A Biomarker Panel Increases the Diagnostic Performance for Epithelial Ovarian Cancer Type I and II in Young Women

PIA LEANDERSSON[1], GRIGORIOS KALAPOTHARAKOS[1], EMIR HENIC[2], HENRIK BORGFELDT[3], MAX PETZOLD[4], GUNILLA HØYER-HANSEN[5] and CHRISTER BORGFELDT[1]

[1]*Department of Obstetrics & Gynecology, Skåne University Hospital, Lund University, Lund, Sweden;*
[2]*Department of Reproductive Medicine, Skåne University Hospital, Lund University, Lund, Sweden;*
[3]*Faculty of Engineering, LTH, Lund University, Lund, Sweden;*
[4]*Health Metrics, Sahlgrenska Academy at University of Gothenburg, Gothenburg, Sweden;*
[5]*Finsen Laboratory, Rigshospitalet, Copenhagen, Denmark*

Abstract. *Background/Aim: To assess preoperative blood levels of a biomarker panel in relation to the new classification system of epithelial ovarian cancer (EOC) type I and II. Patients and Methods: Preoperative plasma levels of B7-family protein homolog 4 (B7-H4), intact and cleaved soluble urokinase plasminogen activator receptor (suPAR), human epididymis protein 4 (HE4) and cancer antigen 125 (CA125) were analyzed in 350 patients with adnexal lesions. Results: The levels of suPAR(II-III), HE4, CA125 were all higher in EOC II than in EOC I, borderline and benign ovarian tumors. B7-H4 was increased in EOC II compared with benign ovarian tumors. The combination of suPAR(II-III), HE4, CA125 and age in premenopausal women discriminates EOC and borderline tumors from benign tumors to higher accuracy compared to the Risk of Ovarian Malignancy Algorithm (p=0.007). Conclusion: The biomarker panel suPAR(II-III), HE4, CA125 and age in premenopausal women improved discrimination of malignant and benign ovarian tumors. The plasma levels of B7-H4 were increased in patients with EOC II compared to those with benign ovarian tumors.*

Predicting whether pelvic masses are benign or malignant has become more important as benign tumors now undergo laparoscopic surgery or conservative management. The suspected malignant tumors are referred to gynecological oncology centers for further evaluation and extensive ovarian cancer surgery, if needed to achieve macroscopic radical tumor reduction, improving survival. Subjective ultrasound evaluation of gray-scale and color Doppler images of pelvic masses, with pattern recognition, in the hands of expert ultrasonographers have shown good prediction of discrimination between benign and malignant adnexal masses (1-3), but the general gynecologist or sonographer does not have that capability, not even after teaching sessions using the International Ovarian Tumour Analysis (IOTA) group scoring system (4) or IOTA simple rules (5).

In suspicion of malignancy, the most commonly used tumor marker, cancer antigen 125 (CA125), is not reliable due to low sensitivity in patients with early-stage ovarian cancer (6). CA125 also has a low specificity since it is often found increased in patients with benign endometriosis. The biomarker human epididymis protein 4 (HE4), alone and in combination with CA125 in the Risk of Ovarian Malignancy Algorithm (ROMA) algorithm, increases sensitivity in the distinction of ovarian carcinoma from benign disease. HE4 is approved by the United States Food and Drug Administration for monitoring recurrence or progressive disease in patients with epithelial ovarian cancer (EOC). We have shown that HE4 is an independent preoperative marker of poor prognosis in serous ovarian cancer (7). Although its physiological functions have not been fully identified, overexpression of the HE4 protein has been found mainly in serous and endometroid ovarian carcinomas (8).

On cancer cell invasion, inflammation is induced and the urokinase plasminogen activator (uPA) system is involved in tissue remodeling. Our group has shown that the combination of plasma soluble urokinase plasminogen activator receptor (suPAR(I-III)) plus suPAR(II-III) and CA125 discriminates between malignant and benign tumors (9). The B7 protein

Correspondence to: Christer Borgfeldt MD, Ph.D., Associate Professor, Department of Obstetrics and Gynecology, Skåne University Hospital, Lund University, SE-22185 Lund, Sweden. Tel: +46 46171000, Fax: +46 46157868, Mobile: +46 709950472, e-mail: christer.borgfeldt@med.lu.se

Key Words: CA125, uPAR, HE4, B7-H4, ROMA, ovarian neoplasm, survival analyses.

family stimulates and inhibits regulation of T-cell responses. B7-family protein homolog 4 (B7-H4) is up-regulated on the surface of cancer cells and immunosuppressive tumor-associated macrophages in several types of human cancer (10). B7-H4 protein has been detected in half of early-stage and two-thirds of late-stage ovarian tumor samples, but not in normal ovarian tissue.

A novel ovarian tumor type and grading system has been proposed based on morphological and molecular genetics, which divides EOC into type I and type II tumors (11, 12). Low-grade serous, and endometrial carcinoma, clear cell cancer and mucinous carcinoma are classified as EOC type I (11). Generally, EOC type I carcinomas behave in an indolent manner and are more often confined to the ovary at diagnosis, with a stable genome and without *TP53* mutations. EOC type II tumors are high-grade serous carcinomas which are more aggressive and genetically highly unstable, and the majority of carcinomas have *TP53* mutations, hypermethylation, or dysfunction of breast cancer gene 1/2. The aggressive EOC type II tumors account for 75% of all EOCs and are responsible for 90% of deaths from the disease. It has also been suggested that type II EOCs develop in the fallopian tube at the conjunction to the ovary or the peritoneum (13).

In this article, we have extended our material and follow-up time in order to evaluate the combination of the four biomarkers, in particular analyzing differences in relation to the type I and type II EOC classification system and prediction in overall survival.

Materials and Methods

Patients. Peripheral blood samples were obtained preoperatively from 350 patients admitted for primary surgery of adnexal masses to the Department of Obstetrics and Gynecology in Lund, Sweden, 1993-2010. Blood was collected in citrate tubes, centrifuged, and the plasma stored at −20˚C until analyzed. The laparoscopic or open surgical procedures in benign cases included resection of the cyst or unilateral oophorectomy, and in the malignant cases abdominal hysterectomy, bilateral salpingo-oophorectomy, and infracolic omentectomy and lymphadenectomy in the pelvis and para-aortic area when indicated. Cytological analysis of ascitic fluid or, when absent, of peritoneal fluid washing was performed. All diagnoses were verified by histopathology of the tumors. Histological type and stage of the disease according to the International Federation of Gynaecology and Obstetrics (FIGO) were available in all malignant cases and re-evaluated according to low-grade and high grade serous ovarian cancer (11). Postoperative adjuvant treatment was given according to clinical standards in patients with invasive cancer. Patents with stage Ic or higher received platinum-based chemotherapy, either alone or combined with paclitaxel. Survival status of all patients, *i.e.* alive or dead including date of death was obtained on December 3, 2013 from the Swedish Population Register (Tumor Registry Center in Lund). Histopathological diagnoses, type and stage of the disease (FIGO) were available in all malignant cases, as shown in Tables I and II. In patients with invasive tumors, the median follow-up time after surgery was 42 months (range=0.2-229 months). The median age was 54.5 years for the whole cohort (range=16-90 years, mean±SD=54.9±16 years), and 63.1 years in the sub-group of patients with invasive cancer (range=31-87 years, mean±SD=64.1±11.9 years).

The study was approved by the Review Board at the Faculty of Medicine, University of Lund, Sweden (DNR 558-2004 and DNR 94-2006)

CA125. Preoperative plasma samples were routinely assayed for CA125 using a commercial electrochemoluminescence immunoassay Elecsys CA125 kit™ (Roche Diagnostics Scandinavia AB, Bromma, Sweden). The assay was performed according to the manufacturer's instructions.

HE4. The HE4 assay (Fujirebio Diagnostics, Gothenborg, Sweden) met normal standard laboratory quality criteria. The inter-assay variation was 1.2% and the intra-assay variation of samples measured in duplicate was 3.0%. Clinical and histopathological data were not available to the technicians performing the assays. Plasma and serum were collected simultaneously and the levels of HE4 matched with an acceptable correlation.

Risk of Ovarian Malignancy Algorithm. ROMA is a predicative probability algorithm that classifies women with pelvic mass or ovarian cyst as being at high or low risk for epithelial ovarian cancer (14), This predicative probability algorithm is based on menopausal status and preoperative levels of HE4 and CA125 and the predictive index (PI) is calculated as follows:

For premenopausal patients:
$PI = -12.0 + 2.38 \times \ln(HE4) + 0.0626 \times \ln(CA125)$
For postmenopausal patients:
$PI = -8.09 + 1.04 \times \ln(HE4) + 0.732 \times \ln(CA125)$
Predicted probability=exp(PI)/[1+ exp(PI)].

uPAR. Three uPAR immunoassays, TR-FIA 1, 2 and 3, have been designed for the specific measurement of uPAR(I-III), uPAR(I-III) + uPAR(II-III), and uPAR(I), respectively (15). The detection limits were 0.3 pmol/l of suPAR(I-III) for TR-FIA 1 and 2 and 1.9 pmol/l of uPAR(I) for TR-FIA 3. The assays were previously validated for use in citrate plasma diluted 1:10 (15). Since the amount of uPAR(I) in citrate plasma diluted 1:10 is close to the limit of quantification, we decided only to dilute our samples 1:5 in assay buffer (DELFIA® assay buffer #1244-111, PerkinElmer, MA, USA). The assays were therefore validated for their use in citrate plasma diluted 1:5. The limit of quantification was determined by spiking suPAR-depleted citrate plasma with purified suPAR and examining the coefficient of variation. suPAR depletion of plasma diluted 1:5 as previously described (9). The amount of suPAR(II-III) was obtained by subtracting the moles of suPAR(I-III) measured in TR-FIA 1 from those of suPAR(I-III) and suPAR(II-III) measured in TR-FIA 2.

B7-H4 (OVR110). The B7-H4 ELISA assay (provided by diaDexus to Fujirebio Diagnostics, Goteborg, Sweden) met normal laboratory quality criteria. The intra-assay variation of samples measured in duplicate was 9.1%. The median levels for the healthy donors were in plasma 0.141 ng/ml (n=20) and in serum 0.126 ng/ml (n=99). Clinical and histopathological data were not available to the technicians performing the assays.

Table I. *Frequency of histological types of ovarian tumor.*

Epithelial ovarian tumor	Functional	Endometriosis	Dermoid	Serous	Endometrioid	Mucinous	Clear cell	Total
Benign	16	39	25	91	0	40	0	211
Borderline	0	0	0	17	1	12	0	30
EOC type I	0	0	0	11	5	14	5	35
EOC type II	0	0	0	74	0	0	0	74
Total	16	39	25	193	6	66	5	350

EOC: Epithelial ovarian cancer.

Statistical methods. Differences between groups regarding plasma levels of the biomarkers were evaluated with the Mann–Whitney *U*-test for unpaired samples, ANOVA with Bonferroni as *post hoc* test and trends across ordered groups were analyzed using linear regression with log-transformed values. Spearmans rho was used as a measure of correlation between the parameters. Receiver operator characteristic (ROC) curves were constructed, and the area under the curve (ROC–AUC) with 95% confidence interval was calculated. The diagnostic performance was expressed as sensitivity, specificity and positive (LR+) and negative (LR–) likelihood ratios. For each logistic regression model, a coefficient for each variable included in the model as well as a model constant was determined. Akaike's Information Criterion was used in selecting the best models including interaction coefficients (16). The markers were used as continuous variables in the univariate and multivariate logistic regression models, the binary outcome being benign disease or ovarian cancer including borderline tumors. Cross-validation of the logistic regression models using a 10-fold-split and leave-one-out approach was performed to obtain the average AUC-ROC values for combinations of biomarkers. Cross-validation reduces the upward bias in estimating AUC-ROC values in the logistic regression model on the set of patients from which the model was initially fitted. The method described by DeLong *et al*. (17) and bootstraps (n=2000) were used for the calculation of the difference between two ROC–AUCs. Overall survival probabilities were calculated using the Kaplan–Meier method and the log-rank test. The Cox proportional hazard model was used in univariate and multivariate survival analyses. Point estimates are reported as hazard ratios (HR) and 95% confidence intervals (CI). Assumptions of proportional hazards were verified graphically where applicable. Significant departures from proportionality were not observed either for dichotomized HE4 nor for other covariates used in the Cox regression analyses. All comparisons were two-sided, and a 5% level of significance was used. The statistical analyses were performed using SPSS™ (22.0.0) (Oracle, CA, USA), MedCalc™ (13.1.1.0) (MedCalc Software bvba, Ostend, Belgium) and R open source statistical packages (The R Foundation for Statistical Computing, Vienna, Austria).

Results

The preoperative plasma levels of B7-H4 were higher in patients with EOC II tumors than in those with benign tumors ($p<0.001$) (Figure 1 and Table III) but no significant differences were found between EOC I or borderline tumors compared with benign tumors. The suPAR(II-III) levels were significantly lower in benign tumors compared to borderline, EOC I and EOC II tumors. HE4 and CA125 levels were significantly different in all comparisons between benign, borderline, EOC I and EOC II (Figure 1 and Table III).

Table II. *Frequency of epithelial ovarian cancer type I and type II in relation to stage.*

	Stage, n				
Epithelial ovarian cancer	I	II	III	IV	Total
Type I	17	3	13	2	35
Type II	8	8	52	6	74
Total	25	11	65	8	109

B7-H4 levels increased with stage ($p_{trend}<0.001$) (borderline tumors excluded) and B7-H4 was higher in patients in stage II-IV compared with benign tumors ($p<0.001$) but not significantly higher than stage I ($p=0.07$) (Figure 2).

The correlation coefficients were moderate between B7-H4 and HE4, and CA125, whereas B7-H4 had strong positive correlation with histological type and stage (Table IV). suPAR(II-III) positively correlated strongly with HE4, histologic type and stage. HE4 had strong positive correlation to CA125, histological type and stage. CA125 had strong positive correlation with histological type and stage. All correlation coefficients shown in Table IV were statistically significant.

The ROC-AUC values comparing benign to malignant tumors including borderline tumors are shown in Table V. Since the menopausal status was unknown in some patients, different menopausal cut-off ages were used in repeated analyzes but changing the cut-off for age did not significantly change the ROC-AUC values. The median menopausal age is 51.8 years in Sweden, which was used in the analyses.

The logistic regression models, which included model, intercept terms and coefficients were calculated. Goodness

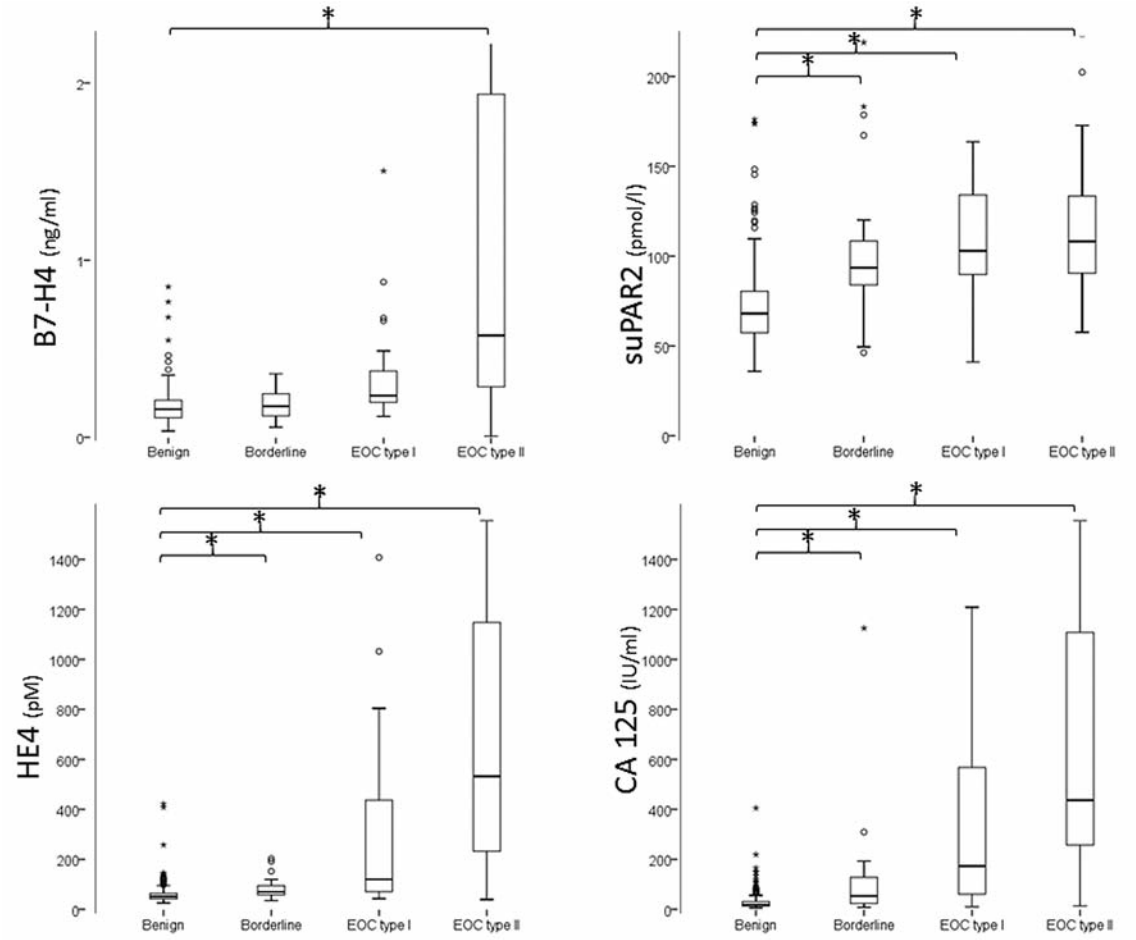

Figure 1. *Peripheral blood concentrations of B7-family protein homolog 4 (B7-H4), soluble urokinase plasminogen activator receptor (suPAR[II-III]), human epididymis protein 4 (HE4) and cancer antigen 125 (CA125) obtained preoperatively in patients with adnexal lesions. The boxes represent the 25th, 50th, 75th percentiles. Bars include highest and lowest values, except outliers (○), which are 1.5- to 3-box lengths from the end of the box, and extremes (*) which are more than 3-box lengths from the end of the box. All values are included in the analyses but some extreme values are excluded from the figures. *Significantly different in ANOVA test with Bonferroni post-hoc test ($p<0.05$).*

Table III. *Mean plasma levels of biomarkers B7-family protein homolog 4 (B7-H4), soluble urokinase plasminogen activator receptor (suPAR[II-III]), human epididymis protein 4 (HE4) and cancer antigen 125 (CA125) in patients with ovarian tumors.*

	Benign		Borderline		EOC type I		EOC type II	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
B7-H4 (ng/ml)	0.17	0.10	0.19	0.09	1.4	6.0	2.4*	7.2
suPAR(II-III) (pmol/l)	73	23	101	39	121	69	123‡	64
HE4 (pM)	61	44	84	43	450	697	1715†	4435
CA 125 (IU/ml)	54	345	117	214	413	593	1340§	2644

In ANOVA with Bonferroni post hoc test:*$p<0.001$ *vs.* benign; ‡$p<0.002$ for all comparisons; †$p<0.020$ for all comparisons; §$p<0.003$ for all comparisons.

of fit assessment was carried out using the Hosmer-Lemeshow test. In premenopausal women, CA125 as single biomarker had the highest individual AUC, which was higher than ROMA (Table V). The model including the three biomarkers, HE4, CA125 and suPAR(II-III), and age was the best model, with AUC=0.96 (95% CI=0.92-0.98). In the cross-validation, 10-fold split of the model including the three biomarkers, HE4, CA125 and suPAR(II-III), and age

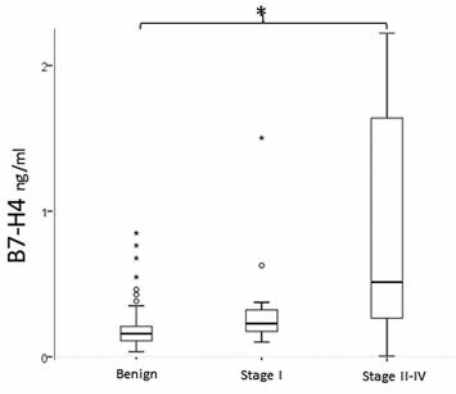

Figure 2. *Peripheral blood concentrations of B7-family protein homolog 4 (B7-H4) obtained preoperatively in patients with adnexal lesions in relation to stage (borderline tumors excluded). The boxes represent the 25th, 50th, 75th percentiles. Bars include highest and lowest values, except outliers (○), which are 1.5- to 3-box lengths from the end of the box, and extremes (*) which are more than 3-box lengths from the end of the box. *Significantly different in ANOVA test with Bonferroni post-hoc test ($p<0.05$).*

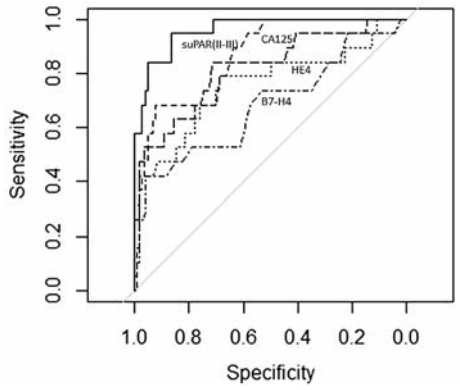

Figure 3. *Area under curves for B7-family protein homolog 4 (B7-H4), soluble urokinase plasminogen activator receptor (suPAR[II-III]), human epididymis protein 4 (HE4) and cancer antigen 125 (CA125), (model including lnHE4 + lnsuPAR(II-III) + lnCA125 + age [solid line]) discriminating malignant, including borderline tumors, from benign ovarian tumors in premenopausal women.*

had an AUC=0.94 (95% CI=0.90-0.98) and in the cross-validation with leave-one-out method AUC=0.93 (95% CI=0.88-0.99). In pairwise comparison, the model including the three biomarkers, HE4, CA125 and suPAR(II-III), and age was significantly better compared with ROMA and CA125 alone (Table V, Figure 3).

The coefficients in the model including suPAR(II-III), HE4, CA125 and age in premenopausal women were:

$$PI=-36.6+3.16\times\ln(HE4) + 7.96\times suPAR(II\text{-}III) - 8.75\times \ln(CA125)+1.64\times age + 2.63\times \ln(HE4) \times \ln(CA125) - 0.37\times \ln(HE4) \times age$$

The diagnostic performance in premenopausal women with the three biomarker panel (suPAR(II-III), HE4, CA125) and age is shown in Table VI. At high sensitivity (99%), the specificity was 71%, and negative likelihood 0.01. At high specificity (95%), the sensitivity was 74%, with positive likelihood 14.7.

In postmenopausal women, the proposed ROMA score gave the highest AUC, which was higher than that for the separate biomarkers B7-H4, suPAR(II-III), HE4 and CA125 (Table V). In postmenopausal women, no model with B7-H4 or any suPAR forms improved the AUC values compared with ROMA.

In the 5-year survival analyses, borderline tumors were excluded and the biomarkers were dichotomized, B7-H4 at 0.40 ng/ml (median in invasive malignant cases), uPAR(I) at 20.2 pmol/l (detection limit), HE4 at 400 pM (median in invasive malignant cases) and CA125 at 400 IU/ml (median in invasive malignant cases) to discriminate between high and low risk for overall survival using univariate Cox regression analysis and Kaplan–Meier curves. In our earlier studies, uPAR(I) had shown higher predictive value for overall survival than suPAR(II-III), which is why uPAR(I) was used. In univariate cox regression analyses, high levels of B7-H4 did not lead any significant differences in the overall survival analyses (Table VII). In univariate cox regression analyses, high levels of uPAR(I) (HR=2.4, 95% CI=1.4-4.1; p=0.001), high levels of HE4 (HR=2.2, 95% CI=1.2-4.1; p=0.008), as well as high levels of CA125 (HR=2.2, 95% CI=1.3-3.8, p=0.005) were associated with shorter survival (Table VII). Patients with high levels of HE4 (>400 pM) or CA125 (>400 IU/ml) had a median survival of 30 months compared with patients meeting neither of these criteria, where the median survival was 42 months (HR=2.0, 95% CI=1.2-3.3; p=0.007).

The short-term survival was analyzed separately at 12 months. The multivariate Cox regression analyses showed that uPAR(I) was the only independent preoperative biomarker indicating poor short time survival (HR=4.62, 95% CI=1.7-12.2; p=0.002) (Figure 4a shows univariate analysis), as well as increased age as a continuous variable (HR=1.06, 95% CI=1.01-1.11; p=0.01). Women above 75 years of age with high uPAR(I) levels had a worse prognosis (HR=8.9, 95% CI=1.7-47; p=0.01) (Figure 4b).

Discussion

We found that a biomarker panel consisting of suPAR(II-III), HE4, CA125 and age in premenopausal women increases the accuracy of discriminating benign from malignant ovarian tumors compared with ROMA. The levels of suPAR(II-III), HE4, CA125 were all higher in patients with EOC type II than in EOC type I, and lower levels were seen in patients

Table IV. *Correlation between biomarkers B7-family protein homolog 4 (B7-H4), soluble urokinase plasminogen activator receptor (suPAR[II-III]), human epididymis protein 4 (HE4), cancer antigen 125 (CA125), histological type (benign, borderline, epithelial ovarian cancer type I and II) and stage (benign=0, stage I, II, III, IV).*

Spearman's rho	suPAR(II-III)	HE4	CA125	Histological type	Stage
B7-H4	0.333	0.484	0.487	0.526	0.514
suPAR(II-III)		0.669	0.475	0.571	0.574
HE4			0.563	0.677	0.682
CA125				0.667	0.679
Histological type					0.974

p-Values for all correlation coefficients were statistically significant ($p<0.05$).

Table V. *Comparison of receiver operating characteristic – area under curves (ROC-AUCs), B7-family protein homolog 4 (B7-H4), soluble urokinase plasminogen activator receptor (suPAR[II-III]), human epididymis protein 4 (HE4) and cancer antigen 125 (CA125) discriminating malignant, including borderline, tumors from benign tumors.*

			Pairwise comparison of ROC–AUC*	
			(DeLong)	(bootstraps 2000)
	AUC	95% CI	*p*-Value	*p*-Value
Pre-menopausal				
B7-H4	0.682	0.532-0.832	0.00009	0.00008
suPAR(II-III)	0.822	0.708-0.936	0.0036	0.0028
HE4	0.761	0.620-0.901	0.0014	0.0012
CA125	0.864	0.783-0.946	0.006	0.006
ROMA premenopausal	0.773	0.633-0.912	0.007	0.005
Model incl lnHE4 + lnsuPAR(II-III) + lnCA125 +age (10-fold split)	0.940	0.902-0.980		
Model incl lnHE4+lnsuPAR(II-III)+lnCA125+age (leave-one-out)	0.933	0.877-0.989		
post-menopausal				
B7-H4	0.795	0.724-0.865		
suPAR(II-III)	0.747	0.670-0.825		
HE4	0.880	0.828-0.932		
CA125	0.889	0.833-0.945		
ROMA postmenopausal	0.914	0.867-0.961		
Model incl lnCA125 + lnHE4	0.910	0.861-0.960		
Model incl lnHE4 + lnCA125 + lnsuPAR(II-III)	0.910	0.861-0.959		
Model incl lnHE4 + lnCA125 + lnB7-H4	0.911	0.862-0.959		
Model incl lnHE4 + lnsuPAR(II-III) + lnCA125 + age	0.912	0.864-0.960		

CI: Confidence interval; ln: natural logarithm. *Model includes lnHE4+lnsuPAR(II-III)+lnCA125+age (leave-one-out) *vs*. other model.

with borderline and benign ovarian tumors. B7-H4 was increased in patients with EOC type II compared with those with benign ovarian tumors but B7-H4 did not improve the biomarker panel differentiating malign from benign ovarian tumors. The combination of high levels of HE4 and CA125 both above their respective cutoffs indicates worse prognosis, and a high uPAR(I) level also indicates worse prognosis, especially in the first year after diagnosis.

The levels of all four biomarkers were higher in patients with EOC type II than in those with benign ovarian tumors. EOC type II is predominately detected when the ovarian cancer is disseminated in the abdominal cavity at advanced stage II-IV. These tumors seem to evolve rapidly and spread to extra-ovarian sites early, without any symptoms or cystic lesion in the ovaries. EOC type II, which consists of high-grade serous ovarian carcinoma, may grow without any sign of cysts, which makes the cancer hard to find with imaging techniques such as ultra-sonography or computed tomographic scan in the early stage. However, biomarker levels have been shown to be increased up to 3 years before diagnosis (18), hence repeated measurement of biomarker panels may be used in a screening setting to detect high-grade serous ovarian cancer.

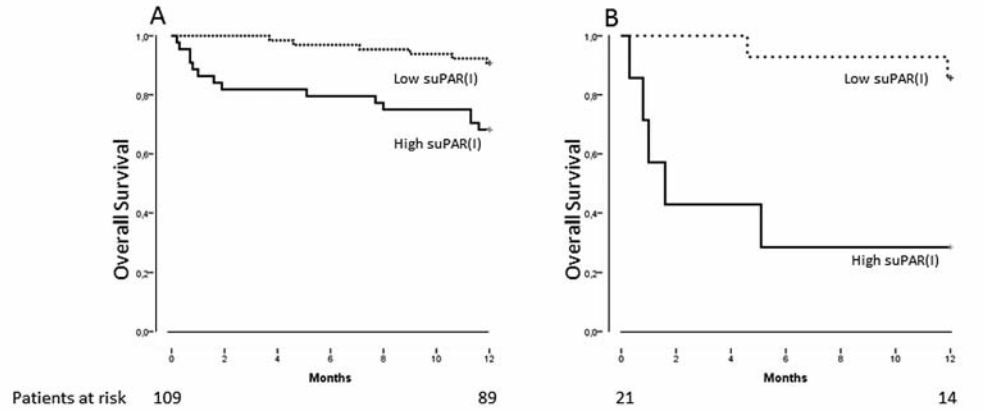

Figure 4. *Kaplan–Meier estimates of 12-month overall survival probabilities using peripheral blood concentrations in patients with ovarian cancer with high soluble urokinase plasminogen activator receptor (suPAR[I]) >20.2 pmol/l vs. low uPAR(I) ≤20.2 pmol/l, dichotomized by the detection limit. A: Whole patient cohort: a high level of suPAR(I) indicated worse short-term survival (hazard ratio=4.01, 95% confidence interval=1.6-10.6; p=0.004). B: Patients aged >75 years: a high level of suPAR(I) and age >75 years indicated a worse short-term survival (hazard ratio=8.9, 95% confidence interval=1.7-47; p=0.01).*

Table VI. *Diagnostic performance in premenopausal women with model including ln human epididymis protein 4 (HE4)+ln soluble urokinase plasminogen activator receptor (suPAR[II-III])+ln cancer antigen 125 (CA125)+age discriminating malignant, including borderline, tumors from benign tumors.*

Pre-menopaual	Sensitivity	Specificity	LR+	LR–
Model lnHE4 +	0.99	0.71	3.4	0.01
lnsuPAR(II-III) +	0.95	0.80	4.7	0.07
lnCA125 + age	0.94	0.90	8.4	0.18
	0.74	0.95	14.7	0.28

LR: Likelihood ratio; ln: natural logarithm.

Table VII. *Univariate Cox regression analyses of overall survival according to preoperative known parameters.*

Overall survival estimating HR	HR	95% CI	*p*-Value
B7-H4 (high *vs.* low)	1.57	0.88-2.77	0.12
uPAR(I) (>21.2 *vs.* ≤20.2 pmol/l)	2.45	1.43-4.17	0.001
HE4 (>400 *vs.* <400 pM)	2.24	1.23-4.07	0.008
Ca125 (>400 *vs.* <400 IU/ml)	2.21	1.28-3.82	0.005

B7-H4: B7-family protein homolog 4; suPAR(II-III): soluble urokinase plasminogen activator receptor (II-III); HE4: human epididymis protein 4; CA125: cancer antigen 125; HR: Hazard ratio; CI: Confidence interval.

In EOC type I the biomarkers suPAR(II-III), HE4, CA125 but not B7-H4 showed increased levels compared to benign tumors. The median levels of suPAR(II-III), HE4, CA125 were all lower in EOC type I compared with EOC type II indicating different tumor biology and biomarker expression in blood in the different types of ovarian tumors. The lower levels of HE4 and CA125 in EOC type I reduces the diagnostic performance of HE4 and CA125 or ROMA score. The EOC type I tumors often grow slow and are more often confined to the ovary at diagnosis as was found in this study. Women more often have symptoms when the tumor is large and the imaging techniques *i.e.* CT scan and ultrasonography find EOC type I tumors easier in an earlier stage with better prognosis.

In patients with borderline ovarian tumors, the plasma levels of suPAR(II-III), HE4 and CA125, but not B7-H4, were increased compared to those in patients with benign tumors. Borderline tumors are more common in women of younger age and borderline lesions have a good prognosis. However, since younger women are interested in fertility-sparing surgery, it is important to find and select those women appropriate for fertility-sparing surgery before progression to a more advanced stage which has slightly worse prognosis (19).

We found that B7-H4 was increased in blood in women with EOC type II and stage II-IV compared with those with benign ovarian tumors, which is in agreement with earlier studies showing that B7-H4 is detected in most patients with late-stage ovarian cancer. We found that B7-H4 was increased in blood in women with higher stage disease, which to some extent may reflect the amount of tumor mass. The B7-H4 protein is expressed on the cell surface of immunosuppressive tumor-associated macrophages and ovarian cancer cells. B7-H4 has shown to be involved in the immune-modulatory signaling mechanism, but also has intracellular effects, such as reducing apoptosis, enhancing proliferation, cell adhesion and migration, and facilitating metastasis. B7-H4 protein has been detected in

half of early-stage and two-thirds of late-stage ovarian tumor samples, but not in normal ovarian tissue. High levels of B7-H4 expression in macrophages in immunostained tumor tissue has been shown to be associated with decreased survival (10, 20). However, in our study, high levels of B7-H4 in blood did not show any association with overall survival.

We found that a model including the three biomarkers HE4, CA125 and suPAR(II-III), and age was the best model in premenopausal women to discriminate benign from malignant ovarian tumors, including borderline tumors, even after cross-validation (AUC=0.94, 95% CI=0.90-0.98). Signs of early-stage ovarian cancer in premenopausal women are especially desirable because the disease is unusual in fertile women and due to the markedly poorer prognosis when ovarian cancer has spread beyond the ovaries. The three biomarkers HE4, CA125, suPAR(II-III) and age show ability to discriminate between malignant and benign adnexal lesions similar to the ultrasound-based predictive models, such as the IOTA Logistic Regression model 2 (LR2) and the Simple Rules (SR) in premenopausal women (21). The widely used Risk of Malignancy Index incorporating ultrasound, CA125 and menopausal status, has been demonstrated to distinguish ovarian cancer from benign ovarian masses, with a sensitivity of 92% and a specificity of 82% (cut-off=200 IU/ml), when used in a tertiary center (22). However, transvaginal ultrasonography is not accessible in primary care and medical diagnostic centers, and therefore requires referral to an experienced gynecologist or trained sonographer before deciding if the patient should be treated at a local Gynecologic Department or at a tertiary center specialized in gynecological oncology.

We did not choose any specific cut-off value in the premenopausal model since in the clinical situation there are several options for fertile women: wait and see, including a new examination in a couple of months; surgery at the local hospital; or referral to a tertiary center. The proposed model shows that adding an inflammatory biomarker known to be involved in carcinogenesis to the established ROMA model increase the accuracy of biomarkers to distinguish between malignant and benign tumors. The most relevant cut-off should be further evaluated and considered from local strategies and capacities. High sensitivity is desirable due to the improved survival in patients referred to and treated by gynecological oncologists (23).

We found that high levels of HE4 or CA125 were associated with poor overall survival. Multiple studies have used a CA125 cutoff of 500 IU/ml as a critical value in analyses of predictability of optimal cytoreducibility (24). However, the CA125 level seems to be a more reliable predictor of the extent of disease and an indicator of the need for radical or upper abdominal procedures to achieve minimal residual disease rather than the ability of the surgeon to perform successful complete cytoreductive surgery. In agreement with our study, another study has shown that an HE4 level above the median value (394 pmol/l) indicates a significantly shorter progression-free interval and survival (25).

In this cohort, a high uPAR(I) level and age above 75 years were associated with very poor short-term prognosis. More than one out of five patients (22%) with ovarian cancer is diagnosed at age 75 years or above (26). A geriatric vulnerability score has been tested and showed promising results, but no randomized controlled trial comparing primary surgery with neoadjuvant chemotherapy has been found in the literature in patients above 75 year of age (27). Especially in older women, preoperative assessment including biomarkers such as HE4, CA125 and eventually uPAR(I) may aid in the decision on extensive primary surgery or neoadjuvant treatment.

In this study, the long-term follow-up time and the consistency in treatment regimens increased the reliability of our results. The Swedish Population Register, which includes all citizens, had complete follow-up of all the patients. Overall survival was chosen as the only end-point, since progression-free survival is dependent on variables such as follow-up intervals and other parameters chosen to indicate progression. Death among patients diagnosed with ovarian cancer is to a large extent related to progression of the malignant disease.

In premenopausal women, the panel of the three biomarkers suPAR(II-III), HE4 and CA125, and age improved discrimination of malignant from benign ovarian tumors compared with ROMA. The levels of suPAR(II-III), HE4, CA125 were all higher in EOC type II than in EOC type I and lower in borderline and benign ovarian tumors. B7-H4 was increased in EOC type II compared with benign ovarian tumors but high levels of B7-H4 in plasma did not improve the biomarker panel and did not predict prognosis. High levels of HE4 (>400 pM) or CA125 (>400 IU/ml) indicate worse overall survival and high uPAR(I) levels indicate very poor prognosis in elderly women the first year after diagnosis.

Competing Interests

None.

Acknowledgements

The study was supported funds from ALF, Lund University and Region Skane and the Swedish Cancer Foundation.

References

1 Kaijser J, Van Gorp T, Van Hoorde K, Van Holsbeke C, Sayasneh A, Vergote I, Bourne T, Timmerman D and Van Calster B: A comparison between an ultrasound based prediction model (LR2) and the risk of ovarian malignancy algorithm (ROMA) to assess the risk of malignancy in women with an adnexal mass. Gynecol Oncol *129(2)*: 377-383, 2013.

2 Valentin L, Ameye L, Testa A, Lecuru F, Bernard JP, Paladini D, Van Huffel S and Timmerman D: Ultrasound characteristics of different types of adnexal malignancies. Gynecol Oncol *102(1)*: 41-48, 2006.
3 Valentin L, Hagen B, Tingulstad S and Eik-Nes S: Comparison of 'pattern recognition' and logistic regression models for discrimination between benign and malignant pelvic masses: a prospective cross validation. Ultrasound Obstet Gynecol *18(4)*: 357-365, 2001.
4 Van Holsbeke C, Daemen A, Yazbek J, Holland TK, Bourne T, Mesens T, Lannoo L, De Moor B, De Jonge E, Testa AC, Valentin L, Jurkovic D and Timmerman D: Ultrasound methods to distinguish between malignant and benign adnexal masses in the hands of examiners with different levels of experience. Ultrasound Obstet Gynecol *34(4)*: 454-461, 2009.
5 Tantipalakorn C, Wanapirak C, Khunamornpong S, Sukpan K and Tongsong T: IOTA simple rules in differentiating between benign and malignant ovarian tumors- Asian Pacific journal of cancer prevention: APJCP *15(13)*: 5123-5126, 2014.
6 Niloff JM, Knapp RC, Schaetzl E, Reynolds C and Bast RC Jr.: CA125 antigen levels in obstetric and gynecologic patients. Obstet Gynecol *64(5)*: 703-707, 1984.
7 Kalapotharakos G, Asciutto C, Henic E, Casslén B and Borgfeldt C: High preoperative blood levels of HE4 predicts poor prognosis in patients with ovarian cancer. J Ovarian Res *5(1)*: 20, 2012.
8 Drapkin R, von Horsten HH, Lin Y, Mok SC, Crum CP, Welch WR and Hecht JL: Human epididymis protein 4 (HE4) is a secreted glycoprotein that is overexpressed by serous and endometrioid ovarian carcinomas. Cancer Res *65(6)*: 2162-2169, 2005.
9 Henic E, Borgfeldt C, Christensen IJ, Casslen B and Hoyer-Hansen G: Cleaved forms of the urokinase plasminogen activator receptor in plasma have diagnostic potential and predict postoperative survival in patients with ovarian cancer. Clin Cancer Res *14(18)*: 5785-5793, 2008.
10 Smith JB, Stashwick C and Powell DJ Jr: B7-H4 as a potential target for immunotherapy for gynecologic cancers: a closer look. Gynecol Oncol *134(1)*: 181-189, 2014.
11 Bodurka DC, Deavers MT, Tian C, Sun CC, Malpica A, Coleman RL, Lu KH, Sood AK, Birrer MJ, Ozols R, Baergen R, Emerson RE, Steinhoff M, Behmaram B, Rasty G and Gershenson DM: Reclassification of serous ovarian carcinoma by a 2-tier system: a Gynecologic Oncology Group Study. Cancer *118(12)*: 3087-3094, 2012.
12 Nik NN, Vang R, Shih I-M and Kurman RJ: Origin and pathogenesis of pelvic (ovarian, tubal, and primary peritoneal) serous carcinoma. Annu Rev Pathol *9*: 27, 2014.
13 Kurman RJ and Shih Ie M: The origin and pathogenesis of epithelial ovarian cancer: a proposed unifying theory. Am J Surg Pathol *34(3)*: 433-443, 2010.
14 Moore RG, McMeekin DS, Brown AK, DiSilvestro P, Miller MC, Allard WJ, Gajewski W, Kurman R, Bast RC Jr. and Skates SJ: A novel multiple marker bioassay utilizing HE4 and CA125 for the prediction of ovarian cancer in patients with a pelvic mass. Gynecol Oncol *112(1)*: 40-46, 2009.
15 Piironen T, Laursen B, Pass J, List K, Gardsvoll H, Ploug M, Dano K and Hoyer-Hansen G: Specific immunoassays for detection of intact and cleaved forms of the urokinase receptor. Clin Chem *50(11)*: 2059-2068, 2004.
16 Akaike: A New look at the Statistical Model Identification. IEEE Trans Automat Contr *19(6)*: 716-723, 1974.
17 DeLong ER, DeLong DM and Clarke-Pearson DL: Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. Biometrics *44(3)*: 837-845, 1988.
18 Anderson GL, McIntosh M, Wu L, Barnett M, Goodman G, Thorpe JD, Bergan L, Thornquist MD, Scholler N, Kim N, O'Briant K, Drescher C and Urban N: Assessing lead time of selected ovarian cancer biomarkers: a nested case-control study. J Natl Cancer Inst *102(1)*: 26-38, 2010.
19 Bendifallah S, Ballester M, Uzan C, Fauvet R, Morice P and Darai E: Nomogram to predict recurrence in patients with early- and advanced-stage mucinous and serous borderline ovarian tumors. Am J Obstet Gynecol *211(6)*: 637 e631-636, 2014.
20 Simon I, Katsaros D, Rigault de la Longrais I, Massobrio M, Scorilas A, Kim NW, Sarno MJ, Wolfert RL and Diamandis EP: B7-H4 is over-expressed in early-stage ovarian cancer and is independent of CA125 expression. Gynecol Oncol *106(2)*: 334-341, 2007.
21 Kaijser J, Sayasneh A, Van Hoorde K, Ghaem-Maghami S, Bourne T, Timmerman D and Van Calster B: Presurgical diagnosis of adnexal tumours using mathematical models and scoring systems: a systematic review and meta-analysis. Hum Reprod Update *20(3)*: 449-462, 2014.
22 Hakansson F, Hogdall EV, Nedergaard L, Lundvall L, Engelholm SA, Pedersen AT, Hartwell D and Hogdall C: Danish 'Pelvic Mass' Ovarian Cancer S. Risk of malignancy index used as a diagnostic tool in a tertiary centre for patients with a pelvic mass. Acta Obstet Gynecol Scand *91(4)*: 496-502, 2012.
23 Fago-Olsen CL, Hogdall C, Kehlet H, Christensen IJ and Ottesen B: Centralized treatment of advanced stages of ovarian cancer improves survival: a nationwide Danish survey. Acta Obstet Gynecol Scand *90(3)*: 273-279, 2011.
24 Ibeanu OA and Bristow RE: Predicting the outcome of cytoreductive surgery for advanced ovarian cancer: a review. Int J Gynecol Cancer *20(Suppl 1)*: S1-11, 2010.
25 Trudel D, Tetu B, Gregoire J, Plante M, Renaud MC, Bachvarov D, Douville P and Bairati I: Human epididymis protein 4 (HE4) and ovarian cancer prognosis. Gynecol Oncol *127(3)*: 511-515, 2012.
26 Cancer Incidence in Sweden 2012. Socialstyrelsen, Stockholm, Sweden, 2014.
27 Falandry C, Weber B, Savoye AM, Tinquaut F, Tredan O, Sevin E, Stefani L, Savinelli F, Atlassi M, Salvat J, Pujade-Lauraine E and Freyer G: Development of a geriatric vulnerability score in elderly patients with advanced ovarian cancer treated with first-line carboplatin: a GINECO prospective trial. Ann Oncol *24(11)*: 2808-2813, 2013.

Received December 26, 2015
Revised February 14, 2016
Accepted February 17, 2016