Two-marker Combinations for Preoperative Discrimination of Benign and Malignant Ovarian Masses

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Abstract. *Background: When caring for patients with ovarian neoplasms, correct preoperative discrimination of benign and malignant disease is deemed vital. In this study, we tested serum biomarkers' alone and in combination, to achieve this aim. Materials and Methods: We measured the concentrations of Cancer Antigen (CA)-125, CA15-3, CA27-29, Carcinoembryonic Antigen (CEA), CA19-9, human chorionic gonadotropin (hCG), Placental Protein (PP)1490, CA72-4, galectin-3, galectin-1 and Human epididymis protein (HE)4 in sera of 133 patients with pelvic masses by ELISA and correlated the results to subsequent histology. We used the area under the curve (AUC) of biomarkers and their combinations and calculated the 95% confidence intervals by using casewise resampling. Results: The best single biomarkers were CA-125 (sensitivity and AUC) and HE4 (specificity). Combinations with HE4 and CA19-9 improved the predictive power of CA-125. The best discrimination was achieved by the combination of CA-125 and HE4, with an AUC of 0.961. Conclusion: A combination of CA-125 with HE4 could facilitate the identification of women at risk for ovarian cancer.*

It has been shown in many studies that treatment by a surgical team specialized in gynecological oncology will lead to an improved outcome for patients with ovarian cancer (1). On the other hand histological diagnosis is only performed during surgery, leaving more than 50% of ovarian cancer patients with inadequate initial cytoreductive surgery because of poor allocation of surgical expertise (2, 3). Therefore, reliable non-invasive predictive tools which allow for the identification of women with pelvic masses seen on vaginal ultrasound, into high- and low-risk groups, are needed (4).

The glycoprotein CA-125 is the most widely used and the most reliable serum marker for the management of ovarian cancer. It is used for preoperative assessment, as well as for postoperative monitoring (5). Unfortunately, only 80% of epithelial ovarian carcinomas express CA-125. Furthermore, elevated serum CA-125 serum levels are only seen in 50-60% of patients presented with early stage ovarian cancer, thus restricting its sensitivity. Moreover many benign gynecological and even non-gynecological diseases can result in elevated levels of CA-125, thereby limiting its specificity (6).

In this study, we explored biomarker combinations for the reliable triage of pelvic masses before surgery. Therefore, we measured biomarkers besides CA-125 in sera, preoperatively taken from patients with pelvic masses. The measured markers were Cancer Antigen (CA)-125, CA15-3, CA27-29, Carcinoembryonic Antigen (CEA), CA19-9, human chorionic gonadotropin (hCG), Placental Protein (PP)1490, CA72-4, galectin-3, galectin-1 and Human epididymis protein (HE)4. Besides CA-125, established biomarkers were CA72-4, which has often been considered as the leading marker in mucinous ovarian carcinoma (7), and Human epididymis-specific protein 4 (HE4), which has been proven to be equal or superior to CA-125 (8).

In order to increase the predictive power and to find markers that might increase the sensitivity and the specificity of CA-125, we calculated all respective marker combinations. Because of the limited number of samples, we confined statistical analysis to two-marker combinations.

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Key Words: Ovarian neoplasms, diagnosis, surgery, blood, marker, preoperative, discrimination, serum CA-125, CA15-3, CA27-29, CA19-9, CEA, hCG, PP1490, CA72-4, galectin 1, galectin 3, HEA, CEA.

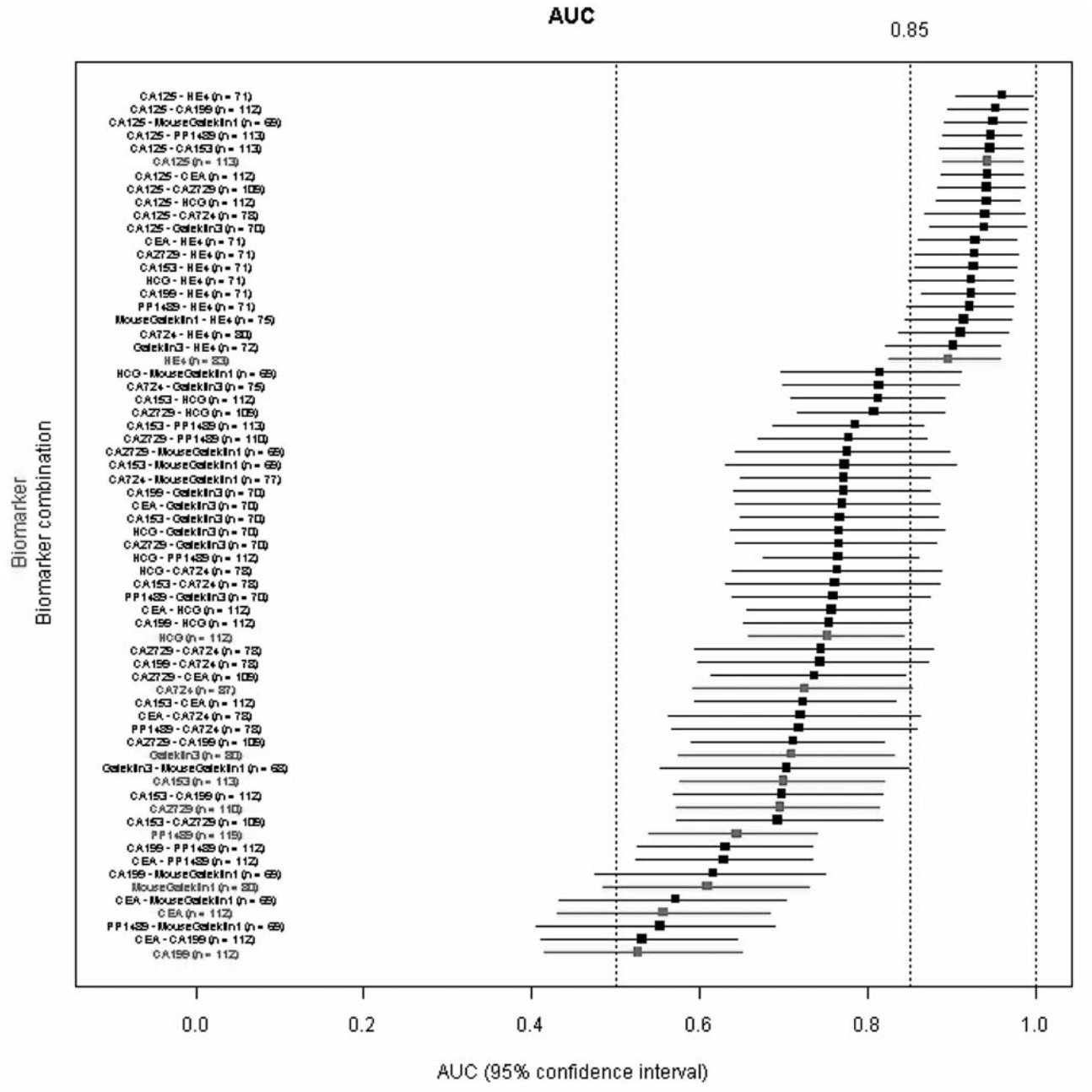

Figure 1. *Area Under the Curve (AUC) of the biomarkers and the biomarker combinations with 95% bootstrapped confidence intervals. Single biomarkers are shown in grey whereas biomarker combinations are coloured in black. In the brackets of each biomarker and biomarker combination, the number of complete cases is provided.*

Materials and Methods

We analyzed serum samples from 133 patients with pelvic masses, diagnosed and cared for between 2003 and 2007 at the Department of Obstetrics and Gynecology in the Ludwig Maximilians University, Innenstadt Campus, Munich, Germany. In order to be eligible for enrollment, patients were required to have a pelvic mass seen on vaginal ultrasound, requiring for surgical intervention. Immediately prior to surgery, blood samples were collected. All patients gave their written informed consent. The institutional Ethics Committee approved the study protocol. Immediately after collection all blood samples were centrifuged and the sera were frozen to –80˚C.

Two specialized gynecological pathologists performed the histological diagnostic evaluation according to the criteria of the Fédération Internationale de Gynécologie et d' Obstétrique (FIGO). This analysis revealed 47 malignant (30 serous, 2 mucinous, 15 endometroid) and 86 benign tumors.

Table I. *Best sensitivity and specificity of CA-125, CA72-4 and Human epididymis protein (HE)4 and resulting cut-off values.*

	CA-125	CA72-4	HE4
Correctly classified	91.2%	82.8%	85.54%
Sensitivty	87.2%	53.3%	62.1%
Specificity	93.2%	98.2%	98.1%
Resulting cut-off	55.1 U/ml	5.8 U/ml	118.9 pM

Table II. *Sensitivity at a set specificity of 95% and Area Under the Curve (AUC) of CA-125, CA72-4 and HE4.*

	CA-125	CA72-4	HE4
Sensitivity	61.5%	53.3%	65.5%
Specificity (rounded)	95%	95%	95%
AUC	0.944	0.726	0.897

Followingly we measured the concentrations of 11 markers: CA-125, CA15-3, CA27-29, CEA, CA19-9, hCG, PP1490, CA72-4, galectin-3, galectin-1 and HE4 at the protein level.

Only limited amounts of serum were available and therefore we were unable to quantify every biomarker in all 133 specimens, resulting in a divergent number of cases, studied for some markers (Figure 1).

Serum CA-125, CA15-3, CA27-29, CA19-9, CEA, hCG and PP1490 concentrations were determined by chemiluminescent immunoassays on an IMMULITE® 2000 automatic analyzer (Siemens Healthcare Diagnostics, Eschborn, Germany). Serum HE4 concentrations were determined using the HE4 assay (Fujirebio Diagnostics, Göteborg, Sweden). Serum CA72-4 and galectin-3 concentrations were measured using the CA72-4 and galectin-3 assay (IBL, Hamburg, Germany), respectively.

As no human-Galectin-1 ELISA monoclonal Antibody is available, we used a mouse-Galectin-1 ELISA (R&D Systems, Minneapolis, MN USA) with Washbuffer (25×; R&D Systems), Reagent Diluent (R&D Systems), Substrat Solution (R&D Systems) and Stop Solution (R&D Systems). Limit of detection was ~1 ng/lane.
All assays were run according to the manufacturer's instructions.

Statistical analyses. Due to the limited sample size, we restricted statistical analyses to combinations of two biomarkers leading to (11×10)/2=55 combinations. All measurements were log transformed and then standardized so that each variable had a mean of zero and a variance of one. Logistic regression for modeling biomarker combinations may yield poor classification performance in case of violations of the assumptions of the model used. Therefore, we quantified the predictive power of all two-biomarker combinations by a less restrictive statistical method which maximizes the area under the curve (AUC) of the receiver operating characteristic curve (ROC curve) following the non-parametric approach of Pepe *et al*. using linear combinations of biomarkers (9). We calculated 95% confidence intervals of the AUCs of the biomarkers and respective combinations by using casewise resampling (bootstrapping) with 9999 replications and by using bias-corrected and accelerated bootstrap confidence intervals (BCa). For each biomarker and biomarker combination we used complete cases. All analyses were carried out using the statistical software R 2.12.1 for Windows (Institute for Statistics and Mathematics, Vienna, Austria). We considered biomarkers and biomarker combinations yielding a point estimate of the AUC of 0.85 or greater as those with acceptable separation performance between benign and malignant ovarian diseases.

Results

Single marker performance. Biomarkers that performed best in univariate analyses were CA-125 (AUC=0.944) and HE4 (AUC=0.897). Other biomarkers and biomarker combinations yielded AUC less than 0.85 (Figure 1).

CA-125 allowed for good separation between benign and malignant ovarian diseases and had a convex curve. The point of the ROC curve which resulted in the highest accuracy was 88.7% for CA-125. A maximum proportion of 91.2% of all patients were correctly classified into the malignant or the benign group by CA-125, yielding a sensitivity of 87.2% and a specificity of 93.2% (Table I). At a set specificity of 95% we retrieved a sensitivity of 61.5% with CA-125 (Table II).

HE4 also allowed for good separation but diagonal elements between a false-positive rate of 0.0 and 0.45 indicated local random performance. The point of the highest accuracy for HE4 alone was 88.7%. A total of 85.54% of all patients would have received the correct diagnosis concerning their tumor, leading to a sensitivity of 62.1%, a specificity of 98.1% and a cut off of 118.9 pM (Table I). At a fixed specificity of 95%, we achieved a sensitivity of 65.5% (Table II).

We gained an AUC of 0.726 with CA72-4, which provides a sensitivity of 53.3% and a specificity of 98.2% (Table I). At a set specificity of 95% we achieved a sensitivity of 53.3% (Table II).

Two-marker combinations: Predictiveness of CA-125 improved by combination with HE4 (AUC=0.961) and CA19-9 (AUC=0.952) and slightly improved with galectin-1 (AUC=0.948), PP1490 (AUC=0.947) and CA15-3 (AUC=0.946) (Figure 1). However, combinations of CA-125 with CA19-9, galectin-1 and PP1490 showed wide, 95%, confidence intervals. The discriminatory power of HE4 was improved by all combinations (AUCs from 0.907 to 0.961) except for the combination with galectin-3 (AUC=0.893). The best performing combination was that of CA-125 and HE4 (Figure 2). Compared to CA-125 alone, the combination of CA-125 and HE4 performed better for a false-positive rate between 0.0 and 0.1 and for a false-positive rate of >0.5. Compared to HE4 alone, the combination performed better for a false-positive rate between 0.05 and 0.4. The point in the ROC with the highest accuracy (94.4%) of the combination of CA-125 and HE4 corresponded to a sensitivity of 92.3% and a specificity of 95.6% compared to the points with the highest accuracy of CA-125 and HE4 alone, which yielded sensitivities of 87.2% and 53.3% and specificities of 93.2% and 98.1%, respectively. Logistic regression of the CA-125 the HE4 biomarker combination lead to an underestimated AUC of 0.948.

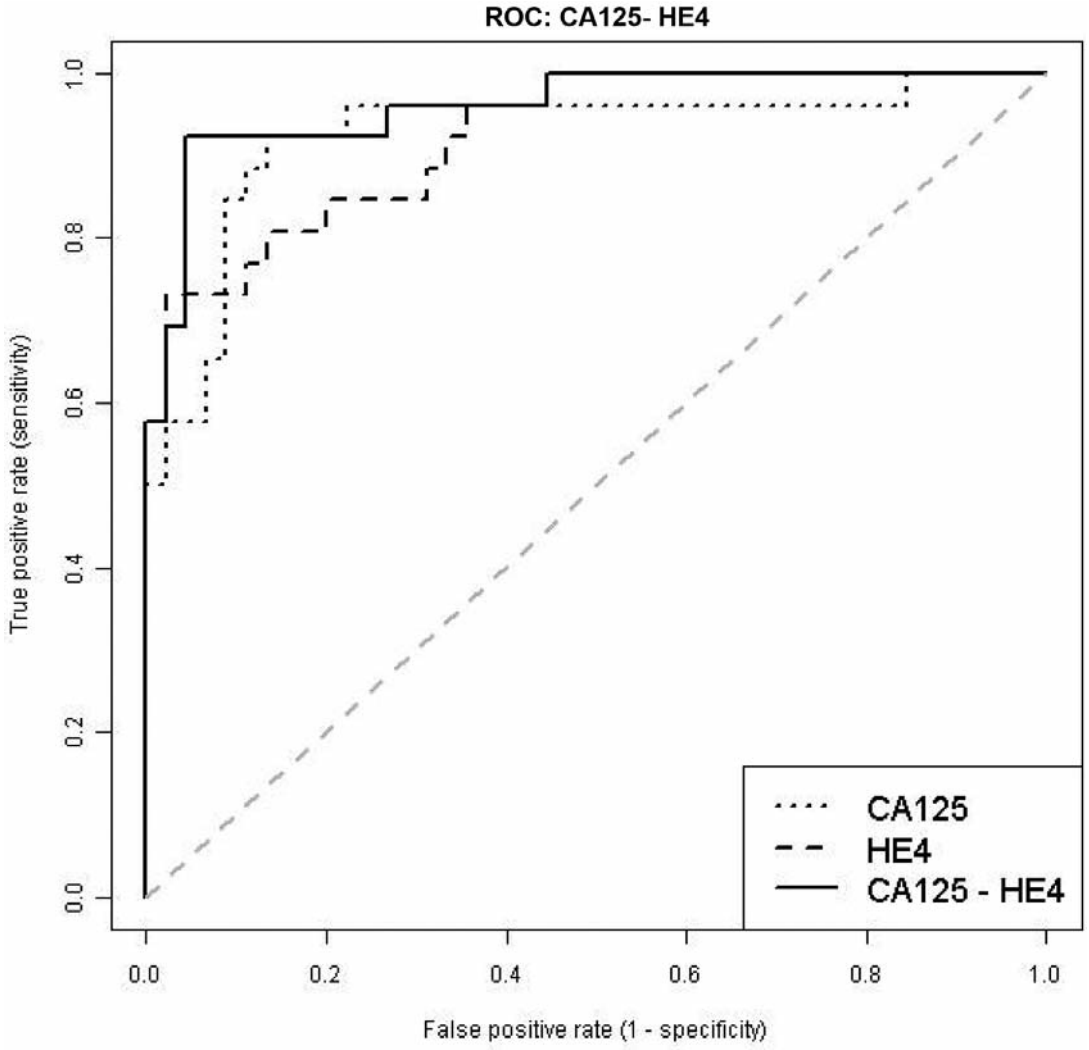

Figure 2. *Receiver operating characteristic curve (ROC curve) of CA-125, Human epididymis protein (HE)4 and the combination of CA-125 and HE4.*

Discussion

Many investigators have focused on early detection and diagnosis of ovarian cancer and glycoproteins, also used as tumor markers, seem to play a vital role in its pathophysiology (10). Bast and co-workers discovered the mucin marker CA-125 in 1981 which since then has been at the center of many trials (11). Indeed, CA-125 is the most utilized biomarker for ovarian cancer, although measurement of CA-125 has known restrictions due to its limited sensitivity and specificity (5). Supporting its leading role in caring for patients with ovarian cancer CA-125 turned out to be the best single marker during our investigations. We achieved an AUC of 0.944 and thereby a good discriminatory power between malignant and benign adnexal masses. We correctly triaged 91.2% of all patients, resulting in a sensitivity of 87.2% and a specificity of 93.2%. The resulting cut-off was 55.1 U/ml and thereby slightly higher than the cut-off that is commonly used for CA-125 (35 U/ml).

Due to the rather small sample size, our study represents an exploratory rather than a confirmatory study. For the same reason, there were no analyses of combinations with three or more biomarkers which might improve the discriminatory performance between benign and malignant ovarian diseases. Optimal cut-off of the biomarker combination of CA-125 and HE4 was determined by using the point with the highest accuracy. However, determining an optimal cut-off should be discussed in terms of subsequent patient morbidity in regard to false-positive and false-negative test results.

For mucinous ovarian tumors some authors report a limited sensitivity of only 50% for CA-125, which is much lower than

that for other histologies (12). The mucin marker CA72-4 is described by some investigators as one possible marker to close this diagnostic gap (7).

In our study, 82.95% of the patients were triaged correctly by CA72-4, which is a surprisingly good result, considering that our collective contained mostly serous tumors. We also achieved a remarkably good specificity of 98.2% with CA72-4, which is in line with other investigators reporting outstanding specificity for this biomarker (13).

The glycoprotein HE4 has been shown to be at least equivalent to CA-125 in different studies, including our own investigations (14). By using HE4 we achieved an AUC of 0.897, a best sensitivity of 62.1% and a best specificity of 98.1%. Although HE4 provided a good discriminatory power within our collective, CA-125 still arises as the slightly superior marker, based on our investigations. This is consistent with the results of other study groups, which also describe CA-125 as their best single marker (1,15). On the other hand, recent publications use HE4 as a marker equivalent to CA-125 (16).

As CA-125 is elevated in many common benign gynecological and non gynecological conditions, HE4 may perfectly complement CA-125 because HE4 is not elevated in many of these conditions and thereby may provide a higher specificity. Our data support that notion. Moreover, roughly 20% of ovarian cancer cases do not express CA-125, while HE4 levels are still elevated in approximately 50% of these cases (17).

These findings make it reasonable that when used in combination, the two markers HE4 and CA-125 complement each other, as each improves the discriminatory power of the other. In 2008, Moore *et al*. published a study of 233 patients with adnexal masses. In this study, the combination of HE4 and CA-125 not only proved to be superior to every single marker but to be the best two-marker combination, with a sensitivity of 76.4% at a set specificity of 95% and an AUC of 0.914. Adding further markers brought only few improvements (18). The study group of Nolen *et al*. achieved similar results (19). Our findings agree with the results of these research groups. The point in the ROC space with the highest accuracy (94.4%) of the combination of CA-125 and HE4 corresponded to a sensitivity of 92.3% and a specificity of 95.6% compared to the points with the highest accuracy of CA-125 alone (88.7%) and HE4 alone (88.7%), which yielded sensitivities of 87.2% and 62.1% and specificities of 93.2% and 98.2%, respectively.

Altogether HE4 provides an alternative tool in the follow-up of patients with CA-125-negative tumors (16). The combined use of HE4 and CA-125 could help to identify women at a high risk of having a malignant pelvic mass in cases of a suspicious ultrasound. More accurate prediction of ovarian cancer cases may contribute to more patients being treated by specialized gynecological oncologists.

Acknowledgements

The work presented here is part of the MD thesis of MKF. We are extremely grateful for wonderful technical assistance by C. Kuhn, S. Schulze and S. Kunze. We cordially thank PD Dr. D. Mayr from the Institute of Pathology for generous assistance.

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Received February 13, 2012
Revised April 17, 2012
Accepted April 18, 2012