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Two novel biomarkers, mesothelin and HE4, for diagnosis of ovarian carcinoma

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Abstract

Introduction—There is a need to improve the diagnosis and prognosis of ovarian carcinoma, particularly the serous type of cancer. Mesothelin and HE4 are two novel biomarkers which are expressed in serous ovarian carcinoma and can be measured in serum and other body fluids, including urine, by using ELISA. The measurement of antibodies to these markers can provide additional useful information.

Areas covered—A literature search was performed to as recent as December 1 2010, using the Internet (e.g. PubMed) on articles concerning mesothelin and HE4 for the diagnosis of ovarian carcinoma. The authors also included up to date recent information from the research performed in their own laboratory.

Expert Opinion—The combination of CA125 with HE4 facilitates the triaging of women with a pelvic mass and detects more stage I/II tumors than CA125, the present ‘gold standard’, when used alone. Assaying urine for HE4 or mesothelin may detect early ovarian carcinoma more often than assaying serum. Antibodies to mesothelin and HE4 are more frequent in women with ovarian carcinoma or with certain types of infertility than in controls. No presently available biomarker or multi-marker panel lends itself to screening large populations of symptomless women to make possible detection of high grade serous ovarian carcinoma at stage I or II. The authors anticipate, within the next 5 years, that a greater emphasis will be given to the fact that the different subtypes of ovarian carcinoma represent different types of disease. Each different type of disease will require a different diagnostic approach and more efforts will focus on high grade serous ovarian carcinoma for which the clinical need is the greatest.

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Article highlights

- There is a need for new assays to complement CA125 to diagnose ovarian carcinoma and improve prognosis.
- An ELISA for mesothelin variants 1 and 3 complements CA125 for detection of ovarian cancer and is the best assay to diagnose mesothelioma.
- An ELISA for HE4 complements CA to diagnose women with pelvic mass and it improves the detection of early ovarian cancer. A combination of a symptom index with assaying may aid diagnosis.
- Assaying urine for mesothelin and HE4 appears to be more sensitive than assaying serum.
- Most patients who are clinically tumor free after treatment for ovarian carcinoma have antibodies to mesothelin. Such antibodies are also detected in some healthy women and more frequently in women with signs of autoimmunity involving the ovary. Their relationship to ovarian cancer, if any, needs to be investigated.
- Novel assays, perhaps involving proteomics, are likely to be needed for mass screening of asymptomatic women to detect sporadic ovarian carcinoma sufficiently early to dramatically improve prognosis.

Keywords

cancer diagnosis; endometrial cancer; ELISA; mesothelioma; ovarian cancer; serological assays; tumor antigen in urine

1. Need for earlier diagnosis

Ovarian cancer is one of the most common causes of cancer-related death in women. There are four major histological types, serous, endometrioid, clear cell and mucinous, which differ in their clinical behavior and molecular characteristics (1-3). Low stage tumors comprise fundamentally different diseases than high stage tumors (4), and high grade serous ovarian carcinoma, which commonly arises from the Fallopian tube (5), is the disease that kills most patients and rarely occurs as stage Ia (3). While the cure rate for non-serous ovarian carcinoma diagnosed at stage I approaches 90%, the 5 year survival of high-grade serous carcinoma is only 35% (6,7). There is thus a great need for improvement, and particularly for high grade serous carcinomas.

Diagnostic techniques which allow early detection are likely to improve prognosis (8). At present, diagnosis of ovarian carcinoma is based on clinical examination, evaluation of symptoms and serological assaying for CA125 in serum/plasma, in many cases together with imaging by ultrasound, CT scanning or MRI. Although CA125 has been the 'gold standard' among serological assays for the past 25 years (9-12), the CA125 levels are elevated (>35 U/mL) in only 50–60% of patients with stage I cancer (13) and are frequently increased in benign gynecological disease (12,14,15) and in women with some unrelated diseases such as liver cirrhosis (16). Since CA125 has longitudinal stability, i.e. its level in serum remains constant unless there is a clinical change, sensitivity can improve by assaying serial samples from the same patient and perform imaging (17) when the CA125 level raises even if it remains within the normal range (13,18).

Much effort has gone into finding assays that can complement and possibly replace CA125, and many novel biomarkers have been identified and compared to CA125 (see, e.g., 19-21). Rosen et al used immunohistology to evaluate the expression of several such markers in sections of ovarian carcinomas which had low or no expression of CA125 in tumors and preoperative serum samples (22). All specimens expressed human kallikrein 10 (HK10), human kallikrein 6 (HK6), osteopontin (OPN), and claudin 3, and a smaller fraction reacted with Mabs specific for DF3 (95%), VEGF (81%), MUC1 (62%), mesothelin (34%), HE4 (32%), and CA19-9 (29%). However, only mesothelin and HE4 showed good specificity for neoplastic versus normal ovarian tissues and may therefore complement CA125. Importantly, both markers are expressed in high-grade serous ovarian carcinoma, as is CA125, while HE4 is not expressed in mucinous carcinoma.

In this article we will review work on mesothelin and HE4, selected because they are among the most promising new biomarkers to complement CA125, particularly for detection of serous ovarian carcinoma. Among them, HE4 has the best specificity and is more sensitive than CA125 to detect stage I/II ovarian carcinoma at a specificity of 95% or 98%. Assaying HE4 also facilitates diagnosis of endometrial carcinoma, as recently discussed in an article that also reviews HE4 for ovarian cancer (23).

2. Mesothelin: molecule and assay development

In the 1990s monoclonal antibodies (Mabs) were made to ovarian carcinomas by immunizing mice with samples obtained directly from patients followed by screening the hybridomas for antibodies binding to neoplastic but not normal cells in histological sections

of ovarian cancers, including most high grade serous ovarian carcinomas. One such Mab, 569, bound to most ovarian carcinoma specimens but not to a variety of normal tissues and was hence further characterized (24). Amino acid sequencing demonstrated that 569 binds to mesothelin, an approximately 40 kD protein which shown by Pastan's group to be expressed by ovarian carcinoma and mesothelioma and proposed as a therapeutic target (25-27). A 69 kDa precursor molecule is synthesized which forms two proteins, the membrane anchored 40 kDa mesothelin and MPF, a soluble megakaryocyte potentiating factor (28). The biological function of mesothelin is not known, but it is noteworthy that mesothelin binds to CA125 to facilitate cellular adhesion (29).

Further work demonstrated that Mab 569 bound to a molecule in supernatants from ovarian carcinoma cultures and in sera from patients with ovarian carcinoma. This led Scholler et al to construct a double determinant ("sandwich") ELISA combining Mab 569 with a second Mab, 4H3, obtained by immunizing mice with material purified from culture supernatants by immunoadsorption (24). In view of reports from Pastan's group that mesothelin is stably expressed at the cell surface (25-27), it was hypothesized that the "soluble" molecule was a new variant of mesothelin which has an 82 bp insert and was referred to as SMRP, soluble mesothelin related peptide (24).

To test this hypothesis, Hellstrom et al (30) made fusion proteins of the 3 known mesothelin variants (Fig. 1), including the one with an 82 bp insert, now called variant 3 (31), and immunized mice to obtain Mabs specific for each variant. Flow cytometry showed that variant 1 is more commonly expressed at the cell surface than the other variants. The published ELISA (24) was found to recognize variants 1 and 3 and have much higher sensitivity (68% versus 15%) than an ELISA for variant 3 (30). Amino acid sequencing of material isolated by immunoadsorption from the ascites of a patient with ovarian carcinoma primarily demonstrated mesothelin variants 1 and 2 (30). Hellstrom et al concluded that the mesothelin molecule primarily detected in body fluids is variant 1 that is shed from the tumor cells (30). Similar results were obtained when serum and plasma samples are analyzed from the same patients.

3. Mesothelin: clinical studies

3.1 Ovarian carcinoma

Using the original "laboratory" assay (24), McIntosh et al (32) evaluated a composite marker that combined CA125 with mesothelin in 'blinded' testing of sera from 52 ovarian cancer cases, 43 patients with benign ovarian tumors, and 220 normal risk controls. CA125, mesothelin and the combined marker were each evaluated for their ability to identify clinical cases and for their temporal stability. The combined marker had better sensitivity than either mesothelin or CA125 alone with a specificity equal to CA125, and it had temporal stability at least as high as CA125. The authors hypothesized that the combined marker would outperform CA125 alone for diagnosis and in a longitudinal screening program (32).

MESOMARK, a commercially available assay (33), was developed by Fujirebio Diagnostics, Inc and was used for all subsequently published studies.

Lowe et al measured serum levels of CA125, mesothelin and HE4 in 155 healthy postmenopausal women at high risk for developing ovarian cancer and examined the relationship between the marker levels and 22 risk factors. The 3 biomarkers were associated with a few ovarian cancer risk factors, but this association was not large. Therefore, incorporation of the risk factors in screening algorithms may not be necessary (34).

Shah et al evaluated the effect of known risk factors for ovarian cancer on the performance of mesothelin, HE4 and CA125, by studying 143 women with ovarian cancer, 124 women with benign gynecological conditions and 344 healthy controls and reached similar conclusions (35).

Moore et al (36) included mesothelin among several ovarian cancer markers which were evaluated to establish a panel to triage women with a pelvic mass. Mesothelin had higher sensitivity than CA125 at both 95% and 98% specificity and complemented CA125 when used as a combination marker, thus supporting the claim by McIntosh et al (32). However, it was inferior to HE4 when used alone or in combination with CA125 and most of the subsequent work by Moore's group has focused on HE4.

Detection of biomarkers in urine should facilitate the frequent testing of subjects to aid the earlier diagnosis of ovarian cancer via longitudinal studies and to monitor responses to therapy and detect relapses. Several markers have been identified in the urine, including a fragment of osteopontin (37). Badgwell et al assayed mesothelin in serum and urine from 28 patients with stage I/II ovarian cancer, 111 patients with advanced ovarian cancer and 19 with tumors of low malignant potential. Marker values were compared to those in healthy controls and patients with benign pelvic masses. Thresholds were set to include 95% of mesothelin values for 127 sera and 89 urines from healthy women. Urine values were normalized to creatinine and with respect to glomerular filtration rate with the latter approach preferred. It is noteworthy that more patients with early stage disease were detected when urine (42%) rather than serum (12%) was assayed. Likewise, 75% of patients with advanced ovarian cancer had elevated mesothelin in urine compared to 48% of patients with increased mesothelin in serum (38).

3.2 Mesothelioma

Most assays using mesothelin as a biomarker have been performed with mesothelioma for which there is no other good biomarker. The findings illustrate the pros and cons of such assays: they complement other diagnostic tools but do not have sufficient sensitivity and specificity for large scale screening of symptom-free subjects.

A “blinded” study, using the originally published “laboratory” assay (24) rather than MESOMARK, was performed by Robinson et al, measuring mesothelin in sera from 44 patients with histologically proven mesothelioma, 68 matched healthy controls, 40 of whom had been exposed to asbestos, and 160 patients with other inflammatory or malignant lung and pleural diseases (39). 37 (84%) of 44 patients with mesothelioma had raised concentrations of mesothelin, compared to 3 of 160 patients with other cancers or inflammatory lung or pleural diseases. None of 28 controls who had not been exposed to asbestos was positive. In patients with mesothelioma, the mesothelin levels correlated with tumor size. Importantly, 7 of 40 asbestos exposed subjects had increased levels of mesothelin and 3 of those 7 developed mesothelioma and one got lung carcinoma within 1–5 years of the positive test. In contrast, none of the 33 subjects with normal mesothelin levels got mesothelioma or lung cancer within 8 years of follow up. The authors concluded that assaying serum for mesothelin can aid the diagnosis of mesothelioma and the monitoring of disease progression, and they suggested that such assays may prove helpful for screening asbestos-exposed individuals for early evidence of mesothelioma.

After the initial report by Robinson et al, further studies all used MESOMARK (33). While they confirmed that mesothelin is the best biomarker for mesothelioma, the degree of specificity/sensitivity has been less than may have been anticipated from Robinson's original publication. It is unclear whether this is due to differences between the ‘laboratory’ assay and MESOMARK or has some other explanation.

Comparing pleural fluid from 52 patients with mesothelioma with such fluids from 140 patients who had pleural effusions of non-neoplastic origin, Creney et al found that MESOMARK had a sensitivity of 67% with a specificity of 98%. In 7 of 10 cases, mesothelin levels were raised in effusions collected 3 weeks to 10 months before diagnosis of mesothelioma and in 4 of 8 cases, mesothelin levels were increased in the effusion but not in the serum (40). An Editorial concluded that measurement of mesothelin cannot replace histocytological diagnosis although it has better sensitivity, and it emphasized that an increased level of mesothelin in pleural fluid raises a strong suspicion of mesothelioma or metastatic (especially ovarian or pancreatic) carcinoma, indicating a need for further investigations (41).

Cristaudo et al measured mesothelin in serum from 107 patients with mesothelioma, 215 patients with lung cancer, 130 patients with benign respiratory diseases and 262 healthy subjects, and constructed ROC curves to evaluate marker performance. Mesothelin levels were significantly higher in patients with mesothelioma or lung cancer than in patients with benign respiratory diseases or healthy controls (42).

Park et al performed a prospective study on 538 asbestos-exposed individuals, and investigated those with elevated serum mesothelin levels by tomography. Among 15 subjects with elevated mesothelin levels, one had lung cancer but none had mesothelioma. Consequently, the false positive rate was too high for screening purposes (43).

Pass et al measured mesothelin in sera from 90 patients with mesothelioma, 170 patients with lung carcinoma and 66 tobacco-matched and asbestos-exposed subjects, and they also studied pleural effusions. The mean level of mesothelin in serum was significantly higher in mesothelioma than in lung cancer, patients with stage 1 mesothelioma had higher levels than asbestos-exposed individuals, and the level increased as the tumor progressed to stages 2-4. Pleural effusions from patients with mesothelioma contained more mesothelin than when derived from patients with other diseases (44).

Davies et al measured mesothelin in 424 pleural fluids and 64 serum samples prospectively collected from 167 patients who presented with pleural effusions. Pleural fluid mesothelin concentrations were significantly higher in patients with mesothelioma than in patients who had metastatic tumors or benign effusions. The MESOMARK assay gave results superior to cytological examination for the diagnosis of mesothelioma and the data were not influenced by inflammatory pleural processes (45).

Segawa et al reported that the major form of mesothelin in patients with mesothelioma is variant 1 and not the molecule (variant 3) with an 82 pb insert (46). This agrees with the findings referred to above from studies on ovarian carcinoma (30).

Wheatley-Price et al collected 165 serial plasma samples from 41 patients with mesothelioma and measured the levels of mesothelin and osteopontin. Among 37 patients with measurable disease, 34 had elevated mesothelin. All of 4 previously treated patients which had no evidence of recurrent mesothelioma had normal baseline levels. In 21 treated patients, a more than 10% change in mesothelin level correlated with radiologic measurements. Preoperative mesothelin levels were elevated in 7 patients whose mesotheliomas were resected and returned to normal after surgery. Increasing mesothelin levels were observed in patients with radiologic evidence of disease progression. There was no association between the clinical state and results from assays for osteopontin (47).

Creaney et al measured mesothelin in stored serum samples from 106 asbestos exposed subjects who later developed mesothelioma, from 99 who did not, and from 109 subjects who had not been exposed to asbestos. Longitudinal determinations of mesothelin levels in

healthy asbestos-exposed individuals over a period of 4 years were stable. Mesothelin concentrations were greater than baseline in serum samples obtained before the diagnosis of mesothelioma from 17 of 106 subjects. Using an increase above the 95% confidence interval of the mean of a given individual's longitudinal mesothelin level, only 33 of 82 subjects (40%) had increasing mesothelin levels before diagnosis (48).

3.3 Pancreatic carcinoma

Johnston et al reported that pancreatic cancers consistently express mesothelin detected by immunohistology, and found circulating mesothelin in 73 of 74 patients with pancreatic cancer as well as in all of 5 patients with benign pancreatic disease while a large group of healthy individuals was negative (49).

4. HE4 gene and protein

The WFDC genes encode a family of stable 4-disulfide core proteins, which include the Wp protein (50), a secretory leukocyte protease inhibitor (51), elafin (52) and human epididymis protein 4, HE4 (53), all of which are secreted molecules. Chromosome 20q12-q13.1 contains a locus with several members of the WFDC family that have homology with whey acidic protein (WAP) (54). The genes are amplified in several cancers, including ovarian, breast, colon, pancreatic cancer and lung adenocarcinoma.

The HE4 protein contains two 50 amino acid sequences containing 4 disulphide bridges, i.e. the N0-WAP and tC-WAP domains. HE4 is unique among the WFDC encoded proteins in that it contains two WAP domains. It is a 20-25 kDa glycoprotein with a 10-12 kDa protein core. The biological function of HE4 is not known.

Studies by Hood's group first demonstrated that the WFDC2 gene is frequently amplified in ovarian carcinoma, whereas its expression in normal tissues, including ovary, is low (55,56), and this work has since been confirmed and extended (Ono, 2000 #2203, 57, 58). The highest normal tissue expression of HE4 is in glandular and respiratory epithelium (54,56,59). Importantly, the HE4 expression is upregulated at mRNA and protein levels in both early and late stage ovarian carcinoma, and the WFDC2 gene is expressed in about 90% of serous ovarian cancer and in most endometrioid and clear cell ovarian cancer (54,60), while mucinous and germ cell ovarian cancer barely express HE4 (54,60). Another WAP gene, which encodes a secretory leukocyte protease inhibitor, is also overexpressed in ovarian cancer (57,61).

In addition to full length HE4 protein, 4 variants of HE4 have been proposed (59). Differences in tissue distribution and clinical utility of the variants is unknown.

5. HE4 assay

About 10 years ago, the HE4 protein was selected as a biomarker to construct an ELISA for ovarian cancer. There were several reasons for this decision. HE4 is secreted rather than shed, its expression in the ovary is restricted to the neoplastic cells, it is expressed by >90% of serous ovarian cancer, its most serious and common form, and in both early and late stage tumors (55,56,62).

To immunize mice and make hybridomas, fusion proteins were constructed based on the gene sequence with the addition of a mouse Ig Fc domain to facilitate uptake by antigen-presenting cells; fusion proteins with a human Fc domain were made for screening the hybridomas (Fig. 2). The proteins were produced in stably transfected CHO cells and purified by Protein A affinity chromatography. Two Mabs, 2H5 and 3D8, which recognize the C-WFDC domain of HE4 (Fig. 3), were selected to construct a sandwich immunoassay

using biotinylated 2H5 for catching the antigen and HRP-labeled 3D8 Mab for detecting it (63). Subsequently, Fujirebio, Inc. (FDI) developed a commercially available kit for clinical use based on the same antibodies. Both the original “laboratory” assay and the FDI kit recognize full length HE4 and splice variants V2 and V3 but not splice variants V1 and V4. Similar results are obtained when serum and plasma samples are analyzed from the same patients. It is unclear whether specificity/sensitivity could be improved by also investigating an assay measuring splice variant 4 or using some different combination of full length/splice variants.

To facilitate assay development, Scholler et al developed a new type of antibody in the form of biobodies from diploid yeast cells transformed with a recombinant plasmid DNA such that, upon secretion, the antibodies were already biotinylated. Biotinylated antibodies with specificity for either mesothelin or HE4 were applied to construct assays which were reported to be as sensitive for detection of ovarian carcinoma as previously published assays (64).

6. HE4: clinical studies

6.1. Ovarian carcinoma

The HE4 “laboratory assay” was first applied in a “blinded” testing of sera from postmenopausal women with ovarian carcinoma and matched controls (63). The specificity and sensitivity of the HE4-based ELISA was shown to be equivalent to that of the CA125 assay. However, the HE4 assay was shown to have an advantage over the CA125 assay by being less frequently positive in patients with nonmalignant disease (63) and may therefore complement CA125. Subsequent to this publication, the commercially available kit from Fujirebio Diagnostics has been used to measure HE4.

Moore et al obtained preoperative serum samples from 259 women undergoing surgery for an adnexal mass and analyzed them for CA125, mesothelin (using MESOMARK), HE4, CA72-4, activin, inhibin, osteopontin, EGFR and Her2 (36). Logistic regression models were estimated for all markers and combinations with cross-validation analysis were performed to obtain the sensitivities at set specificities of 90%, 95% and 98%. As a single marker HE4 had the highest sensitivity for detecting ovarian cancer (36). A combination of CA125 and HE4 yielded the highest sensitivity of 76.4% at specificity 95%, and sensitivity was not increased if further markers were added.

When stage I ovarian cancer was compared with benign disease (36), HE4 was the best single marker. At 95% specificity, 45.9% were positive for HE4 as compared to 15.1% for CA125 and 30.3% for mesothelin, and at 98% specificity 30.8% were positive for HE4 as compared to 7.7% for CA125 and 15.4% for mesothelin. The best sensitivity (38.4%) to detect stage I ovarian cancer at 98% specificity was for a combination of HE4 with CA125, while CA125 plus mesothelin then had a sensitivity of 23.0% (36). While highly encouraging, it is noteworthy that the stage I group comprised 13 patients and that only 4 of them represented the serous subtype.

Havrilsky et al evaluated several biomarkers, including HE4, glycodelin, MMP7, SLP1, plau-R, MUC1, inhibin A, Pai-1 and CA125 in 133 stage I/II ovarian carcinoma patients (of whom 35 were of the serous subtype), 67 stage III patients, and 396 healthy, age-matched controls. Based upon ROC curve analysis, HE4 displayed the highest sensitivity among all single markers for the detection of both early and late state ovarian cancer. For early ovarian cancer, the sensitivity was 82.7% at a specificity of 86.3% and 62.4% at a specificity of 96% (65).

Patients diagnosed with ovarian cancer have improved outcomes when treated at centers experienced in the management of such tumors (66). In view of the ability of a combination of CA125 and HE4 to detect ovarian carcinoma (36), Moore et al performed a prospective study involving 531 patients and 12 sites to assess the risk for ovarian cancer in women with a pelvic mass (67). Preoperative serum levels of HE4 and CA125 were measured, and logistic regression algorithms for premenopausal and postmenopausal women used to categorize patients into low and high risk groups for ovarian cancer. An algorithm utilizing HE4 and CA125 correctly triaged 93.8% of the patients with carcinoma cases into the high risk group.

Montagnana et al measured CA125 and HE4 in pre-surgery serum samples from women with different forms of pelvic masses, including 46 patients with ovarian carcinomas, 53 patients with other gynecological tumors, 40 patients with benign disease and 12 healthy controls. The median CA125 and HE4 serum levels were significantly higher in ovarian cancer patients than in the healthy subjects or patients who had a benign mass or gynecological tumors other than ovarian carcinoma. ROC analysis showed a significantly higher area under the curve for HE4 as compared to CA125 (68).

Palmer et al measured the levels in circulation of 14 biomarkers for serous ovarian cancer and evaluated their performance individually and in combination to characterize samples from women with or without ovarian cancer. The authors concluded that, based on sensitivity and specificity, 4 markers, MUC 16, HE4, mesothelin and MMP7 should be considered for further evaluation (69).

Huhtinen et al analyzed sera for HE4 and CA125 in samples from 129 women with endometriosis, 16 with endometrial cancer and 14 with ovarian cancer as well as in samples from 66 healthy controls. The mean serum concentration of HE4 was significantly increased in patients with endometrial or ovarian cancer but not in patients with ovarian endometriomas or other types of endometriosis and measuring both HE4 and CA125 increased the accuracy of differentiating ovarian cancer from ovarian endometriotic cysts (70).

Abdel-Azzez et al measured CA125, HE4 and mesothelin in serum from 65 patients with pelvic mass, including 41 with ovarian cancer and 24 with benign disease, and also from 25 age and menopausal status-matched healthy women. As a single marker, HE4 had the highest sensitivity for detecting ovarian carcinoma (82.9%) including early disease (76.9%), and a combination of CA125 with HE4 was a better predictor of ovarian malignancy than either alone. Addition of mesothelin to the combination did not improve sensitivity (71).

Andersen et al performed a prospective case-control study to investigate the contributions of a symptom index together with measurement of CA125 or HE4 in serum. Their study comprised 74 women with ovarian cancer (of whom 31 were early stage) and 137 healthy women. A decision rule based on any two of the three parameters being positive had a sensitivity of 84% with a specificity of 98.5% (72). The publication emphasizes the importance of identifying symptoms most commonly associated with ovarian cancer, informing the public about such symptoms, and test those subjects who have symptoms indicative of ovarian cancer with the best available serological assays (CA125+HE4 at this time).

Nolen et al studied 65 ovarian cancer related biomarkers in the circulation of women diagnosed with an adnexal mass, representing either benign diseases or ovarian cancer of early or late stage (73). As individual markers, HE4 and CA125 gave the best discrimination between benign and malignant cases, and the combination of the two was better than either alone. Several multimarker panels were identified that discriminated early and late stage

ovarian cancer from benign cases with sensitivity/ specificity similar to or slightly improved above CA125-HE4, but they did not outperform the CA125-HE4 panel in an independent validation set (73).

Anastasi et al investigated HE4 and CA125 in 32 sera from patients with ovarian cancer and 163 patients with other malignant or benign pathologies. At diagnosis, patients with ovarian cancer had high levels of both markers with 96.9% sensitivity for HE4 and 85.7% for CA125, as compared to 3.7% positivity for HE4 and 21.0% for CA125 in patients with other pathologies. HE4 was also studied as an indicator of recurrent ovarian cancer in treated 8 patients who were followed for 20 months. In 5 patients there was an increase of HE4 that was associated with relapse, and this increase occurred 5-8 months before there was an increase of CA125 (71).

Yurkovetsky et al assayed sera from 139 patients with early and 149 patients with late stage ovarian cancer as well as 1,102 healthy women, using a panel comprising CA125, HE4, CEA and VCAM-I (74). At 98% specificity, the panel had 86 % sensitivity for early and 93% sensitivity for late stage ovarian cancer. These studies were followed by an independent, blinded validation using sera from 44 patients with early and 124 with late ovarian cancer as well as from 929 healthy women and women with other diseases. At 98% specificity, there was 86% sensitivity for stage I/II ovarian cancer and 95% sensitivity for stage III/IV; 33% of women with benign pelvic disease were positive as were 6% of women with breast cancer, 0% of women with colorectal cancer and 36% of patients with lung cancer (74).

Anderson et al performed a nested case-control study to assess the lead time of selected ovarian cancer biomarkers (75). They concluded that serum concentrations of CA125, HE4 and mesothelin may provide evidence of ovarian cancer up to 3 years before clinical diagnosis, but that the likely lead time associated with these markers was less than a year.

Hellstrom et al (76) assayed urines from ovarian carcinoma patients with early (I/II) and late (III/IV) disease and from healthy women matched for age and menopausal state. The data were normalized relative to the creatinine levels in urine. At a specificity level of 94.4%, HE4 was detected in 57/64 (89%) patients with stage III/IV disease, including 34/38 (89.5%) patients with serous ovarian carcinoma, and in 13/15 (86.6%) of stage I/II ovarian cancer patients, including all of 4 patients with the serous subtype. The latter finding is noteworthy, since, at 95% specificity, sera from only 45.9% stage I patients were positive for HE4 in the study by Moore et al (44). Importantly, urines from 11 of 15 women (73.3%) with stage I/II disease were positive at a cut-off when urine from none of 36 healthy women and none of 20 women with benign gynecological disease was positive (100% positivity). Urine and serum samples were not available from the same patients for comparison. However, as discussed above, Badgwell et al showed that urines from 42 % of stage I patients were positive for mesothelin as compared to 12% of sera (38). Assaying urine rather than serum thus appears to be more sensitive. The reason for this is not known. One might speculate that some antigen secreted from tumor cells passes through the fallopian tube and is excreted via urine. If so, sensitivity of the assay may further increase by measuring HE4 and other ovarian cancer antigens in fluids obtained from the fallopian tube by endoscopy, although this is obviously more complicated than obtaining antigen from urine.

6.2. HE4 in endometrioid adenocarcinoma, lung adenocarcinoma and bladder carcinoma

HE4 is also expressed in some tumors other than ovarian carcinoma. So far, the greatest application of the HE4 ELISA beyond ovarian cancer is to aid the diagnosis of endometrial carcinoma. Moore et al assayed preoperative serum samples from 171 surgically staged patients with endometrioid adenocarcinoma of the uterus for levels of HE4, SMRP, CA72-4

and CA125, using control samples from 156 healthy postmenopausal women. Logistic regression models and ROC curves were constructed for each tumor marker and for all combinations with cross-validation analyses to obtain average sensitivities at set specificities of 90%, 95% and 98%. HE4 is elevated in all stages of endometrial cancer and is the dominant biomarker for that tumor by being more sensitive marker than CA125 in early-stage endometrial cancer (77).

Galgano demonstrated HE4 mRNA in lung adenocarcinomas as compared to less than 10% of squamous cell lung carcinomas (54), and Bingle et al performed an immunohistological study which showed HE4 expression by most adenocarcinomas of the lung but not in squamous cell carcinomas (78).

Zhu et al reported that the HE4 ELISA can aid diagnosis of transitional cell carcinoma of the urinary system and be a prognostic marker (79).

7. Auto-antibodies to mesothelin and HE4

Antibodies to tumor antigens have been detected in many cancer patients (80-85) and are sometimes found to correlate with the clinical state. For example, antibodies to HER-2/neu and p53 have been detected by capture ELISA (85,86) in some patients with early stage breast cancer, and anti-MUC1 antibodies have been reported to be more frequent in healthy women with characteristics associated with a decreased risk for ovarian carcinoma (84). Ho et al reported that about 50% of patients whose tumors expressed mesothelin had antibodies to mesothelin, compared to 0-8 % of patients whose tumors did not express mesothelin (87).

To evaluate the potential clinical application of measuring anti-mesothelin antibodies in patients with ovarian cancer, Hellstrom et al constructed an ELISA to measure antibodies to mesothelin and applied it to study sera from patients with divergent clinical outcomes (88). Native mesothelin was used as antigen and was purified either from urine of patients with advanced ovarian carcinoma or from culture supernatants. Anti-mesothelin antibodies, determined as OD450 nm, was seen more frequently in ovarian cancer patients with advanced disease who were free of clinically detectable tumor after therapy (10 of 14) than in similar patients who had clinical evidence of disease (9 of 21) or in healthy women (6 of 23) or women with benign gynecologic diseases (5 of 24). Importantly, 7 of 9 women with pelvic inflammatory disease were positive. Anti-mesothelin antibodies without concomitant mesothelin antigen in serum was seen in only one of 21 patients with clinical evidence of advanced ovarian cancer.

Subsequent studies, using recombinant mesothelin as antigen, have confirmed that anti-mesothelin antibodies in patients who have been treated for advanced ovarian carcinoma are most frequently seen when the patients are (temporarily) without clinical evidence of tumor than in patients with detectable disease. They have also shown that several patients with advanced ovarian carcinoma have high levels of mesothelin and anti-mesothelin antibodies in both serum and ascites. However, because of the high frequency of healthy subjects who have antibodies to mesothelin it is unlikely that measurement of anti-mesothelin antibodies will provide a better way to detect early disease via some screening program.

A similar assay was recently constructed to measure antibodies to HE4 and is presently being evaluated; preliminary findings are similar to those for mesothelin (manuscript in preparation).

There are several reasons why antibodies to mesothelin, HE4 and certain other tumor antigen can have an impact on the development and progression of ovarian cancer. Anti-mesothelin antibodies can prevent binding of mesothelin to CA125 and thereby impact

cellular adhesion (29), and antibodies can be cytotoxic in the presence of complement, mediate antibody-dependent cellular cytotoxicity in the presence of NK cells or macrophages. Furthermore, the generation and expansion of T cell responses to tumor antigens may be impacted by such antibodies as well, both positively and negatively (89). 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, so that changes in antibody levels will influence the amount of mesothelin that is detectable by an ELISA. Furthermore, studies of antibodies formed in response to therapy, including immunotherapy, where mesothelin is a potentially good marker (90), are likely to be aided by measuring assaying for antibodies.

Importantly, women with unexplained infertility and/or recurrent pregnancy loss more frequently than age-matched healthy, fertile women have antibodies to several antigens that are expressed both by normal ovaries and by ovarian carcinomas (91-96). A recent collaborative study (Luborsky et al, manuscript in preparation) indicates that women with unexplained infertility more frequently than controls have antibodies also to mesothelin and HE4 and that the same subject often has antibodies to both those antigens. It is noteworthy that a 20-year longitudinal study of over 12,000 women shows a higher epidemiologic risk for ovarian cancer among women with infertility (97).

Better insight about the relationship between the presence of antibodies to ovarian cancer antigens and tumor formation may be gained by utilizing existing serum banks (such as CARET) to test serial samples from symptom free women who later developed ovarian cancer and from matched women who did not. If women with antibodies to mesothelin, HE4 or other ovarian cancer antigens have a higher risk of developing ovarian cancer, they should be monitored accordingly. We do not know whether the antibodies promote tumor growth to provide some form of immunostimulation (98) or rather reflect an immune response which is commonly successful to prevent tumor formation but sometimes fails.

Studies in animal models may also help elucidating the relationship between circulating antigen, antibodies and tumor occurrence. The high incidence of ovarian carcinoma in egg laying hens (99) makes the hen a useful model for this, and several ovarian carcinoma antigens, including mesothelin and HE4, have homologues which are overexpressed in the chicken tumors (Luborsky et al, unpublished findings). There are also several mouse models of ovarian cancer (100). The most commonly applied models is MOSEC, which was established by Roby et al., who isolated mouse ovarian surface epithelial cells and cultured them in vitro followed by transplantation into mice (101). The ability of MOSEC to form extensive tumors within the peritoneal cavity, similar to those seen in women with Stage III and IV cancer, and the ability of the MOSEC to produce tumors in mice with intact immune systems, makes this model useful for investigations of molecular and immune interactions in ovarian cancer development.

8. Expert Opinion

Prognosis would dramatically improve if ovarian carcinoma, could be diagnosed early, and the need to do so is greatest for serous ovarian carcinoma, which is rarely diagnosed at stage Ia. Screening of asymptomatic women would be needed to accomplish this, since symptoms appear relatively late. Assays measuring tumor markers in serum or other body fluids have the advantage of being non-invasive, simple to perform and relatively cheap. Since one among 2500 postmenopausal women is likely to develop ovarian carcinoma in the USA, an acceptable screening assay would require a sensitivity of 75% and a specificity of around 99.7% to obtain a minimally tolerable positive predictive value of 10% for the detection of ovarian carcinoma. No single marker or multi-marker panel has achieved this, most likely

because the presently known ovarian carcinoma biomarkers are also expressed by certain normal cells from which they are secreted or shed to cause a high background. Much effort should therefore go into identifying women who are at high risk to get ovarian carcinoma and to carefully monitor them for symptoms associated with ovarian cancer, by assays for secreted or shed tumor markers, and, when needed, by imaging.

Until recently, the only FDA approved biomarker for ovarian cancer was CA125, which was discovered almost 30 years ago. This situation has changed significantly over the last few years with a large number of new markers being discovered, among which mesothelin and, particularly, HE4 are among the most promising. HE4, which is the first biomarker after CA125 to be approved by FDA for ovarian cancer, can complement CA125 to triage women with a pelvic mass into groups with high or low probability to have ovarian carcinoma. Furthermore, a combination of CA125 with HE4 detected 38.4% of Stage I ovarian carcinoma at 98% specificity in a study by Moore et al, and the sensitivity, at the same 98% level of specificity, was as high as 86% for stage I/II ovarian carcinoma in a recently published study by Yurkovetsky et al using a panel comprising CA125, HE4, CEA and VCAM-I. Based on experience with CA125, longitudinal studies should further improve the detection of malignancy, as should the imaging of patients whose marker level is increasing. Application of the Luminex technology should facilitate the concomitant measurement of many markers.

Assaying urine for mesothelin or HE4 may further increase the ability to detect early disease and would lend itself to frequent testing (maybe at home) followed by evaluation of those women whose marker level is increasing. Maybe, assaying of antigen secreted or shed into the Fallopian tube will further improve the detection of early tumors. Particular attention should be given to assay women who have symptoms indicative of ovarian cancer or any known risk factor.

It needs to be realized that the different subtypes of ovarian carcinoma are biologically distinct, and that serological testing should include assays which can detect the various subtypes; e.g. mucinous ovarian cancers express CA125 but not HE4. The greatest need for improved diagnosis via serological assays is for high grade serous ovarian carcinomas, and this need has not yet been fulfilled by assaying existing biomarkers, including CA125, HE4 and mesothelin in serum. It remains to be determined whether measurement of antigen secreted or shed into urine or in tubal fluid (obtained by endoscopy which would also provide an opportunity for physical observation) can improve this situation.

Antibodies to mesothelin and HE4 have been demonstrated in many patients and particularly in those who have been treated for ovarian carcinoma and are temporarily clinically tumor free. Since a significant number of healthy women have antibodies to these two antigens, it is unlikely that an antibody assay would be useful for screening to detect early ovarian cancer. Rather, we need to learn whether asymptomatic women who have the antibodies have an increased risk for ovarian cancer, as may be suggested by the finding that infertile women more frequently have antibodies to mesothelin and HE4 and that women with infertility have an increased risk of developing ovarian cancer.

While mesothelin appears to be inferior to HE4 as a biomarker for ovarian cancer, it is the best known marker for mesothelioma and also is a marker for patients with pancreatic disease, and HE4 is an excellent marker for endometrial carcinoma and may also be useful as a marker for lung adenocarcinoma and transitional cell bladder tumor.

We expect that, within the next 5 years, more emphasis will be given to the fact that the different subtypes of ovarian carcinoma represent different types of disease, which need different diagnostic approaches, and that more efforts will focus on high grade serous

ovarian carcinoma for which the clinical need is the greatest. Based on examination of ovaries and Fallopian tubes removed from healthy women with BRCA1/BRCA2 mutations, there is evidence that serous ovarian carcinoma needs to be detected when it is only about 4 mm in diameter to dramatically improve prognosis (3). This is not likely to be achieved by using presently known biomarkers. There is hope that future techniques based on proteomics (102,103) may improve the situation but it will probably take more than 5 years until it will be practically feasible to use such techniques for the mass screening needed to detect sporadic ovarian carcinoma in asymptomatic women. On a more positive note, it is likely that ongoing and planned studies will have demonstrated within the next 5 years that longitudinal assays of serum and other body fluids from women belonging to high risk groups, performed in combination assessment of symptoms and together with imaging and/or endoscopy of the Fallopian tube when there is suspicion of tumor will lead to earlier detection. We also expect that more and perhaps even better biomarkers will be identified and that panels, including CA125, HE4, mesothelin and additional markers will further improve the situation.

We also expect to learn within the next 5 years whether women who have antibodies to mesothelin, HE4, CA125 and/or other ovarian cancer antigens have an increased risk for ovarian carcinoma and should be monitored accordingly, and whether a combined testing for antigen and antibodies aids the monitoring of patients treated for ovarian cancer, including those receiving various forms of immunotherapy.

One may speculate that markers will also be detected during the next 5 years that can be used to assay serum and other body fluids to reflect the presence of stem-like cancer cells and/or emerging populations of tumor cells with increased resistance to certain chemotherapeutic agents.

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Fig 1.

The three variants of mesothelin. The figure indicates binding sites for Mabs 569 and 4H3 and for a Mab applied together with 4H3 to construct an assay for variant 3.

Fig. 2.
Fusion proteins for immunizing mice to obtain and test Mabs to HE4.

Fig. 3. binding specificity of Mabs 3H5 and 3D8 to full length HE4 and its splice variants. Assays based on the 2H5 and 3D8 Mabs recognize full length HE4 and splice variants V2 and V3, while V1 and V4 are not recognized.