# An Autoantibody Signature for the Serologic Detection of Ovarian Cancer

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**ABSTRACT**

Sera from patients with ovarian cancer contain autoantibodies (AAb) to tumor-derived proteins that are potential biomarkers for early detection. To detect AAb, we probed high-density programmable protein microarrays (NAPPA) expressing 5,177 candidate tumor antigens with sera from patients with serous ovarian cancer (n=34 cases/30 controls) and measured bound IgG. Of these, 741 antigens were selected and probed with an independent set of ovarian cancer sera (n=58 cases/60 controls). Twelve potential autoantigens were identified with sensitivities ranging from 13-22% at >93% specificity. These were retested using a Luminex bead arrays using 60 cases and 60 controls, with sensitivities ranging from 0-31.7% at 95% specificity. Three AAb (p53, PTPRA, and PTGFR) had area under the curve (AUC) levels>60% ($p<0.01$), with the partial AUC (SPAUC) over 5 times greater than for a non-discriminating test ($p<0.01$). Using a panel of the top three AAb (p53, PTPRA, and PTGFR), if at least two AAb were positive, the sensitivity was 23.3% at 98.3% specificity. AAb to at least one of these top three antigens were also detected in 7/20 sera (35%) of patients with low CA125 levels and 0/15 controls. AAb to p53, PTPRA, and PTGFR are potential biomarkers for the early detection of ovarian cancer.

## INTRODUCTION

Ovarian cancer is the fifth leading cause of cancer-related mortality of women in the U.S., with over 15,000 deaths per year [1]. Early diagnosis is associated with improved overall survival [2]; however, the majority of patients are currently diagnosed with advanced disease. The five-year survival rate for late-stage ovarian cancer remains less than 30%. Despite the identification of serum CA 125 as a biomarker for ovarian cancer in 1983 [3], there are currently no screening biomarkers recommended for use for the general population.

The utility of CA 125 as a screening test is limited by a low sensitivity of 50% for early stage disease at 99% specificity [4]. Combining CA 125 with transvaginal ultrasound (TVUS) increased the specificity of detection in the UKCTOCS large-scale screening trial [5]. In a joint validation study of 28 potential markers for detecting ovarian cancer in blood [6], the most accurate marker remains CA 125, followed closely by HE4 [7]. Panels of markers demonstrated only marginal improvements over CA 125 alone for the early detection of disease. A recent study showed that the addition of CEA and VCAM-1 to CA 125 and HE4 increased the sensitivity of detection of stage I and II ovarian cancer to 86% at 98% specificity [8], but this remains to be confirmed in a blinded validation study using prediagnostic sera. Biomarkers are needed that complement CA 125 and HE4.

Protein overexpression or mutation can lead to the spontaneous development of autoantibodies (AAb) in the sera of patients with cancer [9]. Tumor antigen-specific AAb have been identified in the sera of patients with cancer, including patients with early-stage disease [10]. There are several potential advantages of AAb biomarkers, including signal amplification by the immune response and persistence of antibodies after antigen is no longer detected. P53-specific AAb, which are associated with p53 mutation and resultant protein stabilization, have been detected in early-stage ovarian cancer [11]. We detected p53-specific AAb in 41.7% of patients with serous ovarian cancer at 91.7% specificity [12]. Unlike CA 125 and HE4, p53-AAb were associated with improved survival [HR=0.56] [12].

We hypothesized that the identification of novel AAb biomarkers beyond p53-AAb would increase the sensitivity of detection of serous ovarian cancer. We used the custom protein microarray technology Nucleic Acid Protein Programmable Arrays (NAPPA), which are generated by printing full-length cDNAs encoding the

target proteins at each feature of the array. The proteins are then transcribed and translated by a cell-free system and immobilized *in situ* using epitope tags fused to the proteins [13]. Sera are added, and bound IgG is detected by standard secondary reagents. NAPPA arrays have been used to identify antibody signatures in early-stage breast cancer [10d, 14].

The objective of this study was to identify novel AAb biomarkers for the detection of serous ovarian cancer. To profile the ovarian cancer immune response, we developed protein microarrays displaying 5,177 full-length candidate antigens. We used a sequential screening strategy to select candidate AAb biomarkers to limit the false discovery rate inherent to large-scale proteomic screening. First, we screened 34 cases of serous ovarian cancer and 30 matched healthy controls (Cohort 1) on all 5,177 candidate tumor antigens, and selected 741 antigens for further testing. Second, we screened 60 cases and 60 healthy controls (Cohort 2) on the 741 antigens, and identified 12 potential candidate AAb biomarkers. Third, we used an independent assay (Luminex bead array) to display these autoantigens, and re-screened sera from women in Cohort 2. Finally, we displayed a smaller set of 7 autoantigens and screened sera from an independent set (Cohort 3) of non-serous cancers (n=30), false-negative CA 125 (n=20), benign ovarian disease (n=30), and healthy controls (n=30). The sensitivity and specificity of each individual biomarker, as well as the biomarker panel, is presented.

## METHODS

### *Patient Sera*

Sera used in these analyses were obtained from the Brigham and Women's Hospital and the Dana-Farber Cancer Institute with support from the NCI Early Detection Research Network and Ovarian SPORE program. Sera derived from ovarian cancer patients were obtained at the time of presentation prior to surgery, and patients received routine post-operative therapy (as described in [12]). One case in Cohort 1 was obtained postoperatively. The non-serous cases were derived from 10 patients with endometrioid cancer, 10 patients with clear cell carcinoma, and 10 patients with mucinous carcinoma. The benign disease samples were derived from 19 patients with serous cytadenomas and 11 patients with mucinous cystadenomas. Sera from age-matched general population control women were obtained from the Brigham and Women's Hospital using a standardized serum collection protocol and stored at -80$^{o}$C until use. Cases and matched controls were processed simultaneously. Women with a personal history of cancer (other than non-melanoma skin cancer) were excluded as controls. Written consent was obtained from all subjects under institutional review board approval. For the serous cases included in the survival analysis, medical records were reviewed and details related to presentation and treatment abstracted.

### *Plasmid repository and high-throughput DNA preparation*

Sequence-verified, full-length cDNA expression plasmids in flexible donor vector systems were obtained from the Arizona Biodesign Institute and are publicly available (www.dnasu.edu). These were converted to the T7-based mammalian expression vector pANT7_GST using LR recombinase (Invitrogen, Carlsbad, CA). The high-throughput preparation of high-quality supercoiled DNA for cell-free protein expression was performed as described [15]. For bead array ELISAs, larger quantities of DNA were prepared using standard Nucleobond preparation methods (Macherey-Nagel Inc., Bethlehem, PA). All 12 selected genes were sequence-confirmed prior to validation studies.

### *Detecting serum antibodies on NAPPA arrays*

Detection of serum Abs on NAPPA arrays was performed as described [16]. Plasmid DNA (1.5 μg/mL), capture antibody (50 μg/mL anti-GST antibody, GE Healthcare Biosciences, Piscataway, NJ) or anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO), protein crosslinker (2 mM, BS3, Pierce, Rockford, IL) and BSA (3 mg/mL, Sigma-Aldrich) were co-printed onto the array surface. All samples were printed using a Genetix QArray2 with 300 μm solid tungsten pins on amine-treated glass slides. The printed DNA was transcribed and translated in situ using reticulocyte lysate according to previously published protocols [14]. Protein expression was detected using anti-GST MAb (Cell Signaling, Danvers, MA) diluted at 1:200. For detecting serum antibodies, the arrays were incubated with serum diluted 1:250-1:600 in 5% PBS milk with 0.2% Tween 20 overnight and detected with anti-human IgG-HRP (Jackson ImmunoResearch Labs, West Grove, PA) with Tyramide (PerkinElmer, Waltham, MA). Slides were scanned with a Perkin Elmer ProScanArray HT. The highly immunogenic EBV-derived antigen, EBNA-1, was included as N- and C-terminal fragments for positive control antigens. Negative controls included empty vectors and no DNA controls. Registration spots for array alignment were printed with purified human IgG proteins.

***Detection of antibodies on Luminex bead arrays.***

In vitro expression and display of target protein antigens on Luminex bead arrays was described in [17]. Briefly, each target gene was expressed as a C-terminal GST-fusion protein using T7 reticulocyte lysate (Promega Corporation, Madison, WI) per manufacturer's recommendations with 500 ng DNA. Vector and p21-GST were also expressed as negative controls. The in vitro transcription/translation (IVTT) products were each captured onto SeroMAP carboxylated microspheres (Luminex Corporation, Austin, TX) coupled with anti-GST antisera. Protein-bound microspheres were pooled together and blocked with 10% each of normal sera from mouse, rabbit, goat, and rat (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), 0.5% polyvinyl alcohol (PVA, Sigma-Aldrich, St. Louis, MO), 0.8% polyvinylpyrrolidone (PVP, Sigma-Aldrich, St. Louis, MO), and 2.5% Chemicon (Millipore, Billerica, MA) in PBS-1% BSA. Test sera were diluted 1:80 in blocking buffer, pre-incubated at room temperature for 1 hr with rotation, and then incubated with the beads overnight at 4°C while shaking. Bound IgG was detected with biotin-conjugated goat anti-human IgG antibody (Jackson

ImmunoResearch Laboratories, Inc., West Grove, PA) and streptavidin-R-PE (Molecular Probes, Inc., Eugene, OR). To control for non-specific and GST-specific autoantibody background, the ratio of MFI for individual Abs to the MFI for the control p21-GST antigen was determined. Protein expression was confirmed with a mouse anti-GST monoclonal Ab (Cell Signaling Technology, Danvers, MA) and PE-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

***Detection of CA 125, and HE4 in sera***

The detection of CA 125 and HE4 in these sera has been reported [12]. CA 125 was detected by immunoassay using two monoclonal antibodies [M 11 and OC 125, Fujirebio Diagnostics]. The upper 95$^{th}$ percentile cutoff for healthy pre- and post-menopausal women is 35 U/mL. [HE4] was detected using a double monoclonal ELISA (RK®, Fujirebio Diagnostics).

***Statistical Analysis***

For the pre-screen, 34 cases and 30 control sera (Test set, Cohort 1) were screened on 5,177 antigens displayed in NAPPA protein array format. Each array was normalized by first removing the background signal estimated by the first quartile of the non-spots and then log-transforming the median-scaled raw intensities to bring the data to the same scale and stabilize the variance across the range of signals. Candidate antigens from the initial 5,177 antigens were selected if they met two different criteria: 1) comparison of the 95$^{th}$ percentiles of the cases and controls using quantile regression [18] and 2) comparison of the proportion of cases with intensities above the 95$^{th}$ percentile of controls to the expected number seen by chance using binomial tests, with a p-value$\leq$0.05.

Independent arrays of these 741 candidate antigens were screened with a fully independent set of age-matched sera consisting of 60 healthy controls and 60 patient sera. We normalized these arrays as follows. First, we removed any duplicate antigen pairs that differed by more than 3 times the median absolute deviation, resulting in removal of 0.2% of spots. Second, we removed background signal by subtracting the first quartile of control spot (no DNA) intensity. Third, to normalize across arrays and 384-well plates we divided the excess

intensity by the median excess intensity for each array and 384-well plate. Two case sera failed our QC measures (more than 20% of spots below background signal) and were excluded from further analysis.

We computed the sensitivity at an approximate 95% specificity for each antigen as follows. We determined a threshold by computing the 95% empirical percentile of the normalized intensity values of the controls. We then computed the sensitivity as the proportion of the cases that exceeded that threshold, and the actual specificity as the proportion of the controls that did not exceed the threshold.

To identify the most sensitive antigens at a high level of specificity, we used receiver operator characteristic (ROC) curve analysis. Specifically, we tested the hypothesis that the partial area under the ROC curve (PAUC) in the region where the specificity > 95% exceeds 0.00125, which is the PAUC for a non-informative diagnostic test. P-values were computed using a normal approximation to the bootstrap sampling distribution and q-values were computed using the qvalue package in R[19]. We used the training set to identify 13 potential antigen biomarkers with q-values < 0.15 (i.e. significant with 15% false discovery rate control). Each of these antigens had a p-value < 0.01; for technical reasons 12 were used for further analysis.

RAPID Luminex ELISA analyses were performed in duplicate. Differences between cases and controls were assessed by chi-square tests. To assess the added value of AAb along with CA 125 and HE4 to discriminate cases from healthy controls, we constructed ROC curves and calculated areas under the curve. Associations between clinical characteristics and AAb detection among cases were tested using logistic regression adjusted for age and Jewish ethnicity. All p-values were two-sided. Statistical analyses were performed using SPSS 14.0 software (SPSS Inc, Chicago, IL) and SAS (SAS Institute Inc., Cary, NC).

## RESULTS

### *Strategy for Biomarker Selection*

Our primary goal was to identify serum AAb biomarkers that would distinguish serous ovarian cancer from healthy controls in order to guide further imaging and monitoring decisions. In order to identify a biomarker panel of AAb in ovarian cancer from 5,177 candidate antigens, sera were tested in sequential stages as described in **Figure 1**. All training and validation case and control sera were gender- and age-matched, collected prior to surgery (for cases), and under standardized collection protocols. Control sera and questionnaires were collected from healthy women in the Boston area with no history of cancer. The serous cases were primarily stage III/IV (95%). **Table 1** shows the age distribution, menopausal status, and sample collection details of the cases and controls selected for these studies. Cases and controls did not differ in age, race, menopausal status, year of blood collection, or length of storage. Only the non-serous cases had a higher frequency of oral contraceptive (OC) use (p=0.003) compared to their matched controls. As expected, they also had lower mean CA 125 levels (85 U/ml) compared to serous cases.

In stage 1 of the biomarker selection, 5,177 antigens were pre-screened with sera (Cohort 1) from 34 cases of serous ovarian cancer and 30 matched controls. For stage 2, 741 selected antigens were screened with sera from 60 cases and 60 matched controls (Cohort 2). Twelve selected antigens were displayed on bead arrays, re-screened with the training set to establish the performance of the assay. Finally, 7 promising antigens were screened by bead arrays using non-serous cases (n=30) and healthy controls (n=30), as well as sera with low CA 125 levels (n=20) and healthy matched controls (n=15). The clinical characteristics of the serous cases in the test, training, and validation sets were similar, but with lower CA 125 levels (mean=647), on average, in the validation set than in the test set (mean=1232).

### *Generation of NAPPA Custom Protein Microarrays for Biomarker Detection*

High-density NAPPA protein microarrays were generated for these studies for biomarker detection as described [14a]. The 5,177 individual cDNAs used on these arrays were derived from the Arizona Biodesign Institute DNA

repository. These cDNAs were all sequence-verified, full length, wild-type genes fused in frame with either a C-terminal GST tag or N-terminal FLAG tag in a vector optimized for mammalian protein expression. The content of these arrays have been described [16] and include the Breast Cancer 1000 gene set [20], selected for their association with breast cancer using bioinformatics and data mining tools. Additional genes included over 300 G-coupled protein receptors (GPCRs), 500 kinases, and 700 transcription factors. The cDNAs were coprinted on glass slides with anti-tag antibodies at a high density (up to 2300 antigens/slide; 3 slides/gene set). Proteins were expressed and captured *in situ* on the arrays using a coupled in vitro transcription-translation system derived from rabbit reticulocyte lysate. DNA content was confirmed by picogreen, and protein expression was confirmed by probing the arrays with anti-GST antibodies (not shown).

***Selection of the Antibody Biomarker Panel***

The goal for Stage 1 was to limit the number of screened antigens by eliminating all of the uninformative antigens (e.g., no difference between case and control). This has the advantages of reducing the false positive rate and the cost of the screen. Thus, 34 cases/30 control sera were screened with sera at 1:250 to 1:600 dilution on 5,177 single antigens, and the arrays were normalized for background intensity (see Statistical Analysis). Protein expression of individual spots on the microarrays was confirmed with anti-GST (not shown), since the spotted cDNAs encode C-terminal GST fusion proteins. The sera were added to the arrays, and bound IgG detected with secondary antibodies. The top 741 antigens (**Supplemental Table 1**) were selected if the $95^{th}$ percentile of signal of cases and controls were significantly different ($p<0.05$) and if the number of cases with signals above the $95^{th}$ percentile of controls was larger than the number expected due to random chance ($p<0.05$).

The goal of the second stage was to identify candidate AAb for further validation. The selected 741 cDNAs were printed in duplicate on single arrays. These arrays were screened with a separate training set of sera from serous ovarian cancer (n=58) and sera from healthy controls (n=60). Two additional patient sera were removed from analysis due to unusually elevated background on the arrays (>5x mean). From these data, 12 antigens were selected as potential biomarkers for further analysis based upon a statistical test of the partial area

under the receiver operator characteristic curve (see Statistical Analysis). The selected antigens had $p < 0.01$ and were significant with a $\leq$15% false discovery rate (FDR).

***Development of a high-throughput Luminex bead array ELISA for AAb detection***

For high-throughput screening of larger numbers of sera, the 12-antigen panel was displayed on a custom Luminex microbead array, which allows for rapid, multiplexed screening of sera in a readily exportable, pre-clinical format. First, the performance characteristics of the 12 target antigens were evaluated using the same set of sera (Cohort 2) used to screen the 741 antigens (n=60 cases/60 controls, **Table 2**). The sensitivities for these antigens are shown, using a cutoff that achieves 95% specificity. AUC and scaled partial AUC (SPAUC) values and their respective p-values are also shown. Overall, these data show that the sensitivity of each individual antigen is low (ranging from 0-31.7%), with AUC levels >60% for p53, PTGFR, PTPRA ($p<0.01$). Further, for these three antigens the partial area under the ROC curve is more than 5 times greater than for a non-discriminating test (SPAUC > 5; $p < 0.01$).

***Detection of autoantibodies in non-serous ovarian cancer***

To determine the performance characteristics of the biomarker panel for non-serous ovarian cancer, the Luminex bead array ELISA was used to determine AAb levels in cases derived from 10 patients with endometrioid cancer, 10 patients with clear cell carcinoma, and 10 patients with mucinous carcinoma (**Table 3**). For this analysis, the top 3 potential antigens (p53, PTGFR, and PTPRA) were selected, as well as 4 additional potential antigens from the validation assay. As expected, p53 AAb were also detected in non-serous ovarian cancer, but at a lower AUC (57.4%), consistent with the lower frequency of p53 mutations (which are strongly associated with AAb formation) in these tumors [21]. In contrast, AAb to PTGFR and PTPRA were not detected in non-serous ovarian cancers. Data on major risk factors for ovarian cancer (parity, ovulatory cycles, breastfeeding) as well as levels of the biomarker, CA15.3, was available on a limited number of subjects in this study. No notable correlations were observed between the markers in Tables 2 and 3 and the epidemiologic factors (data not shown).

***Detection of autoantibodies in the setting of low CA 125***

In the training and validation cohorts, CA 125 is elevated in over 95% of cases, due to selection of patients with serous carcinomas undergoing surgery. To determine if the AAb panel has potential additive benefit beyond CA 125 for the detection of serous carcinomas, 20 sera were identified from patients with serous carcinoma who had low CA 125 levels (median 40, range 15-76.7). These cases were matched by age and stage with 15 sera with high CA 125 levels (median 2116, range 718-23,010) [21]. AAb to at least one of the top 3 antigens (p53, PTGFR, and PTPRA) were detected in 7/20 sera (35%) in the low CA 125 cohort (6 by p53 alone and 1 by both PTGFR and PTPRA) and no controls using cutoff values of mean + 3 SD of the controls. Of the 7 sera with AAb in the low CA 125 cohort, 2 had stage I/II and 5 had late stage III/IV serous carcinoma, with a median CA 125 level median of 39 (range 19-62).

***Detection of autoantibodies in the setting of benign ovarian disease***

One challenge with the CA 125 biomarker as a screening tool is false elevation in the setting of benign ovarian disease. We evaluated the individual performances of p53, PTGFR, and PTPRA AAb in a separate set of sera from 30 serous ovarian cancer patients and 30 age- and gender-matched women with benign ovarian disease (19 patients with serous cytadenomas and 11 patients with mucinous cystadenomas) [21]. The sensitivity of detection of AAb to p53 in cases was 53.3% (AUC 0.86) at 93.3% specificity. The sensitivity of PTGFR was 16.7% (AUC=0.57) and PTPRA was 13.3% (AUC 0.61) at 93.3% specificity.

***Multiplexed Analysis of the Three-Antigen Biomarker Panel***

We examined the utility of these 3 AAb biomarkers as a diagnostic panel from the combined training and validation sets. 27 out of 60 cases (45% sensitivity) scored high (95% specificity threshold for each antigen) on at least one of the 3 antigens, compared to only 8 out of the 60 controls (86.7% specificity). However, due to the rarity of ovarian cancer in the general population and the clinical consequences of a false-positive result, the target specificity of biomarkers for ovarian cancer is 95%-99%. At 95% specificity, the individual sensitivities

of AAb to p53 was 21.7% (AUC=0.6475), PTGFR was 21.7% (AUC=0.6522), and PTPRA was 31.7% (AUC=0.6525). If at least two AAb of the three were positive above the 95% specificity cutoffs, the sensitivity was 23.3% with an improvement in overall specificity at 98.3%.

## DISCUSSION

Using custom protein microarrays, we have identified a panel of 12 AAb biomarkers that were detected in the sera of serous ovarian cancer patients at the time of clinical diagnosis of invasive cancer, but not in healthy women. These individual biomarkers had sensitivities ranging from 13-22% with specificities > 93%. Three of these biomarkers, p53, PTGFR and PTPRA, were consistently selective for serous ovarian cancer with individual AUCs ranging from 64.8-73.8% across two independent serum screenings and two technologic platforms (slide microarrays and bead arrays), involving a total of 94 cases/90 control samples. If at least two AAb of the three were positive, the sensitivity was 23.3% at 98.3% specificity. While the clinical sensitivity is modest, the reproducibility, signal intensity, and clinical specificity across multiple sample sets may provide utility beyond the biomarkers CA 125 and HE4. These biomarkers maintain sensitivity in the setting of false-negative CA 125 levels, and, unlike CA 125, maintain specificity when compared to benign ovarian disease.

This study is the first demonstration of the use of programmable protein microarrays for the proteomic detection of novel AAb biomarkers for ovarian cancer. Almost all of the sera used for this study were from patients with stage III/IV ovarian cancer; evaluation of the performance characteristics of these biomarkers will require testing of pre-diagnostic, prospectively-collected cohorts such as the ROCA or PLCO trials. It is reassuring that of the 5,177 antigens we screened, one of the top 12 AAb biomarkers was the well-established p53-AAb [12]. None of the 12 AAbs were detected in a similar screen for primarily ER+ breast cancer AAb [16], although p53-AAb have been detected in ER-breast cancers which are more commonly mutated in *TP53* [14b]. The top antigen biomarkers did not correlate with known epidemiologic risk factors, such as parity, breastfeeding, or ovulatory years. Many of the top 12 antigen biomarkers we identified have also been described as important in ovarian cancer tumor biology and pathogenesis (**Table 4**).

In addition to p53, which we had previously described, we consistently identified two novel ovarian autoantigens, PTGFR and PTPRA. PTGFR (FP) is the cell surface prostaglandin F receptor that functions to initiate luteolysis in the corpus luteum. It is aberrantly expressed in endometrial carcinoma [22] and stimulation of the receptor triggers MAPK signaling and cell proliferation [23]. PTGFR is strongly expressed in murine ovarian follicles [24] as well as LNCaP prostate cancer cells upon disease progression [25]. PTPRA is a cell surface protein

tyrosine phosphatase that is overexpressed in gastric cancers [26] and prostate cancers [27] and mediates signaling through ERK2. PTPRA is also upregulated in the setting of Her2 inhibition in breast cancer cell lines [28].

Of the other candidate AAb biomarkers, validation testing using our bead-array ELISA and independent sera sets failed to confirm significant selectivity of the biomarkers. This may reflect poor overall performance characteristics of these biomarkers, or decreased sensitivity of the bead arrays for the detection of AAb compared to slide-based microarrays [17]. Many of these potential biomarkers are also associated with cancer pathogenesis. Dihydrofolate reductase (DHFR) is a folate metabolism enzyme and is critical for DNA biosynthesis. DHFR has long been a target for chemotherapy in multiple cancers [29], and gene amplification has been described in ovarian cancer [30]. In our data, DHFR AAb were more frequent in non-serous ovarian cancers. PSMC1 is an ATPase subunit of the 26S proteosomal complex. RAB7L1 is a member of the RAS oncogene family. Elevated serum prolactin (PRL) has been identified in the serum of ovarian cancer patients [31]. CSNK1A1L is a kinase involved in the Wnt signaling pathway. AAb to PSMC1 have been identified in MGUS [32].

Many proteomics-based technologies have been used for the detection of antigen-specific antibodies in ovarian cancer (reviewed in [33]). The Snyder laboratory used serum screening of spotted protein microarrays to identify 94 autoantigens. Although the difference in detection between cases and controls did not reach statistical significance, they found Lamin A and RALBP1 were overexpressed in ovarian cancer tissue [10a]. LC/MS-based approaches can identify native epitopes and post-translationally modified antigens; in one study 100 AAb were identified in at least one patient [34]. Reverse-phase protein microarray [35] can identify PTM-specific Abs, and the S100A7 antigen has been identified [10c]. Phage-displayed antigen microarrays have been used to identify 62 different antigens, including RCAS1, Nibrin, and RPL4 [10b]. Finally, O-glycopeptide epitopes have been identified within MUC1 using glycoprofiling ELISA assays [36].

Additional proteomic methods have been used to identify ovarian cancer autoantigens. Using ascites fluid and protoarrays, 15 candidate AAb's were identified [37]. Using commercial protein arrays, 202 candidate ovarian antigens were identified [38], with DHFR identified in at least one patient [39]. Using 2-D immunoblots of exosomes, ovarian cancer antigens PLAP, survivin, NY-ESO-1, GRP78, and CA 125 were identified [40]. Of the

other 15 AAbs we identified, only casein kinase 1 A has similarity to a previously identified autoantigen, CK1-epsilon [41]. In multiplexed analysis of select antigens (survivin, p53, p16, and cyclins B1, D1, A and E) AAb's had a sensitivity of 62.5% at 90.2% specificity [42]. To our knowledge, other than p53, AAbs to these antigens have not been identified in ovarian cancer. In summary, these studies identify a potential panel of three autoantibody biomarkers for the early detection of serous ovarian cancer using custom protein microarrays populated with cancer-related target antigens.

**Supporting Information**

This material is available free of charge via http://pubs.acs.org.

Supplemental Table 1: List of the top 741 potential ovarian autoantigens, with normalized serum signal intensity for 60 cases and 60 controls.

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**Potential Conflicts of Interest**

Dr. Anderson received travel support from Luminex Corporation in 2011. Drs. Anderson and LaBaer serve on the Scientific Advisory Board with Provista Diagnostics; Dr. Anderson also consults with Provista Diagnostics. The remaining authors report no relevant conflicts of interest.

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**Table 1. Characteristics of cases and controls**

| | Cohort 1 | | | Cohort 2 | | | Non-Serous Set (Cohort 3) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Controls (n=30) n (%) | Serous cases (n=34) n (%) | Fisher's exact p-value | Controls (n=60) n (%) | Serous cases (n=60) n (%) | Fisher's exact p-value | Controls (n=30) n (%) | Non-Serous† cases (n=30) n (%) | Fisher's exact p-value |
| Age | | | | | | | | | |
| <50 | 1 (3.3) | 2 (5.9) | 0.89 | 18 (30.0) | 17 (28.3) | 0.99 | 16 (53.3) | 16 (53.3) | 0.99 |
| 50-60 | 10 (33.3) | 11 (32.4) | | 23 (38.3) | 23 (38.3) | | 9 (30.0) | 9 (30.0) | |
| >60 | 19 (63.3) | 21 (61.8) | | 19 (31.7) | 20 (33.3) | | 5 (16.7) | 5 (16.7) | |
| Menopausal status | | | | | | | | | |
| Pre | 3 (10.0) | 3 (8.8) | 0.87 | 20 (33.3) | 16 (26.7) | 0.55 | 17 (56.7) | 14 (48.3) | 0.60 |
| Post | 27 (90.0) | 31 (91.2) | | 40 (66.7) | 44 (73.3) | | 13 (43.3) | 15 (51.7) | |
| Race | | | | | | | | | |
| White | 28 (93.3) | 32 (97.0) | 0.50 | 60 (100.0) | 53 (94.6) | 0.11 | 30 (100.0) | 26 (86.7) | 0.11 |
| Non-white | 2 (6.7) | 1 (3.0) | | 0 (0.0) | 3 (5.4) | | 0 (0.0) | 4 (13.3) | |
| OC use | | | | | | | | | |
| Never | 17 (56.7) | 20 (60.6) | 0.75 | 20 (33.3) | 23 (41.1) | 0.44 | 6 (20.0) | 18 (60.0) | 0.003 |
| Ever | 13 (43.3) | 13 (39.4) | | 40 (66.7) | 33 (58.9) | | 24 (80.0) | 12 (40.0) | |
| Parity | | | | | | | | | |
| Nulliparous | 3 (10.0) | 3 (8.8) | 0.87 | 9 (15.0) | 13 (23.6) | 0.34 | 6 (20.0) | 15 (50.0) | 0.03 |
| Parous | 27 (90.0) | 31 (91.2) | | 51 (85.0) | 42 (76.4) | | 24 (80.0) | 15 (50.0) | |
| Year of specimen collection | | | | | | | | | |
| 2001-2002 | 9 (30.0) | 11 (32.4) | 0.85 | 18 (30.0) | 11 (18.3) | 0.36 | 8 (26.7) | 8 (26.7) | 0.99 |
| 2003-2005 | 14 (46.7) | 17 (50.0) | | 21 (35.0) | 25 (41.7) | | 13 (43.3) | 14 (46.7) | |
| 2006-2010 | 7 (23.3) | 6 (17.6) | | 21 (35.0) | 24 (40.0) | | 9 (30.0) | 8 (26.7) | |
| Length of storage | | | | | | | | | |
| <5.4 years | 7 (23.3) | 8 (23.5) | 0.92 | 24 (40.0) | 26 (43.3) | 0.10 | 10 (33.3) | 9 (30.0) | 0.99 |
| 5.4-7.6 years | 11 (36.7) | 14 (41.2) | | 13 (21.7) | 21 (35.0) | | 10 (33.3) | 11 (36.7) | |
| >7.6 years | 12 (40.0) | 12 (35.3) | | 23 (38.3) | 13 (21.7) | | 10 (33.3) | 10 (33.3) | |
| CA 125 | | | | | | | | | |
| Mean (95% CI) | | 1044 (550, 1984) | | | 647 (434, 966) | | | 85 (50, 145) | |

* Includes two cases which were excluded from the NAPPA analysis due to background.
† Non-serous cases are 10 mucinous, 10 endometrioid and 10 clear cell cases.

**Table 2. Statistics for 12 Potential Ovarian Cancer Biomarkers**

| Protein | Sens | Spec | Cutoff | AUC | AUC p-value | SPAUC | SPAUC p-value |
|---|---|---|---|---|---|---|---|
| **ACSBG1** | 13.3% | 95.0% | 2.07 | 53.9% | 0.2287 | 1.11 | 0.4351 |
| **AFP** | 15.0% | 95.0% | 1.41 | 54.4% | 0.1971 | 3.56 | 0.0516 |
| **CSNK1A1L** | 10.0% | 95.0% | 2.27 | 52.9% | 0.2819 | 1.56 | 0.2956 |
| **DHFR** | 13.3% | 95.0% | 1.49 | 52.0% | 0.3722 | 3.78 | 0.0392 |
| **MBNL1** | 0.0% | 95.0% | 4.96 | 50.0% | 0.5076 | 0.00 | 1.0000 |
| **p53*** | 21.7% | 95.0% | 9.34 | 64.8% | 0.0024 | 5.56 | 0.0054 |
| **PRL** | 10.0% | 95.0% | 1.29 | 53.9% | 0.2122 | 3.11 | 0.0866 |
| **PSMC1** | 10.0% | 95.0% | 1.71 | 51.6% | 0.3743 | 2.89 | 0.0962 |
| **PTGFR*** | 21.7% | 95.0% | 1.71 | 65.2% | 0.0019 | 8.00 | 0.0002 |
| **PTPRA*** | 31.7% | 95.0% | 1.59 | 65.2% | 0.0019 | 7.11 | 0.0007 |
| **RAB7L1** | 11.7% | 95.0% | 1.96 | 53.9% | 0.2554 | 3.11 | 0.0780 |
| **SCYL3** | 8.3% | 95.0% | 3.91 | 53.4% | 0.2735 | 2.67 | 0.1293 |

**Table 3. Evaluation of 7 Potential Ovarian Cancer Biomarkers for Non-Serous Ovarian Cancers**

| Protein | Sens | Spec | Cutoff | AUC | AUC p-value | SPAUC | SPAUC p-value |
|---|---|---|---|---|---|---|---|
| **DHFR** | 16.7% | 93.3% | 3.02 | 58.7% | 0.1311 | 5.78 | 0.0426 |
| **p53** | 20.0% | 93.3% | 2.42 | 57.4% | 0.1931 | 7.11 | 0.0203 |
| **PSMC1** | 6.7% | 93.3% | 2.78 | 46.1% | 0.6612 | 1.78 | 0.4179 |
| **PTGFR** | 10.0% | 93.3% | 1.99 | 51.4% | 0.4127 | 1.78 | 0.3996 |
| **PTPRA** | 20.0% | 93.3% | 1.92 | 51.0% | 0.4631 | 2.67 | 0.1998 |
| **RAB7L1** | 10.0% | 93.3% | 1.80 | 46.0% | 0.7204 | 1.33 | 0.4548 |
| **SCYL3** | 6.7% | 93.3% | 7.01 | 50.2% | 0.5234 | 2.67 | 0.2815 |

## Table 4. Cellular functions of 12 candidate biomarkers

| Gene | Description | Subcellular Location | General Function | Cancer-related Function |
|---|---|---|---|---|
| ACSBG1 | acyl-CoA synthetase bubblegum family member 1 | cytoplasm, ER | fatty acids metabolism and myelinogenesis | |
| AFP | alpha-fetoprotein | extracellular | a major plasma protein produced by the yolk sac and the liver during fetal life | - certain ovarian tumors with an elevated level of AFP were extremely reponsive to chemotherapy (PMID:19225928)<br>- serological marker for liver cancer |
| CSNK1A1L | casein kinase 1, alpha 1-like | cytoplasm | | |
| DHFR | dihydrofolate reductase | | converts dihydrofolate into tetrahydrofolate | |
| MBNL1 | muscleblind-like (Drosophila) | cytoplasm | RNA processing | |
| P53 | tumor protein p53 | nucleus | tumor suppressor | autoantibodies found frequently in serous ovarian cancer (PMID:20200435) |
| PRL | prolactin | extracellular | peptide hormone/cytokine that regulates development of mammary tissue, lactation, pregnancy | - serum marker for ovarian cancer (PMID:15890779, 18258665)<br>- inhibits apoptosis of ovarian cancer cells (PMID:15700312) |
| PSMC1 | proteasome (prosome, macropain) 26S subunit, ATPase, 1 | cytoplasm | ATPase with a chaperone-like activity | autoantigen in monoclonal gammopathy of undetermined significance (MGUS), the premalignant stage of multiple myeloma (PMID:19587378) |
| PTGFR | prostaglandin F receptor (FP) | plasma membrane | a receptor for prostaglandin F2-alpha (PGF2-alpha), which is known to be a potent luteolytic agent | higher expression in endometrial adenocarcinomas (PMID:14764825) |
| PTPRA | protein tyrosine phosphatase, receptor type, A | plasma membrane | dephosphorylate and activate Src family tyrosine kinases | - higher copy number and expression in gastric cancer (PMID:20187983, 16338072)<br>- shorter isoform induces cellular transformation (PMID:20545765) |
| RAB7L1 | RAB7, member RAS oncogene family-like 1 | membrane | small GTPase | |
| SCYL3 | SCY1-like 3 (S. cerevisiae) | cytoplasm, Golgi | cytoskeletal adaptor protein | |

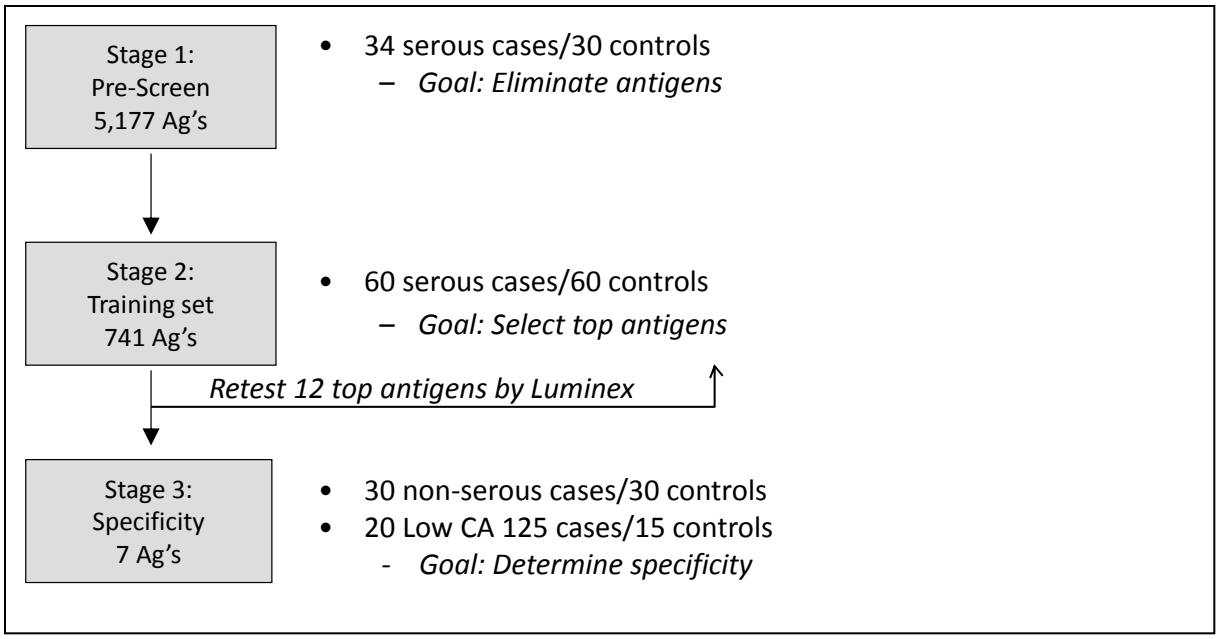


***Figure 1. Schematic of Serum Screening Strategy.*** Ovarian cancer sera were sequentially tested on custom microarrays as shown. Initial screening was performed using arrays expressing 5,177 unique full length cDNAs and case/control sera (Cohort 1). Secondary screening was performed using arrays expressing 741 unique full length cDNAs (Cohort 2), and 12 antigens were re-tested by Luminex ELISA. The specificity of the top 7 antigens were determined using sera from non-serous cases/controls (Cohort 3) and serous cases with low CA125 levels.