Afamin and Apolipoprotein A-IV: Novel Protein Markers for Ovarian Cancer

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Abstract

Comparative proteomics identified the vitamin E-binding plasma protein afamin as a potential novel tumor marker for ovarian cancer. In addition, we observed in a previous small study decreased plasma concentrations of apolipoprotein A-IV (apoA-IV) in preoperative patients with kidney cancer. The aim of this study was therefore to analyze afamin and apoA-IV in a large case-control study to evaluate the diagnostic utility of the two potential novel tumor markers in ovarian cancer patients. We measured plasma concentrations of afamin and apoA-IV by means of a specific sandwich-type ELISA using affinity-purified polyclonal and monoclonal antibodies in 181 ovarian cancer patients of various clinical stages, 399 patients with benign gynecologic diseases, including endometriosis, and 177 controls and compared results with those for the conventional ovarian cancer tumor marker cancer antigen 125 (CA125). Afamin concentrations decreased from a median of 70.7 mg/L (range, 34.6-116.1 mg/L) in healthy controls to 65.2 mg/L (range, 20.2-206.6 mg/L) in patients with benign gynecologic diseases to 56.0 mg/L (range, 4.7-96.0 mg/L) in ovarian cancer patients ($P < 0.001$ for all pairwise comparisons). Similar results were obtained with apoA-IV concentrations decreasing from 13.0 mg/dL (range, 5.5-34.0 mg/dL) in controls to 11.7 mg/dL (range, 2.0-32.3 mg/dL) in benign conditions to 9.4 mg/dL (range, 0.3-29.5 mg/dL) in ovarian cancer (all $P < 0.001$). Receiver operating characteristic analysis for differentiating ovarian cancer patients from healthy controls revealed for a specificity of 90% sensitivity values of 92.4%, 42.4%, and 40.8% for CA125, afamin, and apoA-IV, respectively. Afamin, but not apoA-IV, added independent diagnostic information to CA125 and age for differentiating ovarian cancer from benign and healthy samples; the odds ratio of ovarian cancer was reduced by 44% for each doubling of afamin ($P = 0.032$). The relatively low sensitivity, however, clearly indicates that afamin and apoA-IV alone are not sufficiently suitable as diagnostic markers for ovarian cancer. Afamin contributes, however, independent diagnostic information to CA125, thus establishing its potential as an adjunct marker to CA125. (Cancer Epidemiol Biomarkers Prev 2009;18(4):1127–33)

Introduction

Ovarian cancer is the fifth most common cancer in women and accounts for 4% of all cancer cases and 4.2% of all cancer deaths in women worldwide (1). Despite progress in cancer therapy, ovarian cancer mortality has remained virtually unchanged over the past decades. One major reason for this disappointing observation is the lack of early and tissue-specific diagnostic tools. A steep survival gradient has thus been consistently observed and depends largely on the stage at first diagnosis.

The high frequency and poor prognosis of ovarian cancer emphasizes the need to identify prognostic markers for ovarian cancer. Currently, cancer antigen 125 (CA125) is the only tumor marker with significant effect on the clinical management of ovarian cancer (2). CA125 suffers, however, from low specificity because it is elevated in numerous physiologic and nonmalignant gynecologic conditions such as endometriosis (3) or other cancers such as liver cancer (4). As a result, none of the existing serum markers including CA125 can currently be used individually for screening and may therefore serve only as prognostic markers for therapy monitoring in ovarian cancer patients (5).

Numerous attempts, many using serum proteome analysis, have been made to identify novel, alternative markers for ovarian cancer with improved specificity and sensitivity (6, 7). More than 30 serum markers have been evaluated alone or in combination with CA125 (8), including HE4 (9), mesothelin (10), macrophage colony-stimulating factor (11), osteopontin (12), kallikrein, and the soluble epidermal growth factor receptor. Furthermore, strategies implementing a combination of multiple serum markers to improve diagnostic efficacy in discriminating malignant from benign conditions have been developed and reported (13, 14).

Using a differential proteomics approach, we recently identified the serum glycoprotein afamin as a potential

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novel biomarker for ovarian cancer and validated the finding in an immunoblotting and quantitative immunoassay pilot study (15). Statistically significant decreased serum concentrations of afamin were found in 57 ovarian cancer patients compared with 60 patients with benign gynecologic conditions, 26 patients with borderline ovarian cancer, and 39 healthy controls. Afamin is a previously described member of the albumin multigene family with vitamin E-binding properties shown *in vitro* (16-18). In contrast to other family members, afamin is highly glycosylated and exerts a molecular weight of 87 kDa. It is expressed predominantly in the liver and occurs abundantly not only in human serum but also in extravascular fluids such as follicular, seminal, and cerebrospinal fluid.

In addition, we recently discovered unexpectedly low plasma concentrations (compared with those of healthy controls; ref. 19) of apolipoprotein A-IV (apoA-IV), an important constituent of the plasma lipoprotein family, in a small group of kidney cancer patients (20). This previous study actually aimed to investigate the renal metabolism of the apolipoprotein and therefore used healthy renal tissue from otherwise cancer-affected human kidneys and coincidentally revealed reduced apoA-IV plasma levels.

ApoA-IV is a member of the apoA1/C3/A4/A5 gene cluster located on the long arm of human chromosome 11 (21). Members of this cluster are all involved in lipid and lipoprotein metabolism and thus in many ways associated with cardiovascular disease. ApoA-IV is a 46-kDa glycoprotein that is almost exclusively produced in intestinal enterocytes and secreted into the lymph. It was first identified as a component of chylomicrons and high-density lipoproteins (22). In fasting plasma, apoA-IV is found primarily associated with high-density lipoproteins (23). The physiologic function of apoA-IV seems to be multipurpose: it plays a central role in lipid absorption, transport, and metabolism within the reverse cholesterol transport pathway and may act as a postprandial satiety signal and antioxidant (24). In accordance with these functions, low plasma levels of apoA-IV have been described in several coronary artery disease populations (19) and in patients with Crohn's disease, an inflammatory disorder of the gastrointestinal tract (25). Increased apoA-IV plasma concentrations have been found in various forms of renal disease (26-28), suggesting an active role of the human kidney in the metabolism of apoA-IV (20).

The aim of the current investigation was to validate both afamin and apoA-IV as diagnostic markers for ovarian cancer in a large cross-sectional study in patients with ovarian cancer and benign gynecologic conditions as well as in healthy controls and to compare the achievable diagnostic information with that provided by CA125.

Materials and Methods

Patients. Patient samples were obtained from women presenting with pelvic mass or lower abdominal complaints scheduled between 1985 and 2007 to undergo invasive diagnostics or surgery at the Department of Gynecology and Obstetrics of Munich University Hospital and for whom analysis of CA125 was requested before surgery or further diagnostic procedures. The histologic diagnosis for benign and malignant tumors was recorded along with the surgical stage for the patients diagnosed with a malignancy. Tumors were classified by the department of pathology according to the tumor-node-metastasis classification following International Federation of Gynecology and Obstetrics stage (29). Participants with other carcinomas, past or present, were excluded from analysis.

Samples from healthy individuals, as defined clinically, anamnestically, and by clinical chemistry, were drawn prospectively at the Institute of Clinical Chemistry of Munich University Hospital. The study was approved by the Ethical Committee of the Medical Faculty of the University of Munich.

Patients and controls were in nonfasting state at the time of blood collection. This condition was chosen because apoA-IV plasma concentrations have been reported to be stable in the nonfasting state but decrease substantially during prolonged fasting (30). No studies regarding afamin have been reported thus far; our own investigations[7] indicate no difference in afamin concentrations between fasting and nonfasting states. Blood samples from patients and controls were processed in the same way/location and centrifuged at 3,000 $\times$ *g* for 10 min at room temperature, and serum aliquots were frozen within 1 hour at -80°C. Afamin, apoA-IV, and CA125 were measured in serum samples from 757 participants comprising 177 healthy women, 181 patients with first diagnosis of ovarian cancer before first therapy (45 stage I, 17 stage II, 101 stage III, and 18 stage IV), and 399 patients with active benign gynecologic disease (110 ovarian cysts, 67 uterus myomatosus, 45 benign adnex tumors, 41 endometriosis, 37 cervical dysplasia, 33 nonmalignant but unclear lower abdominal pain, 32 bleeding disorders, 19 cystoma, and 10 localized inflammatory disease).

Laboratory Procedures. CA125 was determined in all samples using an electrochemiluminescent immunoenzymometric assay on an Elecsys 2010 analyzer (Roche Diagnostics).

Afamin was quantified with a double-antibody sandwich ELISA using an affinity-purified polyclonal antibody for coating 96-well microtiter plates and the peroxidase-conjugated monoclonal antibody N13 for detection (16, 18). The coating antibody had been previously biotinylated and bound to streptavidin-coated plates (MicroCoat). Secondary plasma in serial dilutions initially calibrated with a primary standard served as the assay standard. The exact protein concentration of the primary standard was determined by quantitative amino acid compositional analysis. The intra-assay and interassay coefficient of variation for this assay was 3% and 10%, respectively.

ApoA-IV was measured with a double-antibody sandwich ELISA (31). Briefly, affinity-purified polyclonal rabbit antiserum against human apoA-IV was used to coat microtiter plates and prepare a peroxidase-conjugated antibody for detection. Plasma with a known content of apoA-IV (standardized with purified apoA-IV

[7] Our unpublished data.

after phenylalanine quantification by high-performance liquid chromatography) served as calibration standard. The intra-assay and interassay coefficient of variation for this assay was 4.5% and 6.5%, respectively. Multiple freezing and thawing as well as long-term sample storage for up to 2 years has not been shown to significantly influence the analysis (31).

Both ELISA assays were done using the diluter robot Freedom EVO (Tecan) and the microplate spectrophotometer Benchmark Plus (Bio-Rad). Samples from all three study groups (controls, ovarian cancer, and benign cases) were measured in random mixture and blinded with respect to the operating coworker.

Statistical Analyses. ANOVA was used for global tests of whether CA125, afamin, apoA-IV, and participant age differed between healthy, benign disease, and ovarian cancer and between various stages of ovarian cancer. Marker values were logarithmically transformed for these assessments to satisfy normal distribution. Nonparametric Wilcoxon tests were used for pairwise comparison of markers between various disease categories.

Operating characteristics of markers were summarized in terms of their sensitivity and specificity for specified cutoff values, and the receiver operating characteristic (ROC) curve was calculated for ovarian cancer versus healthy controls and ovarian cancer versus benign diseases. Higher values of CA125 and lower values of afamin and apoA-IV are indicative for ovarian cancer. Therefore, for a specified cutoff value, a positive CA125 test is defined as CA125 greater than the cutoff (negative, less than or equal to the cutoff), and for afamin and apoA-IV, a positive test is defined as the marker being less than the cutoff (negative, greater than or equal to the cutoff). For each marker, sensitivity was defined as the proportion of ovarian cancer cases with a positive test and specificity as the proportion of healthy controls with a negative test. The ROC curve was constructed graphically as a plot of 1 minus the specificity versus sensitivity for all cutoff values in the range of the marker. A test of the null hypothesis that the area under the ROC curve (AUC) is 50%, versus the alternative that it exceeds 50%, was done using the Wilcoxon rank-sum test. Statistical comparisons of the AUC for various markers on the same samples were done using the *U* statistics of DeLong et al. (32). The operating characteristic analyses for predicting ovarian cancer were repeated in that control marker values were replaced with benign disease marker values to assess the ability of markers to differentiate ovarian cancer from benign disease.

Pairwise correlations between markers were calculated based on the rank-based Spearman correlation coefficient and hypothesis tests done to assess whether they differed from the null value of zero (no) correlation. To assess the independent diagnostic value of afamin and apoA-IV compared with CA125, stepwise multivariable logistic regression based on Akaike's Information Criterion was employed. The model minimizing the Akaike's Information Criterion is that which has the highest potential to maximize external validation and is hence termed the optimal model. All markers were transformed to the $\log_2$ scale for inclusion in the model so that odds ratios (OR) corresponded to the increase in odds for ovarian cancer from a doubling of the marker

Table 1. Age and marker values for the 757 participants in the study

Participant characteristics (n = 757)	Healthy control (n = 177)	Benign disease (n = 399)	Ovarian cancer total (n = 181)	Ovarian cancer (n = 181), International Federation of Gynecology and Obstetrics stage			
				I (n = 45)	II (n = 17)	III (n = 101)	IV (n = 18)
Age, median (range),* y	37 (21-79)	44 (18-85)	62 (20-88)	56 (20-83)	64 (44-76)	62 (22-88)	61 (26-76)
Afamin, median (10th, 90th percentile) (range),† mg/L	70.7 (52.6-90.9) (29.9-116.1)	65.1 (49.1-90.4) (20.2-206.6)	56.0 (29.7-78.5) (4.7-96.0)	58.1 (39.5-81.5) (27.6-96.0)	56.0 (35.9-81.0) (8.9-90.3)	53.7 (27.1-77.6) (4.7-87.8)	63.8 (28.2-76.6) (12.7-88.3)
apoA-IV, median (10th, 90th percentile) (range),†,‡,§ mg/dL	13.0 (8.3-19.6) (5.5-34.0)	11.7 (6.5-18.5) (2.0-32.3)	9.3 (4.8-16.3) (0.3-29.5)	11.4 (4.9-18.5) (2.6-29.5)	8.9 (4.8-15.7) (4.0-29.5)	8.6 (4.6-14.8) (0.3-24.0)	9.9 (4.9-16.2) (2.8-18.2)
CA125, median (10th, 90th percentile) (range),†,‖,¶ units/mL	12.9 (7.6-23.9) (3.2-68.8)	17 (8.1-81.2) (3.3-1,026.0)	290 (26.7-2,860) (10.3-27,070)	36.6 (15.1-225.5) (10.3-1,370.0)	435.0 (67.5-2,320.6) (35.9-4,380.0)	501.0 (102.0-3,166.0) (24.2-27,070.0)	442.0 (30.6-1,671.2) (10.4-7,307.0)

*P < 0.0001, based on differences between healthy controls, benign cases, and ovarian cancer patients (ANOVA).
†P < 0.1 for difference between stages I and II and III and IV.
‡P < 0.05 for difference between stages I and II and III and IV.
§P < 0.1 for difference among all stages (ANOVA).
‖P < 0.001 for difference between stages I and II and III and IV.
¶P < 0.05 for difference among all stages (ANOVA).

value. Separate multivariable analyses were done to predict ovarian cancer versus healthy controls and ovarian cancer versus benign disease.

All statistical tests were done at the two-sided $\alpha = 0.05$ level of statistical significance.

Results

Characterization of Study Groups. Study characteristics and biomarker distributions are shown in Table 1. Ovarian cancer patients were statistically significantly older than were healthy individuals or benign disease cases ($P < 0.0001$). Afamin decreased from a median of 70.7 mg/L in healthy controls (range, 29.9-116.1 mg/L) to 65.1 mg/L (range, 20.2-206.6 mg/L) in patients with benign gynecologic diseases to 56.0 mg/L (range, 4.7-96.0 mg/L) in ovarian cancer patients ($P < 0.001$ for all pairwise comparisons). Similarly, apoA-IV decreased monotonically from healthy (median, 13.0 mg/dL; range, 5.5-34.0 mg/dL) to benign disease (median, 11.7 mg/dL; range, 2.0-32.3 mg/dL) to ovarian cancer (median, 9.3 mg/dL; range, 0.3-29.5 mg/dL; $P < 0.001$ for all pairwise comparisons). Concurrent with established results, CA125 monotonically increased from healthy to benign disease to ovarian cancer ($P < 0.0001$ for all pairwise comparisons).

Table 1 also shows the distribution of marker values by stage of disease among the 181 ovarian cancer patients. Proceeding from stages I to III, afamin and apoA-IV consistently decreased and CA125 consistently increased, although only the latter trend was statistically significant ($P < 0.0001$). Comparison of stages I and II versus III and IV showed apoA-IV ($P = 0.02$) and CA125 ($P < 0.0001$) to be statistically significantly lower and higher, respectively, whereas afamin did not differ. Most likely attributable to the small sample size in stage IV ($n = 18$), all three markers showed a reverse trend compared with the previous stages.

Age and marker distributions for all ovarian cancer patients, according to their histologic subtype, are given in Table 2. In contrast to CA125 differing statistically significantly among histologies ($P < 0.0001$), afamin and apoA-IV concentrations did not differ between histologic subtypes of ovarian tumors ($P = 0.24$ and 0.59, respectively).

Distribution of age and markers for the 399 investigated benign gynecologic patients across type of benign disease is shown in Table 3. Neither afamin nor apoA-IV differed significantly among subgroups with benign conditions. In contrast, CA125 was different among the various benign disease groups, with patients suffering from inflammation and endometriosis showing the highest median CA125 values confirming well-known earlier reports (2).

ROC curves for the three markers for differentiating ovarian cancer from healthy controls are shown in Fig. 1A. All three markers had respectable operating characteristics with AUC above 70% (all $P < 0.0001$ for testing against the null of no predictive value). CA125 obtained the highest AUC [96.7%; 95% confidence interval (95% CI), 94.8-98.3%] followed by afamin (AUC, 75.4%; 95% CI, 70.4-80.4%) and lastly apoA-IV (AUC, 72.0%; 95% CI, 67.6-78.1%). The AUC for CA125 was statistically significantly greater than that for afamin and apoA-IV (both $P < 0.0001$), and there was no statistically significant difference between the AUC for afamin and apoA-IV ($P > 0.05$). In contrast to afamin and apoA-IV, the sensitivity of CA125 remains high, even at low false-positive rates (Table 4A). At a 5% false-positive rate, CA125 detects 84.0% of cancers versus only 28.7% and 29.8% for afamin and apoA-IV, respectively.

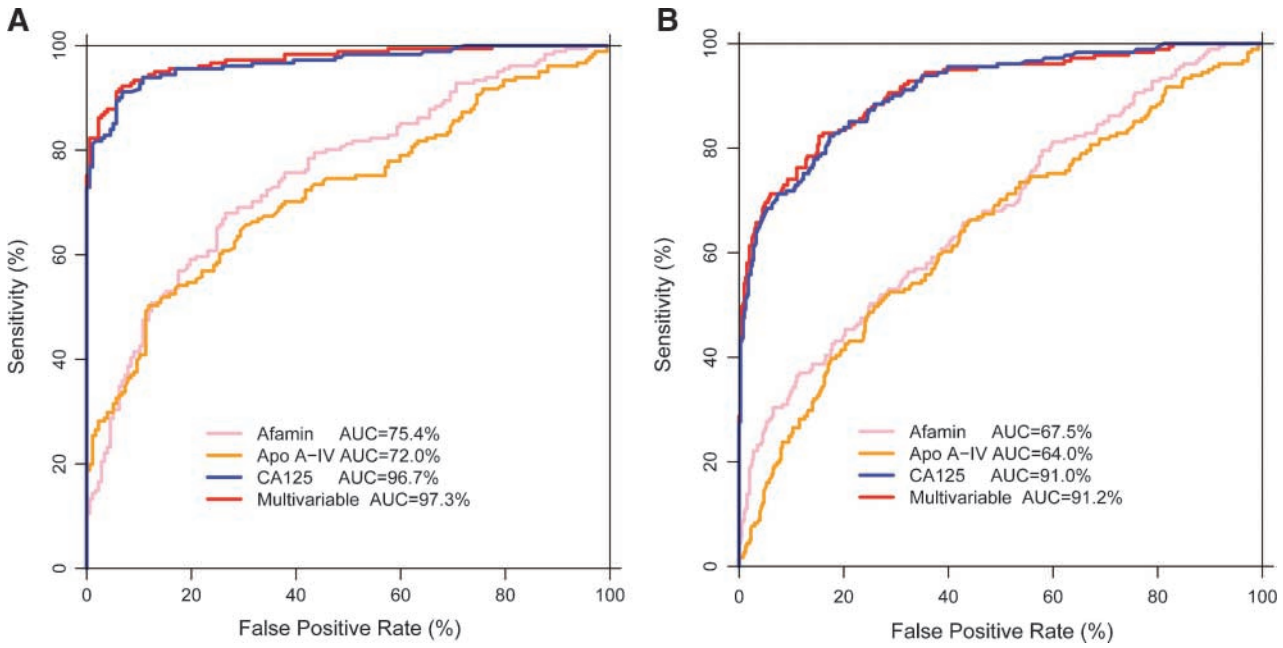

Figure 1. ROC curves for afamin, apoA-IV, and CA125 and the optimal multivariable models for differentiating ovarian cancer patients from healthy participants (**A**) and from participants with benign disease (**B**).

Table 2. Age and marker distributions for all 181 ovarian cancer patients (differentiated by histology)

Median (10th, 90th percentile) (range)	Histology (n = 181)			
	Age (y)	Afamin (mg/L)	ApoA-IV (mg/dL)	CA125 (units/mL)*
Serous-epithelial (n = 55)	62.6 (43.4-75.6) (22.7-80.7)	54.8 (27.8-74.0) (4.7-90.3)	7.7 (4.0-16.3) (0.3-29.5)	400.0 (38.6-2,565.4) (15.8-13,060.0)
Endometrioid-epithelial (n = 12)	65.6 (48.0-75.5) (41.2-82.5)	59.0 (42.4-78.4) (40.2-83.8)	9.1 (5.1-14.7) (4.8-21.6)	181.8 (25.4-1,517.7) (24.4-1,899.0)
Mucinous-epithelial (n = 9)	59.1 (50.6-71.1) (47.9-76.5)	55.8 (26.3-70.4) (16.9-74.7)	7.0 (4.7-15.7) (4.0-17.0)	48.4 (27.5-144.7) (10.3-212.0)
Undifferentiated carcinoma (n = 9)	73.8 (42.3-76.7) (40.4-77.4)	52.7 (34.5-78.5) (24.4-85.2)	8.9 (4.9-12.2) (3.2-14.0)	383.0 (157.0-2,962.4) (65.0-9,908.0)
Adenocarcinoma (n = 31)	62.4 (44.6-76.6) (35.2-84.5)	58.2 (34.5-66.6) (23.6-78.1)	9.4 (6.1-15.8) (1.5-19.1)	1,050.0 (150.0-3,039.0) (35.9-8,261.0)
Others † (n = 22)	58.9 (36.1-76.9) (25.6-81.3)	66.8 (39.1-85.5) (27.6-88.3)	10.7 (5.2-22.6) (3.9-26.0)	115.5 (13.2-1,335.7) (10.4-7,000.0)
Unknown (n = 43)	57.8 (39.1-78.0) (20.7-88.2)	55.1 (31.0-79.6) (12.7-96.0)	10.5 (4.8-16.4) (1.7-18.2)	196.0 (23.1-2,189.0) (13.6-27,070.0)

*P < 0.0001 for difference among all subtypes (ANOVA).
†Mucinous adenocarcinoma, teratoma, gonadal stroma- and granulo-cellular tumors, clear-cell carcinoma, and mixed Mullerian tumor (all n < 4) and special forms (n = 11).

For differentiation of ovarian cancer from benign disease (Fig. 1B), all three markers showed diminished performance. CA125 retained a high AUC (91.0%; 95% CI, 88.2-93.4%) that was statistically significantly different from 50% and markedly higher than that for afamin or apoA-IV (all P < 0.0001). Afamin (AUC, 67.5%; 95% CI, 62.8-72.3%) and apoA-IV (64.0%; 95% CI, 59.0-68.8%) each performed better than 50% (P < 0.0001) but similar to each other (P > 0.05). With increased cutoffs to retain 95% (128 units/mL) and 90% (82 units/mL) specificity for identifying ovarian cancer from benign disease, CA125 still detected 67.4% and 71.8% of cancers, respectively (Table 4B). At 95% specificity, afamin detected 26.0% of cancers and apoA-IV detected 14.9% of cancers.

Independent Diagnostic Information of Afamin and apoA-IV to CA125. Having established that afamin and apoA-IV have moderate predictive ability for ovarian cancer, multivariable analyses were done to assess whether they provide independent diagnostic value to CA125. All three markers were weakly correlated in healthy controls and benign disease cases, with correlations ranging from −0.19 to 0.15. Among ovarian cancer cases, the correlations between CA125 and afamin, apoA-IV, respectively, were −0.27 and −0.23, and the correlation between afamin and apoA-IV was 0.40, indicating that they may provide independent diagnostic value in addition to CA125. The optimal multivariable logistic model for predicting ovarian cancer from healthy individuals included as predictors age, CA125, and apoA-IV and an interaction term between CA125 and age (afamin failed to meet the minimal predictive criterion for inclusion in the model), but neither age, apoA-IV, nor the interaction term was statistically significant (all P > 0.05). The odds for ovarian cancer

Table 3. Age and marker distributions for 399 benign patients (differentiated by benign stages)

Median (10th, 90th percentile) (range)	Benign subtypes (n = 399)			
	Age (y)*	Afamin (mg/L), P = 0.06	ApoA-IV (mg/dL), P = 0.06	CA125 (units/mL)*
Cervical dysplasia (n = 37)	37.2 (25.9-69.9) (19.6-85.2)	68.3 (43.3-93.1) (20.2-109.2)	13.7 (5.2-16.9) (4.1-22.1)	13.5 (9.0-43.6) (3.3-381.0)
Cystoma (n = 19)	57.0 (37.2-67.7) (25.3-81.4)	62.9 (48.4-93.6) (47.9-206.6)	9.8 (6.5-18.4) (5.4-20.4)	25.9 (12.5-81.6) (10.4-184.0)
Inflammation (n = 10)	44.0 (27.2-71.8) (23.9-73.7)	72.8 (34.5-86.2) (28.1-91.6)	7.6 (4.2-13.6) (3.7-21.5)	60.0 (8.4-47.0) (4.9-232.0)
Bleeding disorder (n = 32)	51.1 (34.8-72.1) (20.4-84.6)	62.2 (43.0-90.9) (41.2-120.6)	11.8 (6.4-17.1) (4.1-22.9)	12.9 (8.4-47.0) (4.9-232.0)
Uterus myomatosus (n = 67)	43.1 (33.1-66.8) (21.6-75.7)	64.0 (50.7-86.2) (44.5-103.6)	11.3 (6.5-18.1) (2.0-20.3)	18.1 (8.8-73.2) (4.0-157.0)
Ovarian cyst (n = 115)	46.5 (25.9-73.7) (20.0-84.4)	69.1 (51.4-98.4) (32.7-130.0)	11.7 (7.9-20.3) (3.1-32.3)	14.7 (7.4-52.2) (5.0-1,026.0)
Endometriosis (n = 41)	36.2 (26.3-51.5) (21.7-75.0)	58.8 (48.5-79.5) (28.0-94.0)	11.4 (5.0-20.6) (2.3-28.6)	34.9 (11.0-125.0) (8.1-371.0)
Benign adnex tumor (n = 45)	48.6 (26.0-74.3) (18.5-84.1)	68.5 (52.2-90.5) (39.3-127.2)	11.1 (7.6-16.2) (3.3-20.6)	19.8 (9.4-104.9) (4.1-293.0)
Nonmalignant unclear lower abdominal pain (n = 33)	45.9 (25.7-69.5) (23.5-79.3)	65.0 (50.6-88.2) (35.0-135.6)	13.2 (8.5-19.6) (2.2-31.4)	12.0 (5.4-29.6) (4.3-122.0)

*P < 0.0001 for difference among all subtypes (ANOVA).

Table 4. Sensitivity of CA125, afamin, and apoA-IV at cutoffs obtaining specificities 60% to 90% for differentiating ovarian cancer patients from healthy individuals (A) and from individuals with benign disease (B)

	CA125		Afamin		ApoA-IV	
Specificity (%)	Cutoff (units/mL)	Sensitivity (%)	Cutoff (mg/L)	Sensitivity (%)	Cutoff (mg/dL)	Sensitivity (%)
(A)						
95	38.1	84.0	45.8	28.7	7.2	29.8
90	24.4	91.2	52.7	41.4	8.3	40.3
80	19.0	95.6	60.9	59.1	10.2	54.7
70	16.2	96.1	65.8	69.1	11.2	65.7
60	14.6	97.2	68.4	75.7	11.8	70.2
(B)						
95	128.0	67.4	44.3	26.0	5.2	14.9
90	82.0	71.8	49.1	33.1	6.5	24.9
80	38.7	83.4	53.8	44.2	8.6	41.4
70	26.2	90.1	57.6	53.0	9.7	52.5
60	19.9	95.6	62.0	61.3	10.7	60.2

increased ~8-fold when doubling CA125 (OR, 7.74; 95% CI, 4.10-14.61; $P < 0.0001$). The AUC for the multivariable model, although an optimistic estimate because it is evaluated on the same data used to derive the model, was hardly increased from the AUC of CA125 alone, 97.3% versus 96.7%, respectively, showing that the added risk factors age and apoA-IV did not improve diagnostic accuracy (Fig. 1A). The multivariable stepwise logistic regression for predicting ovarian cancer in individuals with benign disease included as predictors age, CA125, and afamin and an interaction effect between age and CA125. The effect of age was not statistically significant in this comparison. The odds of ovarian cancer increased >2-fold for a doubling of CA125 (OR, 2.30; 95% CI, 1.94-2.72; $P < 0.0001$) and were reduced by 42% for each doubling of afamin (OR, 0.58; 95% CI, 0.34-0.99; $P = 0.044$). The AUC for the multivariable model was 91.2% compared with 91.0% for CA125 (Fig. 1B). The multivariable stepwise logistic regression for predicting ovarian cancer from healthy or benign individuals included as predictors age, CA125, and afamin and an interaction effect between age and CA125. Neither age nor apoA-IV was statistically significant and the interaction effect was only marginally statistically significant and had a small effect size (OR, 1.01; 95% CI, 1.00-1.02; $P = 0.040$). The odds for ovarian cancer increased >2-fold when doubling CA125 (OR, 2.51; 95% CI, 2.11-2.97; $P < 0.0001$) and were reduced by 44% for each doubling of afamin (OR, 0.56; 95% CI, 0.33-0.95; $P = 0.032$). The AUC for the multivariable model was again only slightly higher than that for CA125, 93.0% versus 92.7%, respectively.

Discussion

The aim of this study was 2-fold: first is to confirm the suitability of afamin as a novel tumor marker for the specific diagnosis of ovarian cancer as shown previously in a small pilot study (15) in a sufficiently large population of different ethnic origin. As shown in the previous study, ovarian cancer patients had significantly lower serum concentrations of afamin, a member of the albumin gene family (17), than did healthy controls. In contrast, our data also revealed significant differences between median afamin values in the benign patient group and those in both ovarian cancer and control groups possibly due to the much larger samples sizes in the present study. As in the previous study, patients were substantially and significantly older than were controls. Because age has only a very small, nonsignificant influence on afamin concentrations (ref. 15; $P = 0.834$ in this analysis), this age difference is very unlikely to explain the differences in afamin concentrations between patients and controls.

Interestingly, the lower serum concentrations of afamin and apoA-IV were uniformally diminished among all investigated histopathologic subtypes of ovarian cancer. This was in clear contrast to CA125, which differed significantly among those subtypes ($P < 0.0001$). The expression of CA125 and of most other established or candidate tumor markers for ovarian cancer differs significantly between histologic subtypes but does not vary across stages within each subtype (33). This is particularly evident for the mucinous subtype, which was confirmed in our study (where CA125 levels were close to normal). Although there is a justified debate about the usefulness of ovarian cancer subtype-specific diagnostic markers, we see, nevertheless, also a broad application need for using a uniform marker recognizing all major ovarian cancer subtypes.

Furthermore, we observed median afamin concentrations that were ~10 mg/L higher in all groups investigated (controls, patients with ovarian cancer, or benign diseases) in the present study than in the previous one. This relatively large difference is currently not completely explainable and might most likely be attributable to the small sample size in the previous study (15).

The second major aim of this study was to investigate a further serum protein as a potential tumor marker for ovarian cancer. This protein, apoA-IV, belongs to the apoA-I/apoC-III/apoA-IV/apoA-V gene cluster located on chromosome 11 (21). apoA-IV and the other members of this cluster are all involved in various functions within the lipid/lipoprotein transport machinery and therefore play various roles in the development of atherosclerotic complications and diseases.

The rationale for this investigation came from an unexpected finding of very low plasma concentrations of apoA-IV in a small patient group with kidney cancer (20). Our working hypothesis was that apoA-IV is

decreased also in ovarian cancer and that both decreased apoA-IV and afamin levels could contribute to a more specific diagnosis of ovarian cancer.

The present study also showed decreased plasma concentrations of apoA-IV in ovarian cancer patients that were of similar magnitude as those detected with afamin.

The ROC of both afamin and apoA-IV were significant although inferior to those of CA125. Given the current progress regarding combinatory multiple ovarian cancer markers (13, 14), the finding in this study that neither afamin nor apoA-IV budge the AUC from CA125 for differentiating ovarian cancer from either controls or benign disease, although they add statistically significant information in terms of logistic regression, makes them unlikely candidates as potential adjunct markers for panels involving CA125 and/or other promising markers for ovarian cancer.

Because CA125 has been reported to be elevated in numerous other benign and malignant diseases (8, 34), it will be necessary to investigate afamin and apoA-IV also in various other malignancies to see whether organ specificity might be superior than for CA125.

At present, the reason for decreased circulating concentrations of afamin and apoA-IV is unknown. Afamin and apoA-IV are expressed predominantly in liver and small intestine, respectively, and there is little evidence that expression in ovarian tissue contributes significantly to circulating protein levels. Detailed analysis of tumor tissue is certainly required to clarify this issue. It will also be interesting to look at possible accumulations of both proteins in the ascites fluid of the tumor. In this context, it is noteworthy to speculate about possible antioxidative properties of both proteins (18, 24) and their subsequent putative response to tumor growth.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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