# Ovarian Cancer

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# Part I
# Ovarian Cancer Detection and Pathogenesis

# Potential and Limitations in Early Diagnosis of Ovarian Cancer

**Nicole Urban and Charles Drescher**

## 1 Ovarian Cancer Screening May Reduce Mortality in the Future but Many Challenges Remain

Five-year survival rates for invasive epithelial ovarian cancer have changed little in recent decades, remaining constant at about 30% when cancer has spread outside the ovaries, and about 90% when disease is confined to the ovaries. Ten-year survival for ovarian carcinoma varies greatly according to the stage at diagnosis (1) and survival is best when cancer is confined to the ovary at the time of diagnosis (Fig. 1); even patients with high-grade serous tumors do well if they are diagnosed while the tumors are confined to the ovary (Fig. 2).

The goal of screening is to reduce mortality by detecting cancer early. The potential reduction in mortality is great, because currently fewer than 25% of cases are confined to the ovary at diagnosis. Interest in diagnostic markers that can be measured in blood products is particularly high, as several promising marker panels have been reported in the last decade (2, 3). However, using these markers to detect ovarian cancer early enough to reduce mortality remains challenging because screening needs to identify cancer before symptoms occur, early enough that the disease is still curable. It is well established that the best screening tests detect cancer *before* it becomes invasive, by identifying precursor lesions and enabling prevention of invasive cancer through early intervention.

In considering the challenges inherent in ovarian cancer screening, it is helpful to distinguish among diagnostic, early detection, and risk markers. Figure 3 depicts the behavior of three hypothetical markers as cancer progresses through a precursor lesion stage, an early invasive stage, metastasis, and death. Markers A, B, and C are equally elevated at the time of diagnosis, but they are not equally good early detection markers because their behavior prior to diagnosis varies. Marker A performs well as a diagnostic marker because it is highly elevated in women with cancer who present clinically with symptoms, but it does not provide signal until the disease is well advanced. Marker B is a better early detection marker because it elevates while the disease is still potentially curable, signaling preinvasive as well as invasive disease. Marker C elevates even earlier; hence, it might be useful as a risk marker to predict disease in the future especially if precursor conditions are unknown or

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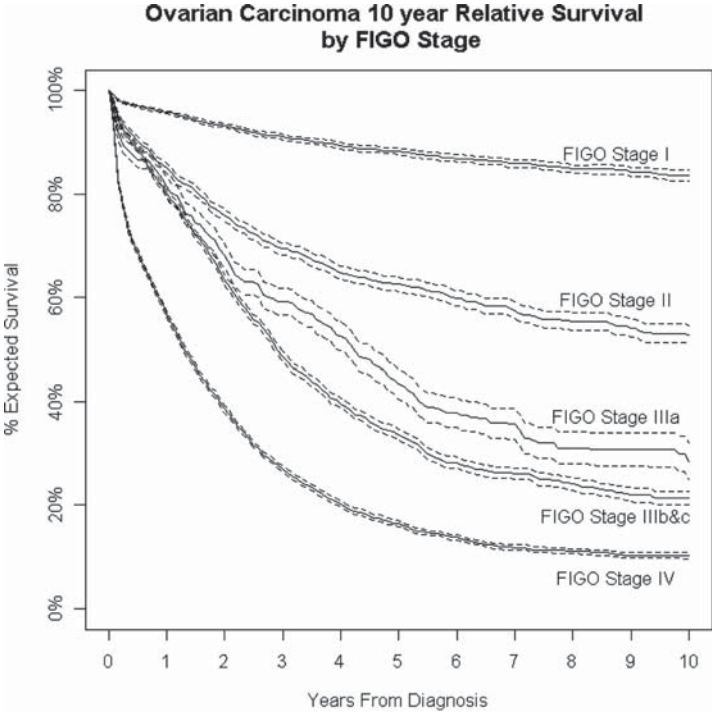


**Fig. 1** Ten-year survival for ovarian cancer varies greatly according to FIGO stage at diagnosis, only when the cancer is confined to the ovary is long-term survival above 80%

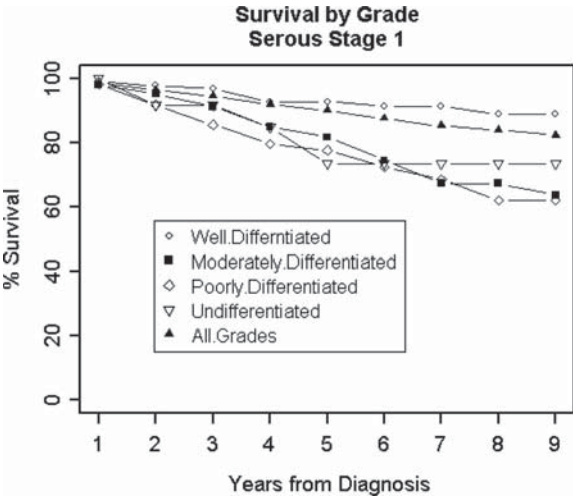


**Fig. 2** Ten-year survival is over 60% when the cancer is confined to the ovary at the time of diagnosis even for serous ovarian cancers that are poorly differentiated

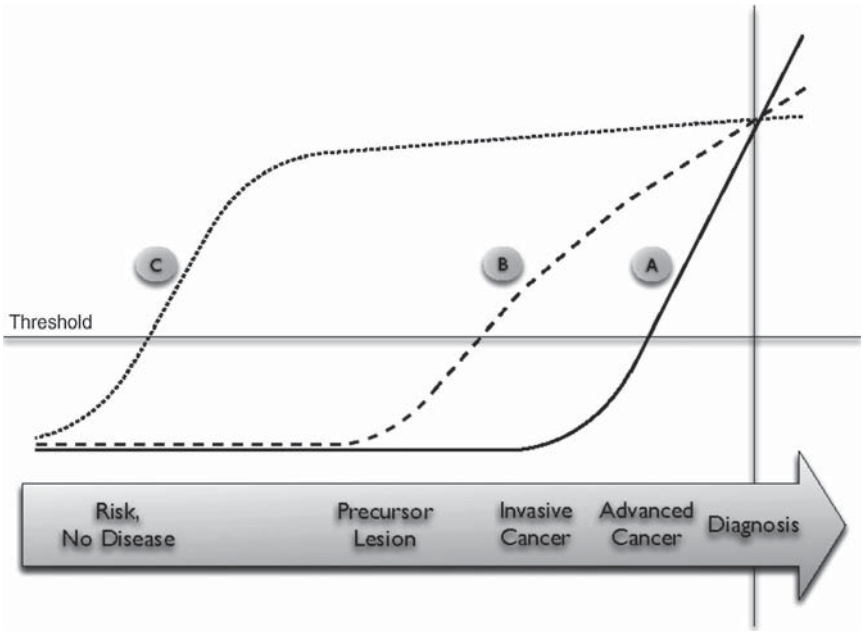


**Fig. 3** Conceptual framework for determining the clinical utility of a serum marker. The signal provided by a screening test prior to symptoms and clinical diagnosis determines its utility as a diagnostic (a), early detection (b), or risk (c) marker. Reproduced from (4) with permission from Future Medicine Ltd

undetectable. Screening for elevated risk can reduce disease incidence if preventive treatment is available; for example, screening for and treating high cholesterol/triglycerides and high blood pressure effectively reduces the adverse events associated with cardiac disease. A similar use of screening for ovarian cancer risk markers may be important to explore because of the many challenges to early detection of curable invasive lesions.

## 2 Good Early Detection Serum Marker Candidates Complement CA125 and Show Stability Over Time

The potential for reducing ovarian cancer mortality through earlier diagnosis and treatment is great, but available screening approaches such as CA125 and transvaginal sonography (TVS) often fail to detect early, asymptomatic disease; in addition they can lead to unnecessary surgery. The hope for early detection remains high, however, because emerging technologies are facilitating identification of

novel markers that complement CA125. Many serum biomarkers have been identified for ovarian cancer, including CA125 (5), prolactin (6), mesothelin (7), HK11 (8), osteopontin (9), HE4 (10), B7-H4 (11), and SPINT2 (12).

To date, only CA125 has been shown to detect ovarian cancer prior to symptoms. CA125 above 30 U $mL^{-1}$ was used to select postmenopausal women for ultrasound screening in a pilot trial in the UK. Prevalence screening (22,000 women) yielded sensitivity of 85% and 58% at 1-year and 2-year follow-up, respectively, and specificity of 99.6%. Results of the 2-arm RCT (11,000 per arm) suggest that survival was better in the screened group (72.9 vs. 41.8) and that the positive predictive value was acceptable at 20% (13). For a disease as rare as ovarian cancer, specificity of 99.6% is needed in a screening test with 80% sensitivity to achieve a positive predictive value (PPV) of 10%. A high PPV is important because definitive diagnosis requires major abdominal surgery. Results of the pilot trial in the UK suggest that use of a marker panel including CA125 to select women for TVS and/or surgery may be a cost-effective screening strategy. These results are consistent with predictions of a microsimulation model of ovarian cancer screening (14) that uses three interrelated components to estimate screening outcomes. Assumptions were made regarding the natural history of ovarian cancer in the absence of diagnosis and treatment; disease detection as a function of characteristics of the woman, her cancer, and detection modalities used; and survival as a function of age of the woman and the stage of her disease at the time of diagnosis. The model predicted that using *rising* CA125 to select women for TVS is a cost-effective approach to screening, and that frequent screening may be needed to realize benefits if the disease progresses quickly from a curable to an incurable condition.

On the basis of these and other observations, statistical methods have been developed for using marker history to improve screening performance. Methods such as the Risk of Ovarian Cancer algorithm (ROCA) (15) or the Parametric Empirical Bayes (PEB) decision rule (16) are particularly useful when marker levels rise (or fall) as cancer develops relative to an individual woman's usual marker levels. As illustrated in Fig. 4, a marker's levels in the absence of cancer may vary more among women than within an individual woman over time, rising (or falling) significantly relative to a woman's usual level only in the presence of cancer. This is characteristic of many potentially useful markers including CA125.

Testing for change over time in a marker can improve sensitivity without loss in specificity. In the PEB approach (16), at each screen, a woman's serum is tested for deviance from her own normal value of the marker. The threshold for positivity can be set such that a targeted percent of women are referred for further work-up at each screen, so that sensitivity is maximized within desired specificity. Women's characteristics such as age are accounted for using the PEB rule. The risk-of-ovarian cancer algorithm (ROCA) is similar but tests specifically for exponential rise in CA125 using call-backs for repeat testing (15).

Markers that are specific to malignancy are needed to avoid identification of benign ovarian conditions that are much more common than ovarian cancer. Several such markers are under evaluation. For example, the human epididymis protein 4 (HE4/WFDC2) (17) has been studied independently by several institutions and is

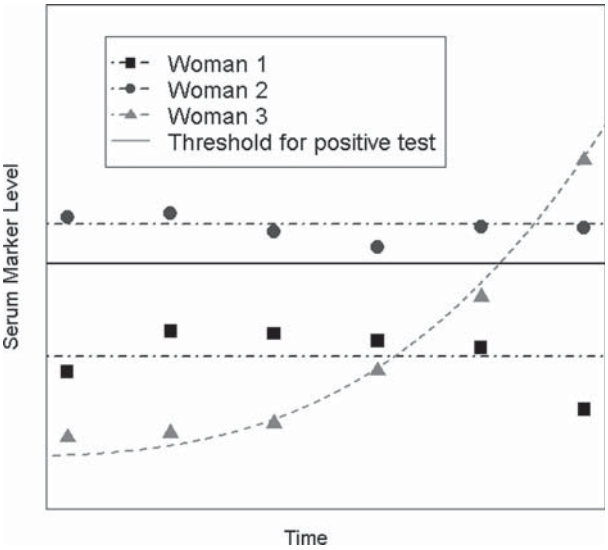


**Fig. 4** Conceptual framework for developing decision rules for markers such as CA125. When marker levels vary less over time within an individual woman than among women in a screening population, change over time in a marker can signal cancer earlier than a single threshold rule

found to be promising as a marker for ovarian carcinoma (10). It is a secreted glycoprotein that is overexpressed by serous and endometrioid ovarian carcinomas (18). It is one of the several genes showing *in silico* chromosomal clustering and displaying altered expression patterns in ovarian cancer (19). Evaluation in serum suggests that HE4 is as sensitive as CA125 and more specific in that it detects fewer benign tumors; it is also stable over time in healthy women (10).

Similarly, mesothelin (MSLN) has been shown to be a soluble protein present in serum, and is potentially useful in a diagnostic panel including CA125. Mouse monoclonal antibodies were used in a sandwich ELISA to measure MSLN in serum (7). Receiver operating characteristics (ROC) curve analysis was used to evaluate the value added of MSLN to a composite marker including CA125, using 53 cases and 220 controls (20). Logistic regression was used to define a composite marker including CA125 and MSLN. The composite marker is a linear combination of the markers in the panel. Marker levels were converted to logs and standardized. Logistic regression was used to estimate the weights for each marker (21), controlling for menopausal status: CM = 1.4 × CA 125 + 1.0 × MSLN. The CM can be analyzed as if it were a single marker in ROC curves (Fig. 5) and in longitudinal algorithms such as the PEB for use in screening.

HE4, MSLN, CA125, and 15 other candidate markers were further evaluated in 200 blinded serum specimens from ovarian cancer cases and healthy women, including 41 healthy controls from a screening study (20 contributed blood two times one year apart), 47 otherwise healthy women undergoing pelvic surgery without tubal/

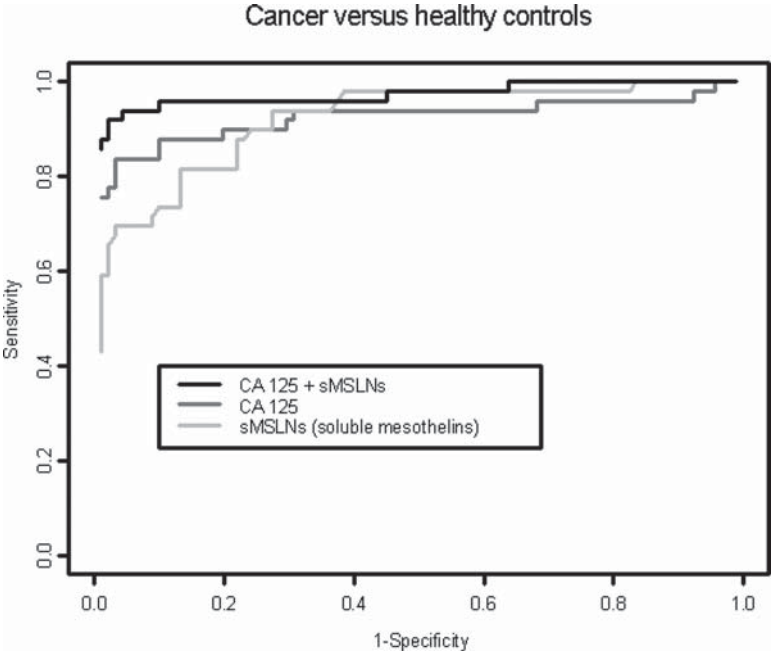


**Fig. 5** Addition of MSLN to a panel that includes CA125 improves detection of ovarian cancer

ovarian pathology, 24 surgical controls with benign ovarian conditions and 68 cases including 11 stage 1, 5 stage 2, 39 stage 3, 11 stage 4, and 2 unstaged ovarian cancers. All epithelial cancer histologies were represented, including 34 serous, 7 endometrioid, 3 mucinous, 2 clear cell, 17 other and 5 undifferentiated. Several good marker candidates were identified, including 8 markers with sensitivity > 50% at 80% specificity. Three had sensitivity >50% at 85% specificity, two had sensitivity >50% at 90% specificity, and one had sensitivity >50% at 99% specificity as well as sensitivity >75% at 95% specificity.

Markers that performed well as individual diagnostic markers were further evaluated in clinical samples for their contribution to a panel including CA125, at Fred Hutchinson Cancer Research Center (FHCRC) in Seattle; some of these and other novel markers were evaluated similarly at Dana Farber Cancer Center (DFCC) in Boston. At both institutions, clinical samples were obtained from women with ovarian cancer at the time of diagnosis, prior to any treatment including surgery. Markers that showed univariate sensitivity of at least 30% at 95% specificity included seven markers from the FHCRC panel and five markers from the DFCC panel. Six markers improved the sensitivity of CA125 at 95% specificity. Eight markers showed correlation >0.5 for samples taken 1 year, apart from the same woman, suggesting stability over time within women. The screening performance of these markers can be improved using a longitudinal screening

algorithm such as the PEB (22). Research to identify the markers that provide signal early, when the disease may still be curable, is currently underway but results are not yet available.

## 3 The Best Candidates for Use in an Early Detection Panel Provide Signal Prior to Symptoms, Early in the Disease Process

Estimates of the lead times of candidate markers are needed to accurately predict the markers' contribution to an effective early detection panel for use in a screening program. Promising serum markers have been evaluated independently in clinical samples at several institutions, but their lead times remain unknown. To address this need, a 2-year validation study has been initiated by the National Cancer Institute (NCI) in the US to evaluate candidate markers, using the serum repository of the NCI Prostate, Lung, Colorectal, and Ovarian Cancer (PLCO) screening trial, a repository that contains serial preclinical samples from over 100 women who have been diagnosed with ovarian cancer as well as serial samples from healthy comparable controls. Preclinical samples are needed to estimate the markers' lead times and the probability that cancer will be diagnosed within an arbitrary period such as 2 years, as a function of marker levels and change. The PLCO trial is a large, multicenter randomized controlled screening trial that includes collection and storage of 6 serum samples collected one year apart from 37,000 healthy women randomized to the screened arm, as well as 10 years of follow up for cancer diagnosis for all 74,000 women participating in the trial (Table 1).

It has long been recognized that collaboration is needed to identify and validate the best diagnostic and early detection panels, as promising results from single-institution studies have seldom been reproducible. The NCI collaboration is a Phase II/III Validation Study (23) of a Consensus Panel of Early Detection Serum Markers led by Dan Cramer (DFCC) and Nicole Urban (FHCRC). A 2-year study began in

**Table 1** The Prostate, Lung, Colon, and Ovary (PLCO) trial provides serial serum samples from healthy post-menopausal women with follow up for cancer. Reproduced from (4) with permission from Future Medicine Ltd

| | |
|---|---|
| Centers collaborating | 10 |
| Arms | 2 |
| Study population | Women aged 55–74 |
| Endpoint | Cause-specific mortality |
| Size | 74,000 total (37,000 in each of two arms) |
| Power | 88% for 35% mortality reduction (1-sided test) |
| Enrollment period | 3 years |
| Duration of screening | 4 screens 1 year apart[a] |
| Duration of follow-up | Minimum of 10 years postrandomization |
| Screening protocol | Annual TVS, CA-125, bimanual pelvic exam |

[a] The PLCO design was revised to continue screening for an additional 2 years using only CA125

August 2005, which includes investigators from five Ovarian Cancer Specialized Program of Research Excellence (SPORE) sites (DFCC, FHCRC, Fox Chase Cancer Center, MD Anderson Cancer Center, and University of Alabama, Birmingham), three Early Detection Research Network (EDRN) sites (FHCRC, DFCC, U Pittsburgh), and the PLCO trial at NCI. In the first year, a new set of Phase II (clinical) specimens will be used to evaluate the most promising diagnostic markers including HE4 and MSLN as well as an expanded panel of markers measured by bead-based assays. In the second year, the best diagnostic markers will be evaluated in PLCO (preclinical) specimens to predict their utility as early detection or risk markers. Using data from analysis of PLCO preclinical blood samples, diagnosis of ovarian cancer within 2 years (or another arbitrary period) of a blood draw can be predicted. Because the women who contributed blood samples were all participating in screening, cancer could have been detected by CA125, TVS or symptoms, or symptoms. Blood samples were not collected from women allocated to the control group of the PLCO trial.

The NCI collaborative study will test the hypothesis that a panel of biomarkers will have better performance characteristics than any single marker, and yield a longer lead time than CA125 alone. Over 20 putative biomarkers have been evaluated by SPORE and EDRN investigators using bead-based (Luminex®) assays as well as standard ELISA. In the first year, candidate biomarkers will be evaluated in a new set of 160 cases (80 early-stage and 80 late-stage), 160 surgical controls, 480 general population controls, and serial samples collected 1 year apart in 40 healthy controls. Samples will be provided by five ovarian cancer SPORE institutions for blinded measurement of assays at three laboratory sites: DFCC, FHCRC, and U Pittsburgh. A consensus panel will be identified including the biomarkers that are most informative on their own or most complementary when used together, within specimen volume constraints. For as many markers in the consensus panel as possible, bead-based assays will be developed and evaluated for their reproducibility, validity, and performance relative to standard ELISA. Bead-based assays, multiplexed if possible, will be used in PLCO specimens to preserve PLCO specimen volume for future studies.

In the second year, PLCO preclinical samples from approximately 100 cases and 1,000 matched controls will be used to estimate the lead time of each individual marker and establish the best marker combination. Markers that show elevation within a year prior to diagnosis will be evaluated using the entire preclinical history to estimate the lead time for each marker and the marker panel. A small amount of serum from prediagnostic specimens from the PLCO cases and controls will be made available for the study. Some will be allocated for testing bead-based (Luminex® platform) assays, and the remainder will be used for high-priority markers that can be measured only by standard ELISA. Any remaining sera from false positive and false negative cases will be used to discover additional biomarkers that complement the existing panel, using novel high throughput proteomic discovery platforms.

A research challenge is that specimen quantities are limited in the stored samples from the PLCO trial. Accordingly, to the extent possible, bead-based assays will be used to measure candidate markers in the PLCO samples to minimize specimen

requirements. In preparation for this and other validation studies using preclinical specimens, bead-based assays have been developed for top marker candidates, including CA125 and HE4. For CA125, four commercially available monoclonal antibody pairs were tested on a bead-based platform to select the best pair with respect to assay feasibility (affinity) and accuracy in assessing known antigen concentrations. Two CA125 bead-based assays were optimized and evaluated in serum samples using the two best pairs of CA125 antibodies, and one HE4 assay was similarly optimized using the only available monoclonal antibody pair. These three bead-based assays were then measured blinded in a triage set of 64 cases, 55 screening controls, and 70 surgical controls, most of which had been previously characterized for CA125 and HE4, using ELISA. Each bead-based assay was evaluated for reproducibility, validity, and screening performance (24).

The best CA125 bead-based assay uses antibodies from RDI, with a correlation between replicates of 0.99 overall and 0.83 in screening controls. Its correlation with CA125II is 0.95 overall and 0.64 in screening controls. The HE4 bead-based assay showed correlation between replicates of 0.95 overall and 0.86 in healthy controls, and its correlation with ELISA was 0.95 overall and 0.86 in screening controls. A composite marker (CM) was constructed for CA125 and HE4, defined as a linear combination of the HE4 and CA125 (RDI antibody pair) bead-based assays. Using published methods (21), marker levels were converted to logs and standardized, and logistic regression was used to estimate the weights for each marker: $CM = 0.56 \times CA125 + 1.20 \times HE4$. Its diagnostic performance was measured by the area under the curve (AUC) for the ROC curve estimated using the triage set described earlier. Performance for the CM using bead-based assays for cases vs. all controls (AUC = 0.91) was better than that of the CA125II RIA assay used alone (AUC = 0.87), the bead-based CA125 assay used alone (AUC = 0.85), or the HE4 bead-based assay used alone (AUC = 0.89) (24). Interassay CVs for the bead-based assays were found high by commercial ELISA standards but have been recently improved by normalizing across plates.

These analyses suggest that bead-based assays for HE4 and CA125 combine to form a panel that performs better than either marker used alone, particularly at the very high specificities needed in screening programs. Multiplexed bead-based assays may reduce specimen requirements even further. The availability of assays that require 15 μL or less of serum, such as those described earlier, may make it possible to explore the behavior of candidate markers in stored samples from the Women's Health Initiative (WHI) (25) as well as those from the PLCO. The WHI population is restricted to women aged 50–79 at entry and represents the average-risk, postmenopausal population from which the majority of ovarian cancers arise. A total of 68,000 women were randomized in the clinical trial (CT) and 93,000 women were enrolled in the observational study (OS). The women provided self-reported demographics, reproductive, medical, and family history, and lifestyle data as well as blood samples at baseline and either 1 year (CT) or 3 years (OS) later. Table 2 reports the number of women for whom samples are currently available for biomarker validation from the OS, reported by months elapsed between the blood draw and the cancer diagnosis.

**Table 2** Women's Health Initiative (WHI) samples are appropriate for estimating lead time and best clinical use of each candidate marker: Samples are available for over 250 women of whom 70 provide two samples 3 years apart, and the second sample obtained within 2 years of the cancer diagnosis

| Months from draw to diagnosis | Number of cases after baseline blood draw ($n$ = 250) | Number of cases after 3-year blood draw ($n$ = 100) |
|---|---|---|
| 0–6 | 11 | 20 |
| 6–12 | 19 | 14 |
| 12–18 | 25 | 16 |
| 18–24 | 19 | 20 |
| 24–30 | 17 | 5 |
| 30–36 | 20 | 7 |
| 36–42 | 24 | 7 |
| 42–48 | 26 | 4 |
| 48+ | 88 | 7 |

*Note:* This table contains information on incident ovarian cancer cases in the OS through August 2003 currently available for biomarker validation work

Although they are less well-suited to describing marker behavior over the preclinical phase of the disease in individual cases, preclinical samples from the WHI have several advantages over the PLCO samples. First, the WHI is larger: As of August 2006, 374 and 243 cases of ovarian cancer have been diagnosed in the observational study (OS) and the clinical trial (CT) components of the WHI, respectively, providing samples that could potentially be used both to develop and to validate a screening decision rule. Second, samples from the WHI allow unbiased estimation of markers' lead time relative to clinical detection and diagnosis, whereas in the PLCO many of the cases were detected by screening using CA125 or TVS or both. Third, because some of the blood samples were obtained many years prior to diagnosis, and follow up of over 10 years has been completed, the relative risk of a cancer diagnosis within 5 or 10 years can be estimated from data generated by the WHI and the behavior of each marker as cancer develops (Fig. 3) can be determined.

The availability of preclinical samples from the PLCO and WHI trials will greatly improve our understanding of the behavior of candidate markers during the preclinical phase of epithelial ovarian cancer. However, analysis of these samples cannot reveal the presence or absence of invasive disease at the times prior to diagnosis when the preclinical serum samples were obtained. Accordingly, these samples may be most useful for estimating the relative risk of subsequent ovarian cancer diagnosis on the basis of marker levels or changes in marker levels. Knowledge of the presence or absence of disease at the time a marker first provides signal requires a prospective screening study in which surgical intervention is triggered by the marker. Until such a study is initiated and completed, it may be useful to invoke a different screening paradigm focusing on markers that predict, rather than detect, disease (4). Particularly useful would be markers that could predict

5-year or 10-year risk that would provide indications for risk-reducing surgery. Women identified as high risk would not be expected to have invasive disease at the time of surgery, but some might have premalignant changes that could be confirmed using markers detectable in ovarian or tubal tissue.

## 4 Significant Progress Can be Expected in the Future

We can reduce ovarian cancer mortality through screening if (1) cancer detected early can be cured, (2) biomarkers in the blood can signal early cancer, (3) available technology can identify biomarkers, (4) appropriate research can be conducted to demonstrate screening efficacy, and (5) biomarkers can be used cost-effectively for cancer screening. In the last decade, methods have been developed for discovering and prioritizing candidate markers, predicting the cost-effectiveness of alternative screening strategies, combining markers for use in a panel, using marker history in a longitudinal decision rule for early detection, and evaluating specimen-efficient bead-based assays for use in validation research. Several candidate markers have been identified that perform well as diagnostic markers, and studies are underway to evaluate their potential as early detection markers. It is likely that additional markers will be needed to detect ovarian cancer early enough to reduce mortality through screening, including risk markers that detect precursor lesions or signal developing disease several years before it becomes invasive and potentially incurable. New proteomics technologies that make discovery in serum possible are likely to revolutionize the field in the near future.

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# SMRP and HE4 as Biomarkers for Ovarian Carcinoma When Used Alone and in Combination with CA125 and/or Each Other

**Ingegerd Hellstrom and Karl Erik Hellstrom**

## 1 There is a Need for Biomarkers to Detect Ovarian Carcinoma by Assaying Serum and/or Other Body Fluids

Assays measuring tumor antigens in serum have the advantage that they are noninvasive, quick, and relatively inexpensive. Early detection as well as monitoring of disease in treated patients requires high specificity and sensitivity and constant levels of circulating marker unless there is a change in the patient's clinical status.

CA125 is the present "gold standard" for diagnosis of ovarian carcinoma using serum samples (1–4). However, it is elevated in several nonmalignant conditions, which can lead to false-positive results (5). There is a need for additional markers to improve sensitivity with retained or better specificity, and many new biomarkers have been introduced and continue to be evaluated. Our group has focused on soluble mesothelin-related proteins (SMRP) and on HE4, a protease that is secreted into serum. In immunohistological studies of ovarian cancer samples with little or no detectable CA125 expression, mesothelin and HE4 stood out as the most promising markers, when reactivity with normal tissues was taken into account (6). Other biomarkers in this study included HK4, HK6, OPN, claudin 3, DF3, VEGF, MUC1, and CA19-9.

## 2 SMRP as Marker for Diagnostic Assays of Serum and Urine

With the goal to obtain monoclonal antibodies (MAbs) for therapy, our group immunized mice with human ovarian carcinoma cells in the mid-1990s. This work resulted in MAb569, which reacts with ovarian carcinomas and has low reactivity with normal tissues except for the mesothelium. N-terminal amino acid sequencing of the antigen recognized by MAb 569 showed identity with the sequence of mesothelin, a tumor marker first described by Pastan's group (7), except for the lack of a 24 bp insert. By following our standard procedures for characterizing antigens detected by MAbs (8), we found the MAb569-defined antigen in supernatants of antigen-positive tumor cells and subsequently in malignant effusions, suggesting that it may be a marker for serum-based diagnosis. This finding was surprising

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because studies by Pastan's group had indicated that mesothelin is stably expressed at the cell surface and not released in to tumor culture supernatants or body fluids from cancer patients (9).

To develop a double determinant (sandwich) immunoassay, as our group had done for other tumor antigens in the past (10), additional MAbs were generated by immunizing mice with Mab569 immunoaffinity-purified antigen and applied MAbs to two different epitopes to construct a "sandwich" ELISA specific for mesothelin (11). In the initial study, a SMRP variant with an 82 bp insert was also detected. In view of Pastan's finding indicating that mesothelin is not soluble, we speculated that this variant is the molecule that is measured with the original ELISA (11).

A "blinded" study was performed in collaboration with B. Robinson's group from the University of Western Australia in Perth. We demonstrated the value of our SMRP-specific ELISA for the diagnosis of patients with mesothelioma (12). Eight of 40 individuals who had been exposed to asbestos but were clinically cancer free had increased levels of circulating SMRP. Importantly, three of those individuals subsequently developed mesothelioma within 15, 26, and 69 months, dying after 3, 6, and 6 years, respectively, and one developed lung carcinoma. In contrast, none of the 32 subjects with normal SMRP levels got mesothelioma or lung cancer within 6 years of follow up, suggesting a potential predictive value of the assay.

Another "blinded" study was performed together with Dr. N. Urban and her colleagues at the Fred Hutchinson Cancer Research Center in Seattle. It showed that SMRP has similar sensitivity and specificity as CA125 for diagnosis of ovarian carcinoma and that a combination of CA125 with SMRP has higher sensitivity than either assay alone. Like CA125, SMRP has temporal stability, suggesting that repeated studies on the same high risk subjects may facilitate earlier diagnosis (13).

A third study was performed in collaboration with Dr. N. Sardesai and his colleagues at Fujirebio Diagnostics, Inc. It indicated that SMRP is released into urine of patients with ovarian carcinoma and that the measurements of SMRP in urine, using the original ELISA, offer promise for detection of ovarian carcinoma. If confirmed by ongoing studies, the ease by which urine can be obtained would facilitate frequent studies on subjects that have high genetic risks of developing ovarian cancer.

As illustrated in Fig. 1, three mesothelin variants have been identified (14): one without inserts (variant 1), one with a 24 bp insert (variant 2), and one with an 82 bp insert (variant 3). To explore which variants are released into the circulation from ovarian carcinoma cells, we created recombinant fusion proteins of the three variants, immunized the mice with them, and obtained specific MAbs. Flow cytometry on live cells was performed with MAbs to the different mesothelin variants and showed that a MAb to variant 1 identifies as many tumors as a MAb to all three variants, while variants 2 and 3 are expressed infrequently (15). The published ELISA (11) was found to recognize variants 1 and 3 and has much higher sensitivity (68% vs. 15%) and specificity than a newly constructed ELISA specific for variant 3 (15). SMRP released into ascites from a patient with ovarian carcinoma was shown to have a molecular weight of approximately 40 kDa.

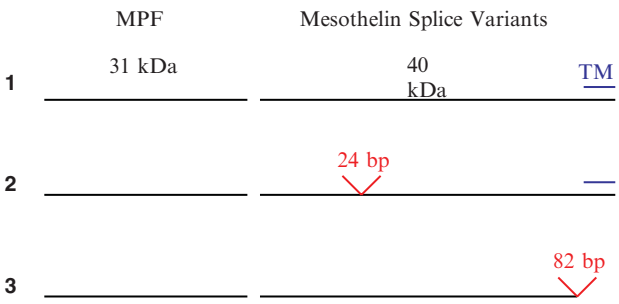


**Fig. 1** Mesothelin variants

According to amino acid sequencing, variants 1 and 2 were found in the ascites, and variant 3 could not be excluded (15). A standard curve was constructed to measure SMRP with a limit of detection of 200 pg/ml, and an assay for clinical use is marketed by Fujirebio Inc, in Europe and Australia, for monitoring mesothelioma patients. Today, there is an agreement between Pastan's and our group that mesothelin is released from antigen-positive tumor cells as a useful diagnostic marker for serum assays (16).

## 3 Autoantibodies to Mesothelin

Autoantibodies to tumor-associated antigens have been detected in many cancer patients (17–22) and are sometimes found to correlate with the clinical state. We have started to investigate whether patients with ovarian cancer form antibodies to mesothelin, whether such antibodies can also be found in healthy subjects, and whether the presence of anti-mesothelin antibodies provides clinically useful information. Native mesothelin was purified by Mab569 immunoaffinity chromatography from urine of patients with ovarian cancer and used to coat ELISA plates. Sera were added from patients with ovarian cancer at various stages, as well as from control donors, and bound autoantibodies were detected with anti-human IgG antibody as a probe. Antibodies were detected in a fraction of sera from both patients with ovarian cancer (Fig. 2) and healthy women (data not shown). Experiments are ongoing to find out whether the presence of these antibodies provides information on diagnosis or monitoring of ovarian cancer.

There are several reasons why antibodies to mesothelin can have important functions that relate to the development and progression of ovarian cancer. Anti-mesothelin antibodies have been shown in vitro to prevent binding of mesothelin to CA125 and thereby impact cellular adhesion (23). Furthermore, antibodies can be cytotoxic in the presence of complement, mediate antibody-dependent cellular cytotoxicity in the presence of NK cells or macrophages, and the generation and expansion of T cell responses to tumor antigens may be impacted by such antibodies as

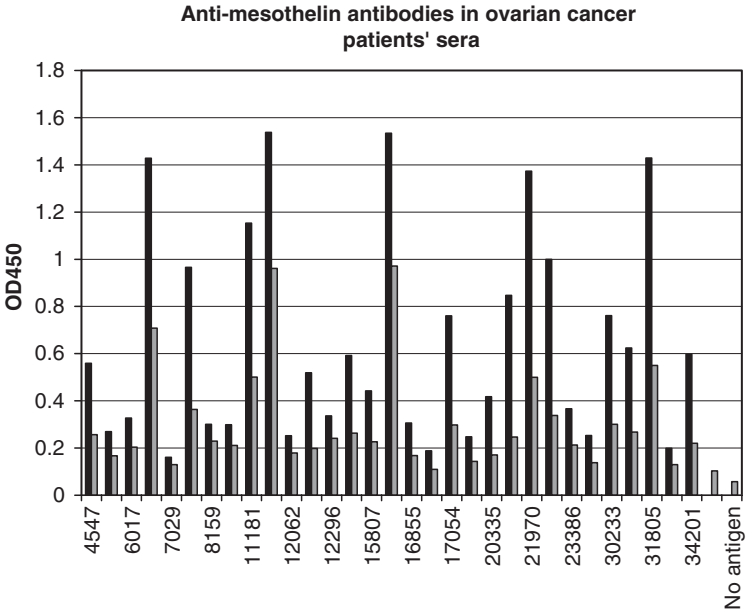


**Fig. 2** Data from an ELISA detecting antibodies in the sera from patients with ovarian cancer (31 patients studied). Values above 0.4 are considered positive. Patients studied at two serum dilutions (1:20 *first*, 1:80 *second bar*)

well (24). Anti-cancer therapy is likely to influence antibody formation, not only by decreasing the number of tumor cells releasing antigen but also by acting directly on antibody forming cells, as in the case of cytotoxic drugs, and changes in antibody levels are likely to influence the amount of SMRP, which is detectable by ELISA.

## 4 HE4 as a Marker for Ovarian Carcinoma

The *WFDC2* (HE4) gene (25), which is a member of the disulfide-core family of secreted proteins, is amplified in ovarian carcinoma (26). On the basis of this already published information, we decided to evaluate HE4 as a biomarker for ovarian cancer and hence made fusion proteins, immunized mice, and constructed a Sandwich ELISA. "Blinded" studies on sera from postmenopausal women with ovarian carcinoma and controls were then carried out in collaboration with Dr. N. Urban and her colleagues at FHCR. They showed the sensitivity of the HE4-based ELISA to be equivalent to that of CA125, but that HE4 was found to be less frequently positive in women with nonmalignant disease, i.e., to be more specific. Therefore,

HE4 may complement CA125 for diagnosis and monitoring of ovarian cancer (27). Like CA125 and SMRP, HE4 has temporal stability, which should make longitudinal studies possible to facilitate earlier diagnosis.

According to an ongoing, collaborative study with Dr. E. Friedman's group in Israel on 329 sera from 111 patients with clinical evidence of ovarian carcinoma, 68% of the patients were positive for CA125, 57% for HE4, and 65% for SMRP. A combination of all three markers detected 85% of the patients (E. Friedman et al., unpublished data).

## 5 Detection of Other Tumors by Assaying for SMRP or HE4

Mesothelin is overexpressed by carcinomas of the pancreas (unpublished findings in collaboration with Dr. P. Goedegebuure), indicating that assays for SMRP in serum and other body fluids should be evaluated as possible aids to diagnose and monitor patients with that tumor. Recent immunohistological studies have demonstrated expression of HE4 in most adenocarcinomas of the lung. This suggests that it may be a biomarker for serum assays also for those tumors, a matter that needs to be studied further (28).

## 6 Summary

Assays measuring SMRP (mesothelin) and HE4 (a secreted protease) in serum and other body fluids (including urine for SMRP) are likely to be clinically useful for patients with ovarian cancer, as data indicate that they complement CA125 for diagnosis and monitoring of patients. Both markers have temporal stability, as does CA125, which may be utilized to facilitate earlier diagnosis by performing longitudinal studies on high risk subjects. Preliminary data show autoantibodies to native mesothelin in some patients with ovarian carcinoma and in some healthy women. We are presently studying their relationship to the patients' clinical state to learn whether measurements of antibody levels provide information that can aid diagnosis and monitoring of treated patients. Prospective studies are needed to establish the clinical relevance of our findings.

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