, including CA-125 and implemented a novel platform that could simplify the test and decrease potential variability. The use of multiplex provides numerous advantages as a platform for the diagnostic implementation (17). The ability to measure multiple markers in a small sample volume makes the multiplex platform suitable for large validation studies (19). The use of six biomarkers is adequate enough to provide the sensitivity and specificity required but not sizeable enough to impede and complicate evaluation.

The biological significance of the described biomarkers provides for a better understanding of the test. The proteins identified in this study, with the exception of CA-125 and MIF, are all related to the normal physiology of the ovaries. These proteins are produced either by the surrounding supportive cells or as a response to signals originating from the ovary. IGF-II is the primary IGF in the human ovary, acting as mediator of gonadotropin action (20, 21). Prolactin on the other hand, has been shown to participate in the regulation of steroidogenesis in ovarian follicles, particularly the inhibition of progesterone secretion in the early stages of the follicular growth and its enhancement in the luteal phase (22, 23). There is strong evidence that links leptin and the hypothalamic gonadotropin-releasing hormone secretion, which affects the hypothalamic-pituitary-ovarian axis (24). The levels of expression of these proteins are maintained by a delicate balance between each of the cellular components of the ovary.

We hypothesize that the presence of abnormal cells may alter this intercellular communication and disrupt the axis, resulting in the abnormal expression levels observed in cancer samples. The proteins identified in this study may not represent factors produced by the tumor but instead represent the organ/body's response to the presence of neoplastic cells. We propose that significant levels of tumor products could only be detected in peripheral blood at later stages of tumor development; however, the body is able to recognize and respond to early stages of the tumorigenicity, and this is reflected in peripheral blood proteins. Based on this premise, the protein panel identified in this study is able to detect early stages of the disease.

An important aspect of this study is the use of different cohorts for the training and the test sets. As shown in our study, the use of the same samples for the training and test evaluation generate results that are not representative of the test's real function. If we use the same samples used for the training, we observed a high specificity and sensitivity for each of the markers; however, when we evaluate in a different cohort, the sensitivity and specificity of each marker decrease, although they remain higher than the standard CA-125.

Although the sensitivity of the test for stage I and II is 91.6%, it is significantly higher than the only available test CA-125, which has a sensitivity less than 60% in early disease (6, 7). Consequently, the use of this test will enhance the potential of treating ovarian cancer in its early stages and therefore, increases the successful treatment of the disease. In terms of the PPV for the general population, the test is above the suggested 0.10 necessary used as a screening test (6, 7).

Conclusion

In summary, we have shown the specificity of this test for detection of ovarian cancer using three different platforms, four types of statistical analysis, and two different sample cohorts. Therefore, the results described in this study are not the product of a "unique sample population," or a potential artifact of a technique, or the result of a sophisticated algorithm.

The sensitivity of the test fulfills the last requisite of the proposed road map: a viable application for screening with a limited risk for false-positives results. The implementation of this test for early detection of ovarian cancer may prove beneficial for the management of this deadly disease.

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