

Research Article

Serum Human Epididymis Protein 4 and Risk for Ovarian Malignancy Algorithm as New Diagnostic and Prognostic Tools for Epithelial Ovarian Cancer Management

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Abstract

Background: The aim of this work was to analyze the diagnostic and prognostic value of serum human epididymis protein 4 (HE4) and Risk for Ovarian Malignancy Algorithm (ROMA) in epithelial ovarian cancer (EOC).

Methods: Preoperative serum samples of 419 women (140 healthy controls, 131 ovarian benign cysts, 34 endometriosis, and 114 EOC) were tested for CA125 and HE4 using fully automated methods (Abbott ARCHITECT) and validated cutoff values.

Results: For the discrimination of benign masses from EOC, in premenopausal women, the sensitivity and specificity were 92.3% and 59.4% for CA125, 84.6% and 94.2% for HE4, and 84.6% and 81.2% for ROMA, whereas in postmenopausal women, the sensitivity and specificity were 94.3% and 82.3% for CA125, 78.2% and 99.0% for HE4, and 93.1% and 84.4% for ROMA. In patients with EOC, elevated CA125, HE4, and ROMA levels were associated with advanced Federation of Gynaecologists and Obstetricians (FIGO) stage, suboptimally debulking, ascites, positive cytology, lymph node involvement, and advanced age (all $P \leq 0.05$). Elevated HE4 and ROMA (both $P \leq 0.01$), but not CA125 ($P = 0.0579$), were associated with undifferentiated tumors. In multivariable analysis, elevated HE4 and ROMA (all $P \leq 0.05$) were independent prognostic factors for shorter overall, disease-free, and progression-free survival.

Conclusions and Impact: This study underlines the high specificity of HE4 in discriminating endometriosis and ovarian benign cysts from EOC and the high sensitivity of CA125 in detecting EOC. We showed HE4 and ROMA as independent prognostic factors. Multicenter studies are needed to draw firm conclusions about the applicability of HE4 and ROMA in clinical practice. *Cancer Epidemiol Biomarkers Prev*; 20(12); 2496–506. ©2011 AACR.

Introduction

Epithelial ovarian cancer (EOC) is the most frequent cause of death from gynecologic cancer. It has the highest fatality-to-case ratio of all gynecologic malignancies, being characterized by early widespread metastasis and high-grade malignancy at diagnosis. The 5-year survival rate is about 80% to 90% for patients with stage I disease and only 30% for patients with stage III or IV. Although

survival has improved with the use of maximal cytoreductive surgery along with platinum- and taxol-based chemotherapy, nearly 80% of ovarian cancers relapse and patients inevitably succumb to the development of chemotherapy-resistant disease (1).

At the moment, serum CA125 is the commonly used biomarker for EOC diagnosis. Jacobs and colleagues (2) developed the widely used Risk of Malignancy Index (RMI), an algorithm that uses ultrasound findings, architectural features of pelvic mass, CA125 levels, and menopausal status to stratify patients into high- and low-risk groups. However, as CA125 is associated with a high false-positive rate among benign gynecologic conditions, such as endometriosis that affects mainly women in premenopause, its use for EOC detection is almost exclusively reserved for postmenopausal cases (3–6). Furthermore, CA125 has low sensitivity in identifying patients with early EOC disease, being increased in only 50% of patients with stage I (7). CA125 is also used to monitor response to therapy and in early detection of ovarian cancer recurrence after treatment (8–11), but the value of preoperative

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CA125 is not associated with prognosis of patients with EOC (12, 13).

Clinicopathologic features known to be prognostic variables for EOC are age, surgical stage [International Federation of Gynaecologists and Obstetricians (FIGO) stage], histologic subtype, differentiation grade, ascites, lymph node involvement, and residual tumor after cytoreductive surgery. According to the 3-yearly analysis of the FIGO Annual Report on the Results of Treatment in Gynaecological Cancer, stage, grade, and residual tumor have the greatest prognostic value (14). However, these factors provide an insufficient picture of EOC biology and they are frequently interrelated.

Therefore, there is a pressing need to develop new methods for EOC diagnosis and prognosis. First, the preoperative diagnosis of EOC would refer patients to centers specialized in optimal tumor debulking and complete surgical staging, as it has been shown that optimal surgery treatment improves overall survival (OS) in patients with EOC (15–20). Moreover, the prediction of disease outcome in patients with EOC could be useful for developing individually tailored and possibly more effective post-surgical treatments.

Serum analysis is a low-cost, noninvasive technique and it is not subjected to operator variability, such as imaging analysis. Therefore, considerable efforts are underway to identify new serum biomarkers that alone or in combination with CA125 could improve EOC diagnosis (7, 21–36).

In the majority of studies, HE4 has emerged as one of the most promising new serum biomarker in EOC diagnosis. Previous reports evaluated the clinical utility of the HE4 and CA125 combination (ROMA algorithm) to assess the risk of EOC pathology in patients presenting with a pelvic mass (32, 37–42). However, some recent articles (39, 41, 42) showed that diagnostic accuracy of ROMA compared with CA125 and HE4 alone is still controversial. At present, the prognostic value of HE4 has been investigated by only one study (43) in patients with advanced EOC. This report showed HE4 as an independent prognostic factor for progression-free survival (PFS). However, in such study, the prognostic analysis was conducted in a small sample size, the comparison with prognostic value of CA125 was not evaluated and patients with EOC were not dichotomized by median value of biomarker, as usually conducted in prognostic studies.

The aim of this work was to analyze diagnostic and prognostic value of serum HE4, CA125, and ROMA in a large number of patients, using fully automated methods for biomarker determination that guarantee a higher reproducibility and robustness of assay results (41).

Initially, we analyzed the diagnostic performance of HE4 compared with CA125 in discriminating among subjects with EOC, ovarian benign cysts, endometriosis, and healthy controls. Then, we analyzed ROMA algorithm, compared with CA125 and HE4 alone, for the differential diagnosis between benign pelvic mass and EOC.

Finally, we investigated the role of HE4 and ROMA, in comparison with CA125 and established prognostic factors, in predicting OS, disease-free survival (DFS), and PFS in patients with EOC.

Materials and Methods

Patients' characteristics

A total of 419 patients referred to the Gynaecologic Oncology Department of the University of Brescia from 2003 to 2010 were included in the study. All patients signed an informed consent approved by the Institutional Review Board. Patients with a past or concomitant history of malignancy were excluded from the study. Cohorts of patients in pre- and postmenopause were balanced as regards the number and age. Premenopausal women included 39 healthy controls (age: mean, 39 years; range, 21–53), 34 patients with endometriosis (age: mean, 36.5 years; range, 25–51), 35 patients with ovarian benign cysts (age: mean, 41.5 years; range, 18–59), and 26 patients with EOC (age: mean, 44.7 years; range, 33–54). Postmenopausal women included 101 healthy controls (age: mean, 63.3 years; range, 40–76), 96 patients with ovarian benign cysts (age: mean, 64.0 years; range, 46–89), and 87 patients with EOC (age: mean, 66.3 years; range, 46–87). One patient with EOC had an unknown menopausal status and thus was included only in prognostic analysis. Women were considered in postmenopause if they reported no menstrual periods within the 12 months before blood collection.

EOC patients' charts were reviewed to obtain all clinical and pathologic features at the moment of the diagnosis treatment and during follow-up. Low malignant potential tumors were excluded from this study. Standard treatment for EOC consisted in complete pelvic surgery with cytoreductive surgery in advanced stages and platinum-based chemotherapy. Cytoreductive surgery included total abdominal hysterectomy, bilateral salpingo-oophorectomy, omentectomy, and pelvic and paraortic lymph node sampling, with cytologic evaluation of ascites or peritoneal washing. The staging procedure was conducted according to the FIGO system standards. Histologic subtype and differentiation grade were assigned according to World Health Organization criteria.

A group of 98 patients with EOC was evaluated for survival analysis. The remaining 16 patients with EOC were excluded from survival analysis because 2 refused chemotherapy, 4 were not eligible for primary surgery because of their poor medical conditions, and 10 had incomplete follow-up.

Patients were followed up from the date of surgery until death or November 30, 2010 (median follow-up, 19.5 months; range, 1–85 months).

HE4 and CA125 immunoassays

Blood was drawn before any surgical or chemotherapeutic treatment and centrifuged within half an hour for serum collection. Serum samples were stored at -80°C

until analysis. Levels of CA125 and HE4 were measured by chemiluminescent microparticle immunoassays (CMIA) on the fully automated ARCHITECT instrument (Abbott Diagnostics Division) at the III Laboratory Service, Spedali Civili di Brescia, Brescia, Italy. According to the indications of the HE4 manufacturer, the normal ranges were ≤ 70 pmol/L for premenopausal state and ≤ 140 pmol/L in menopausal state.

ROMA algorithm

ROMA uses the results for HE4 and CA125 to generate a predictive index (PI) for EOC (32), calculated by the following formulas:

For premenopausal women:

$$PI = -12.0 + 2.38 \times \ln(\text{HE4}) + 0.0626 \times \ln(\text{CA125})$$

For postmenopausal women:

$$PI = -8.09 + 1.04 \times \ln(\text{HE4}) + 0.732 \times \ln(\text{CA125})$$

Then, ROMA value is calculated as follows: ROMA value (%) = $\exp(\text{PI}) / [1 + \exp(\text{PI})] \times 100$.

According to the indications of the HE4 manufacturer, indexes of at least 7.4% and 25.3% indicate a high risk for the presence of EOC in pre- or postmenopause, respectively.

Statistical analysis

The Wilcoxon–Mann–Whitney test and the Kruskal–Wallis test were used to compare biomarkers distributions across 2 and more than 2 subgroups of patients, respectively.

Differences between the proportions of patients with level of biomarkers above thresholds have been compared within the same subgroup of patients with McNemar test.

Area under the receiver operating characteristic (ROC) curves were used to quantify each biomarker's ability to discriminate between diagnostic groups. Areas under the ROC curves (AUC) were compared with the method described by DeLong and colleagues (44).

For survival analysis, 3 endpoints (cancer relapse, cancer progression, and cancer-related death) were used to calculate DFS, PFS, and OS, respectively. DFS was defined as the time interval between the date of surgery and the date of identification of disease recurrence; PFS was

defined as the time interval between the date of surgery and the date of identification of progressive disease (disease not treatable with curative intent); and OS was defined as the time interval between the date of surgery and the date of death. For all 3 endpoints, the last date of follow-up was used for censored subjects.

Survival curves were calculated using Kaplan–Meier method, and differences in survival between subgroups of patients were tested using the log-rank test.

The effect of HE4, CA125, and ROMA serum levels on prognosis was evaluated categorizing them on the basis of the median values, computed on the whole cohort (Low; High).

Univariate Cox proportional hazard models were fitted to evaluate the role of CA125, HE4, and ROMA and established prognostic factors on the considered outcomes. Multivariable Cox regression models were used to estimate the effect of biomarkers adjusted for FIGO stage, residual tumor, and histologic subtype, the most important established prognostic factors.

All *P* values were 2-sided. A *P* value less than 0.05 was considered to indicate statistical significance. All the analyses were conducted using STATA 11.0 software (Stata Corporation).

Results

CA125, HE4, and ROMA diagnostic performances

Comparison of CA125 and HE4 levels between pre- and postmenopausal healthy controls showed that CA125 is significantly higher in premenopausal than in postmenopausal status (14.9 vs. 10.1 U/mL, *P* = 0.0001), whereas HE4 is inversely significantly higher in postmenopausal than in premenopausal status (41.2 vs. 35.2 pmol/L, *P* = 0.001). For this reason, CA125 and HE4 diagnostic performances were analyzed separately in pre- and postmenopausal women. CA125 and HE4 values detected in healthy controls and in patients with endometriosis, ovarian benign cysts, and EOCs are represented in Fig. 1.

The levels of HE4 and CA125 were significantly higher in patients with EOC than in healthy controls, endometriosis, and ovarian benign cysts, independently from

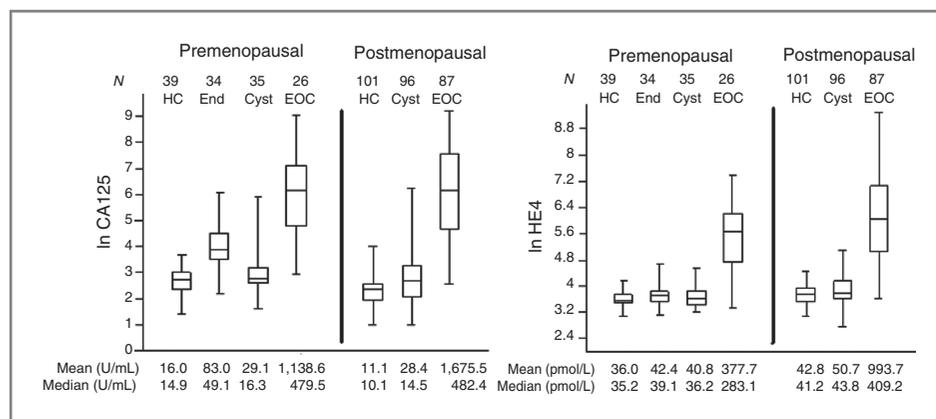


Figure 1. Serum CA125 and HE4 levels detected in healthy controls (HC) and in patients with endometriosis (end), ovarian cysts (cyst), and EOC.

menopausal status (all $P < 0.0001$). Both CA125 and HE4 levels were slightly higher in patients with ovarian cysts than in healthy controls, but these differences reached the statistical significance only in postmenopausal women (14.5 vs. 10.1 U/mL, $P < 0.0001$ for CA125; 43.8 vs. 41.2 pmol/L, $P = 0.0381$ for HE4) and not in premenopausal ones ($P = 0.1561$ for CA125; $P = 0.2718$ for HE4). In premenopausal women, HE4 and CA125 showed different ability in discriminating endometriosis from healthy controls and ovarian benign cysts. CA125 was significantly higher in patients with endometriosis (49.1 U/mL) than in healthy controls (14.9 U/mL) and ovarian benign cysts (16.3 U/mL; both $P < 0.0001$). On the contrary, HE4 showed a marginally significant increase in endometriosis (39.1 pmol/L) toward healthy controls (35.2 pmol/L; $P = 0.0447$) and a not statistically significant increase in endometriosis toward ovarian benign cysts (36.2 pmol/L; $P = 0.5015$).

To evaluate the differences in diagnostic abilities between CA125 and HE4, we used the reference value indicated by standard clinical use for CA125 (35 U/mL) or proposed by the manufacturer for HE4. In patients with EOC, CA125 and HE4 levels were above the cutoff point in 82 of 87 (94.3%) and in 68 of 87 (78.1%) postmenopausal patients, respectively, and in 24 of 26 (92.3%) and in 22 of 24 (84.6%) premenopausal patients. The difference between CA125 and HE4 was statistically significant only in postmenopausal women ($P = 0.0002$). In patients with ovarian benign cysts, CA125 and HE4 levels were above the cutoff values in 17 of 96 (17.7%) and in 1 of 96 (1.0%) postmenopausal women, respectively ($P = 0.002$), and in 3 of 35 (8.5%) and 2 of 35 (5.7%) premenopausal women, respectively ($P = 0.6547$). In patients with endometriosis, all in premenopausal status, CA125 and HE4 were above the cutoff point in significantly ($P = 0.0001$) different percentages: 25 of 34 (73.5%) and 2 of 34 (5.8%), respectively. Moreover, at these cutoff points, 2 of 140 (1.4%) healthy controls were identified as positive by CA125, whereas no healthy control (0%) was positive for HE4.

The overall abilities of CA125 and HE4 to discriminate among subjects belonging to the 4 cohorts were also evaluated by ROC curves (Table 1). In postmenopausal status, CA125 ROC-AUC was significantly higher than HE4 ROC-AUC when comparing EOCs with healthy controls; in premenopausal status, CA125-AUCs were significantly higher than HE4-AUCs when comparing endometriosis with ovarian benign cysts or healthy controls. Other differences between CA125-AUCs and HE4-AUCs did not reach statistical significance.

ROMA algorithm was calculated in 278 patients presenting with pelvic mass (endometriosis, ovarian benign cysts, and EOC). Distribution of patients with EOC, endometriosis, and ovarian benign cysts according to their positivity and negativity for CA125, HE4, ROMA and diagnostic performances of the 3 serum markers are reported in Table 2.

Of note, CA125, HE4, and ROMA detected 15 (6 in pre- and 9 in postmenopause), 11 (4 in pre- and 7 postmeno-

pause), and 14 (4 in pre- and 10 postmenopause) of 21 patients with EOC with stage I of disease.

CA125, HE4, ROMA serum levels and clinicopathologic features of patients with EOC

Relationships between CA125, HE4, and ROMA levels and clinicopathologic characteristics are illustrated in Table 3. Elevated CA125, HE4, and ROMA levels were associated with advanced FIGO stage, suboptimally debulked tumor, ascites, positive cytology, lymph node involvement, and advanced age (all $P \leq 0.05$). Elevated HE4 and ROMA levels ($P \leq 0.01$), but not CA125 levels ($P = 0.0579$), were associated with undifferentiated tumors. Finally, CA125, HE4, and ROMA levels were associated with histologic subtypes (all $P \leq 0.0001$). Indeed serous papillary, undifferentiated, and mixed type showed higher levels of CA125, HE4, and ROMA than endometrioid, clear cell, and mucinous subtypes.

CA125, HE4, and ROMA prognostic performances

At the time of the last follow-up, 42 (42.8%) patients were alive without evidence of disease, 11 (11.2%) patients were alive with disease, 33 (33.7%) patients died of disease [median OS, 46 months; 95% confidence interval (CI), 32 to not estimable] and 12 patients were alive with unknown status. The number of events for OS, DFS, and PFS were 33, 47, and 38, respectively.

Survival analyses of OS, DFS, and PFS on the basis of high versus low CA125, HE4, and ROMA levels were significantly different (all $P \leq 0.0001$). The 2-year OS, DFS, and PFS rates for patients with EOC with low CA125 levels were 76.3% (95% CI, 60.3%–86.6%), 65.0% (95% CI, 47.9%–77.7%), and 71.1% (95% CI, 54.6%–82.5%), respectively, and decreased to 63.2% (95% CI, 44.4%–77.2%), 13.9% (95% CI, 4.0%–29.6%), and 44.9% (95% CI, 27.3%–61.1%) for patients with high levels. The 2-year OS, DFS, and PFS rates for patients with EOC with low HE4 levels were 90.1% (95% CI, 75.8%–96.2%), 71.3% (95% CI, 54.0%–83.1%), and 82.4% (95% CI, 66.5%–91.3%), respectively, and decreased to 47.3% (95% CI, 29.6%–63.2%), 8.9% (95% CI, 1.8%–23.5%), and 32.6% (95% CI, 1.7%–49.1%) for patients with high levels. The 2-year OS, DFS, and PFS rates for patients with EOC with low ROMA levels were 85.9% (95% CI, 71.3%–93.5%), 69.2% (95% CI, 51.9%–81.3%), and 77.9% (95% CI, 61.6%–87.9%), respectively, and decreased to 50.4% (95% CI, 32.0%–66.2%), 8.4% (95% CI, 1.6%–22.6%), and 35.7% (95% CI, 19.4%–52.4%) for patients with high levels. The Kaplan–Meier curves for OS on the basis of high versus low CA125, HE4, and ROMA levels are shown in Fig. 2.

Univariate and multivariable analyses for survival were reported in Table 4. We could not analyze the prognostic impact of tumor grade, due to extremely unbalanced number of events available for the analyses in the 3 subgroups (G1, G2, and G3). As expected, clinicopathologic features known to be prognostic variables for EOC such as FIGO stage, residual tumor after cytoreductive

Table 1. Comparisons of the ROC-AUCs for CA125 and HE4 across the groups enrolled in this study

	CA125 ROC-AUC (95% CI)	HE4 ROC-AUC (95% CI)	P
Postmenopause			
EOC vs. HC	0.9940 (0.9866–1.0000)	0.9576 (0.9279–0.9873)	0.0147
EOC vs. cysts	0.9602 (0.9353–0.9852)	0.9400 (0.9039–0.97599)	0.2439
Cysts vs. HC	0.6671 (0.5898–0.7444)	0.5856 (0.5055–0.6656)	0.1420
Premenopause			
EOC vs. HC	0.9773 (0.9457–1.0000)	0.9177 (0.8279–1.0000)	0.1233
EOC vs. cysts	0.9549 (0.9053–1.0000)	0.9016 (0.8068–0.9965)	0.1867
EOC vs. end	0.8473 (0.7397–0.9549)	0.8925 (0.7907–0.9944)	0.3211
Cysts vs. HC	0.5960 (0.4644–0.7275)	0.5744 (0.4390–0.7097)	0.8280
End vs. HC	0.8982 (0.8183–0.9781)	0.6369 (0.5055–0.7682)	0.0011
End vs. cysts	0.8361 (0.7307–0.9416)	0.5471 (0.4075–0.6867)	0.0019

NOTE: "Cysts" refers to patients with ovarian benign cysts and "end" refers to patients with endometriosis.
Abbreviation: HC, healthy controls.

surgery, histologic subtype, presence of ascites, positive cytology, lymph node involvement, and age showed a statistically significant association with OS, DFS, and PFS in univariate analyses, proving the validity of the patients cohort recruited in this study (all $P \leq 0.02$). In addition,

univariate analyses showed that elevated levels of CA125, HE4, and ROMA were significantly associated with shorter OS, DFS, and PFS (all $P \leq 0.028$).

Multivariable analysis was conducted to estimate the effect of biomarkers adjusted for FIGO stage, residual

Table 2. Distribution of patients with EOC, endometriosis, and ovarian benign cysts according to their positivity for CA125, HE4, and ROMA and diagnostic performances

	Premenopause		Postmenopause	
	EOC (N = 26)	Cysts and endometriosis (N = 69)	EOC (N = 87)	Cysts (N = 96)
CA125^a				
Positive	24	28	82	17
Negative	2	41	5	79
Accuracy (95% CI)	68.4 (58.1–77.6)		88.0 (82.4–92.3)	
Sensitivity (95% CI)	92.3 (74.9–99.1)		94.3 (87.1–98.1)	
Specificity (95% CI)	59.4 (46.9–71.1)		82.3 (73.2–89.3)	
HE4^b				
Positive	22	4	68	1
Negative	4	65	19	95
Accuracy (95% CI)	91.6 (84.1–96.3)		89.1 (83.6–93.2)	
Sensitivity (95% CI)	84.6 (65.1–95.6)		78.2 (68.0–86.3)	
Specificity (95% CI)	94.2 (85.8–98.4)		99.0 (94.3–100.0)	
ROMA^c				
Positive	22	4	81	15
Negative	13	56	6	81
Accuracy (95% CI)	82.1 (72.9–89.2)		88.5 (83.0–92.8)	
Sensitivity (95% CI)	84.6 (65.1–95.6)		93.1 (85.6–97.4)	
Specificity (95% CI)	81.2 (69.9–89.6)		84.4 (75.5–91.0)	

NOTE: "Positive" indicates values above the cutoff and "negative" indicates values below the cutoff.

^aFor CA125, cutoff value was 35 U/mL.

^bFor HE4, cutoff values were ≤ 70 pmol/L for premenopausal state and ≤ 140 pmol/L in menopausal state.

^cFor ROMA, cutoff values were $\leq 7.4\%$ for premenopausal state and $\leq 25.3\%$ menopausal state.

Table 3. Association between serum CA125, HE4, and ROMA levels and clinicopathologic characteristics of patients with EOC

	N	CA125 median	P	HE4 median	P	ROMA median	P
Stage							
Early stages (I–II)	33	88.3	<0.0001	116.5	<0.0001	47.6	<0.0001
Late stages (III–IV)	80	658.3		482.1		97.0	
Unknown	1						
Grade							
G1 + G2	20	133.9	0.0579	101.7	0.0019	47.0	0.0101
G3	83	542.9		438.0		94.6	
Unknown	11						
Residual tumor, cm							
0	46	129.7	<0.0001	167.1	<0.0001	73.5	<0.0001
>0	44	1,040.3		629.9		97.2	
Unknown	24						
Histologic subtype							
Serous papillary	51	769.7	<0.0001	488.1	<0.0001	97.4	<0.0001
Endometrioid	17	122.6		168.2		73.5	
Clear cell	10	228.6		200.2		80.0	
Mucinous	8	73.1		66.3		20.6	
Undifferentiated	8	1,553.2		476.25		95.6	
Mixed	15	746.7		491.50		90.2	
Unknown	5						
Presence of ascites							
No	44	112.9	<0.0001	154.2	<0.0001	64.2	<0.0001
Yes	58	1,110.8		760.8		98.3	
Unknown	12						
Cytology							
Negative	19	141.7	0.0001	168.2	0.0042	73.5	0.0005
Positive	63	961.2		488.1		97.0	
Unknown	32						
Lymph nodal involvement							
Negative	35	171.0	0.0009	169.4	0.0040	73.5	0.0004
Positive	34	764.1		460.6		96.4	
Unknown	45						
Age, y							
<50	27	171.5	0.0311	169.3	0.0022	74.2	0.002
≥50	87	563.8		438.0		95.3	

tumor, and histologic subtype, the most important established prognostic factors. Interestingly, among the 3 biomarkers analyzed, we found that HE4 and ROMA (all $P \leq 0.039$), but not CA125 (all $P \geq 0.082$), are independent prognostic factors for OS, DFS, and PFS. Among the established prognostic factors, only residual tumor has been found as an independent prognostic factor for DFS (HR = 3.05; 95% CI, 1.29–7.21; $P = 0.011$) and for PFS (HR = 3.00; 95% CI, 1.15–7.83; $P = 0.025$).

Discussion

EOC is often detected at an advanced stage and is characterized by poor survival. As a result, it is the most frequent cause of death from gynecologic cancer. At the

moment, transvaginal ultrasonography and CA125 assay are mainly used for EOC diagnosis. Two longitudinal randomized screening trials, PLCO and UKCTOCS, are ongoing to determine their potential role in early detection of EOC. However, transvaginal ultrasonography requires specialized knowledge to reduce interoperator interpretation variability and, like CA125, it is only partially effective in discriminating benign and malignant lesions. Therefore, there is a pressing need for clinical practice to develop new, easy, low-cost, and standardized serum analysis methods for an accurate differential diagnosis between benign and malignant lesions. In fact, it has been shown that discrimination between malignant and benign pelvic masses improves care and outcome of patients with EOC (15–20). Moore and colleagues (32)

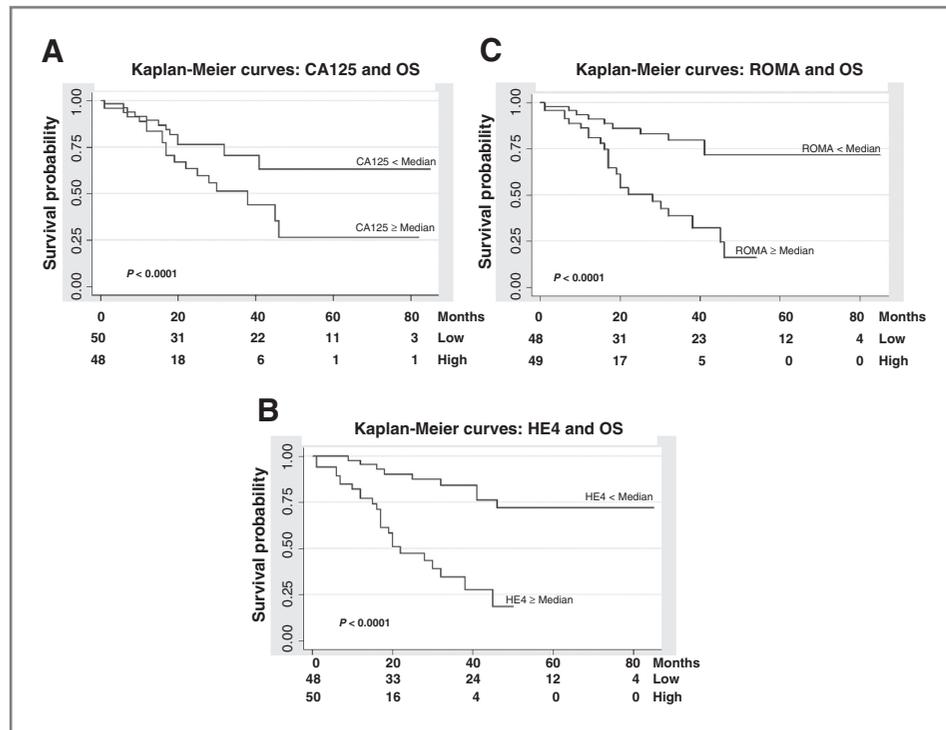


Figure 2. Kaplan–Meier survival curves in relation to serum CA125, HE4, and ROMA levels for patients with EOC. Serum CA125 levels and OS (A), serum HE4 levels and OS (B), and ROMA levels and OS (C).

proposed ROMA algorithm for the differential diagnosis between benign and malignant lesions, and Nolen and colleagues (33), among 65 biomarkers tested, reaffirmed the superiority of the CA125/HE4 combination. Moreover, Moore and colleagues (37) showed that ROMA algorithm has better diagnostic performance than the widely used RMI. However, diagnostic accuracy of ROMA compared with CA125 and HE4 alone is still controversial. This is mainly due to the fact that different assays for CA125 and HE4, different thresholds for HE4 and ROMA, and different patient selection criteria were used in each study to compare diagnostic performances of HE4, CA125, and ROMA (32, 37–42). In the present study, to report consistent results about HE4, CA125, and ROMA diagnostic performances, we enrolled a large number (419) of women, selecting only invasive EOC as malignant ovarian pathology. Moreover, we analyzed HE4 and CA125 using fully automated methods and using biomarkers' thresholds largely validated by manufactures or by clinical practice. With these thresholds, CA125 classified correctly 98.6% of healthy controls, whereas HE4 classified correctly 100% of healthy controls; consequently, these thresholds seemed adequate for our study.

Our analysis conducted on healthy controls revealed that HE4 and CA125 are differentially expressed in pre- and postmenopause, in agreement with the literature (3, 45). As the expression of the markers and the risk of developing benign and malignant pathologies correlated with the menopausal status, we analyzed separately pre- and postmenopausal women.

The biologic function of HE4 has not been identified yet. HE4 is a member of the whey acidic protein (WAP)

4-disulfide core gene cluster that harbors 15 small serine protease inhibitor genes. Comparative genomic hybridization studies showed that WAP gene cluster is among the most frequent amplified chromosomal regions in EOC (46–49), suggesting the potential presence of oncogenes in this region.

This study, according to previous reports (5, 6, 39, 50), proved the high specificity of HE4 in differential diagnosis between lesions of malignant and benign nature, as it is less frequently increased in patients with benign cysts or endometriosis. In our experience, 17.7% of postmenopausal patients with ovarian benign cysts and 73.5% of patients with endometriosis exceeded the threshold value for CA125, whereas only 1.0% of the same postmenopausal patients with ovarian benign cysts and 5.8% of patients with endometriosis exceeded the threshold value for HE4 (CA125 vs. HE4: $P \leq 0.002$). According to the fact that CA125 is frequently increased in patients with endometriosis, when we compare endometriosis with healthy controls, we observed that CA125 ROC-AUC was significantly higher than HE4-AUC (0.8982 vs. 0.6369, $P = 0.0011$).

There are conflicting results about CA125 sensitivity. Prior studies (23, 50, 51) showed that HE4 is more sensitive than CA125 in detecting EOC, being elevated in patients with EOC who do not express CA125. However, the current study, in agreement with other observations (38, 52, 53) showed that CA125 has greater sensitivity than HE4 in detecting EOC. Indeed, in postmenopausal EOC, CA125 exceeded the threshold value in 94.2% of patients, whereas HE4 exceeded the threshold value in only 78.1% of patients (94.2% vs. 78.1%, $P = 0.0002$).

Table 4. Univariate and multivariable survival analyses in relation to serum CA125, HE4, ROMA levels and clinicopathologic prognostic variables for patients with EOC

	OS			DFS			PFS		
	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
Univariate analyses									
CA125	2.20	1.09–4.45	0.028	3.57	1.87–6.81	<0.001	2.43	1.24–4.76	0.010
≥Median vs. <median									
HE4	5.69	2.55–12.67	<0.001	4.73	2.40–9.33	<0.001	5.80	2.66–12.64	<0.001
≥Median vs. <median									
ROMA	4.21	1.96–9.04	<0.001	4.76	2.43–9.31	<0.001	4.58	2.16–9.68	<0.001
≥Median vs. <median									
FIGO stage	5.28	1.81–15.36	0.002	6.66	2.53–17.5	<0.001	6.49	2.18–19.35	0.001
Late vs. early									
Residual tumor	4.13	1.84–9.25	0.001	5.97	2.92–12.21	<0.001	5.27	2.41–11.5	<0.001
≥0 vs. 0									
Histologic type	2.43	1.15–5.12	0.020	2.57	1.40–4.72	0.002	2.50	1.25–5.01	0.009
Serous vs. nonserous									
Presence of ascites	3.99	1.77–8.97	0.001	5.03	2.48–10.20	<0.001	4.70	2.11–10.45	<0.001
Yes vs. no									
Cytology	8.48	1.97–36.47	0.004	7.52	2.28–24.77	0.001	8.87	2.07–38.02	0.003
Positive vs. negative									
Lymph nodal involvement	10.33	2.22–47.89	0.003	5.67	2.2–14.58	<0.001	6.96	2.19–22.15	0.001
Yes vs. no									
Age	1.05	1.02–1.08	0.001	1.03	1.00–1.05	0.016	1.04	1.01–1.06	0.006
≥50 vs. <50									
Multivariable analysis (adjusted for FIGO stage, residual tumor, and histologic type)									
CA125	1.40	0.61–3.21	0.424	1.88	0.92–3.85	0.085	1.35	0.64–2.88	0.433
≥Median vs. <median									
HE4	3.98	1.35–11.75	0.012	2.46	1.09–5.56	0.030	2.77	1.12–6.85	0.028
≥Median vs. <median									
ROMA	3.22	1.21–8.57	0.019	2.67	1.25–5.72	0.011	2.76	1.15–6.59	0.022
≥Median vs. <median									

Notably, we did not find any patient with EOC positive for HE4 and negative for CA125, whereas 16 (including 5 stage I) patients with EOC were positive only for CA125. As CA125 was more sensitive than HE4 in detecting patients with EOC, when we compare EOCs with healthy controls by ROC curves, CA125-AUC was significantly higher than HE4-AUC (0.9940 vs. 0.9576, $P = 0.0147$).

Among premenopausal women, we observed that CA125 is more sensitive in detecting EOCs, whereas HE4 is more specific in discriminating ovarian cysts from EOCs. However, this analysis did not reach statistical significance, possibly because of the small sample size.

Concerning ROMA, some previous reports showed that CA125/HE4 combination yielded a higher diagnostic accuracy than single markers (23, 32), whereas other articles did not reach the same conclusions (38, 39). In our experience, ROMA inherits the strengths and the weakness of CA125 and HE4 alone. Indeed, ROMA is more sensitive than HE4 but less sensitive than CA125 and it is more specific than CA125 but less specific than

HE4. Unfortunately, because of the heterogeneity of previous studies, it is not possible to pool data and to conduct a meta-analysis to obtain univocal indications about the clinical application of this algorithm in EOC diagnosis.

Ovarian cancer management could also be improved through the development of new methods for EOC prognosis. Therefore, the need for additional prognostic data to calibrate therapeutic tools on an individual basis in women with EOC seems obvious. To find out how CA125, HE4, and ROMA influence EOC biology, we analyzed the association between the biomarkers and the clinicopathologic characteristics of the patients. Remarkably, elevated HE4 and ROMA levels (both $P \leq 0.01$), but not CA125 levels ($P = 0.0579$), were associated with undifferentiated tumors, suggesting that they are correlated with cancer aggressiveness. Moreover, as elevated levels of CA125, HE4, and ROMA were found in patients suboptimally debulked, we could suppose that these biomarkers, associated with others parameters (such as imaging analysis), would be useful in preoperative assessment of residual

disease and, eventually, in the evaluation of a neoadjuvant treatment. Univariate analyses showed that CA125, HE4, and ROMA, as well as established clinicopathologic prognostic variables for EOC, were significantly associated with shorter OS, DFS, and PFS (all $P \leq 0.05$). However, when multivariable analysis was conducted, we found that only HE4 and ROMA were independent prognostic factors for OS, DFS, and PFS. Moreover, multivariable analysis proved that HE4 and ROMA appear to be better prognostic factors than FIGO stage, residual tumor, and histologic subtype for OS, DFS, and PFS. These data suggest that HE4 and ROMA could reflect intrinsic tumor aggressiveness that established prognostic factors were not able to identify in our cohort of EOCs. Thus, elevated HE4 and ROMA levels, in association with the traditional prognostic factors, could be clinically useful in identifying patients with high-risk EOC for a more aggressive tailored therapy, consisting in consolidation of treatment with chemotherapeutic drugs or novel biologic agents. To date, we are the first group that showed HE4 and ROMA as independent prognostic factors for OS, DFS, and PFS in EOC. Differently, the Peak study showed that HE4 is an independent prognostic factor only for PFS and only in patients with advanced (IIIc–IV) stage EOC. This could be explained by the fact that this latter analysis has been carried out in a small group of women, and the effect of HE4 serum levels on prognosis was evaluated by not categorizing HE4 on the basis of the median values but on the basis of cutoff value proposed by Moore and colleagues (23) for diagnostic purpose.

Recently, our group has reported the prognostic value of serum HE4 levels in patients with poorly differentiated endometrial carcinoma. Higher serum HE4 levels were correlated with a more aggressive tumor phenotype and worse outcome (54) and similar results have been shown for breast and lung cancer (55, 56). Moreover, it has already been shown that Leukocyte Protease 1 (SLPI), one of the best-studied member of the WAP protein family, contrary to what is expected for a protease inhibitor, promotes malignant potential of cancer cells (57). On the basis of the similarity of HE4 and SLPI,

it is tempting to speculate that HE4, like SLPI, could directly enhance malignant potential of tumor.

In conclusion, this study confirms the diagnostic role of HE4 and ROMA already suggested by other reports, but it adds a clinically relevant information, as for the first time, we showed that high levels of HE4 and ROMA are promising prognostic factors in EOC, identifying a subgroup of patients with poor survival and at higher risk of death and, subsequently, directing to a more aggressive adjuvant therapy. Further multicenter studies with homogeneous laboratory procedures for HE4 and CA125 assays, as well as patients' selection criteria, are needed to draw firm conclusions about the applicability of HE4 and ROMA in clinical practice.

Disclosure of Potential Conflicts of Interest

C. Galli is currently employed by Abbott Diagnostics as the Scientific Affairs Manager. No potential conflicts of interest were disclosed by other authors.

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