Use of lysophosphatidic acid in the management of benign and malignant ovarian tumors

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Summary

Objective: To establish whether LPA determination improves the differentiation of benign from malignant ovarian tumors.

Methods: Total LPA and LPA species in the serum were determined using a novel method in 142 patients with ultrasound (US) suspecious ovarian tumors and in 78 healthy women. All women underwent determination of CA125 in the serum, a vaginal US examination and morphology scoring of the tumor.

Results: The levels of total LPA and its species in women with ovarian tumors were significantly higher from those in healthy women ($p < 0.001$). No significant difference was found in the levels of total LPA or any of its species between the women with benign and those with malignant ovarian tumors.

Conclusions: Determination of serum LPA would be an appropriate test for ovarian tumor presence, especially in women of reproductive age. The method however does not differentiate benign from malignant ovarian tumors.

Key words: Lysophosphatidic acid; Benign and malignant ovarian tumors; Predictive value.

Introduction

Ovarian tumors occur in all age groups. According to some authors, five out of ten women will undergo ovarian tumor surgery once in a life-span [1]. About 85% of all ovarian tumors are benign; two out of three are detected during reproductive age [2]. The incidence of epithelial cancer, which represents 90% of ovarian cancers, increases with age and is usually of borderline malignancy in women aged 30-50 years. The incidence of malignant ovarian tumors is the highest in women aged 60-74 years [3].

Before ultrasound became routinely used in gynecology, every palpable ovarian tumor had to be removed for histological determination of its benign/malignant nature. Today, however, a precise ultrasound (US) examination permits indirect evaluation of the tumor, morphology score being the most reliable [4-7]. Another possibility is to follow the changes in protein structure (proteomes) in body fluids [8].

In 1998, lysophosphatidic acid (LPA) was added to the suggested potential biomarkers for the detection of ovarian cancer [9].

Phospholipids represent the main structural component of cell membranes and play an important role in cell regulation. LPA is the simplest glycerophospholipid exerting its biological activity in many diseases [10]. It influences cell proliferation, cell apoptosis, elimination of cytokines, smooth muscle contractions and neurite retractions [11]. LPA can be detected in various body fluids, such as serum [12], plasma [9], saliva [13], follicular fluid [14] and ascites due to peritoneal carcinomatosis [15]. The LPA level in the plasma of healthy women is almost undetectable (< 0.6 μM) and far lower than that in the serum (2-5 μM) [9, 12].

So far, the studies on LPA have shown significantly higher plasma levels of LPA in women with ovarian cancer compared to healthy women [9]. The LPA level was also significantly increased in 90% of patients with Stage I disease. In the same study, LPA proved to be more sensitive than CA125 with specificity and sensitivity of 91.1% and 96.3%, respectively [9, 16].

LPA levels were also increased in patients with benign gynecological diseases, but were statistically lower compared to the levels in cancer patients [9]. However, the data on LPA levels in women with benign gynecological tumors is scarce.

The aim of this study was to determine the range of LPA serum levels in healthy women and in patients with either benign or malignant ovarian tumors. We wanted to examine whether the determination of LPA, in addition to the established US score and CA125 assessment, improves the differentiation of benign from malignant ovarian tumors.

Materials and Methods

Study population

In the study 220 patients were enrolled.

The study group consisted of 142 women with US findings suspicious of adnexal tumor regardless of their age, fertility or menopausal status. US criteria for a suspected tumor were tumor size > 5 cm in diameter, mixed or hyperechogenous tumor, inclusions, septa or ascites.

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The control group consisted of 78 women volunteers of different ages, fertility or menopausal status. The inclusion criterion was the absence of any tumor in the pelvis on US examination. The exclusion criterion was CA125 level > 35 kU/l.

All women included in the study underwent the following diagnostic procedures: medical history, bimanual examination, vaginal US (Toshiba Sonolayer Alfa SSA 250 A) and CA125 level determination.

For objectivity and evaluation of the morphologic score, all tumors were evaluated following the criteria selected by means of the established scoring systems [5, 6, 17, 18]. One point was attributed to any of the following: tumor size ≥ 5 cm or more, multifocality, septa, inclusions, ascites and echogenicity. Two points were given to echogenicity. The US score was the sum of individual morphologic features. Tumor size < 5 cm and absence of the listed features was scored zero.

In all women the tumors were surgically removed and histologically examined; 20 women were excluded from further statistical analysis due to intraoperative or histological evidence that the tumor was not ovarian. Of the 20 non-ovarian tumors excluded from the analysis, seven were benign tumors of the uterus (myomas) and 13 were malignant tumors (5 tumors of the uterus, 4 tumors of fallopian tubes and 4 metastatic bowel tumors).

Blood markers

A sample of 5 ml of venous blood was taken from the women in both groups into a non-EDTA (red top) test tube. To achieve complete coagulation, the blood was incubated for 30 minutes at room temperature before it was centrifuged for 15 minutes at 1500 x g (Rotixa/RP, Hettich Zentrifugen, Tuttlingen, Germany).

Serum levels of CA125 were determined according to the manufacturer's instructions (LIAISON®, Byk-Sangtec Diagnostica, Germany). The remaining serum was stored and frozen at –27°C in a 1.7 ml siliconized microcentrifuge tube (Sigma-Aldrich St. Louis, USA) until further analysis. LPA and its species (C16:0, C18:0, C18.2, C18:1, C20:4) were analyzed with a novel method described below. The species were labeled with the number of carbon atoms in the fatty acid chain and the number of non-saturated centers (the first and the second number, respectively).

LPA sample preparation and method description

The frozen sera were thawed and 300 µl of each sample was transferred into a glass centrifuge tube. Internal standard (LPA 17:0) and methanol-chloroform mixture were added to the sample. The tube was mixed vigorously for 15 sec on a vortex-mixer and incubated at 4°C for 20 min. After incubation the samples were warmed to room temperature and centrifuged at 2600 x g for 10 min. The clear supernatant was transferred into a new glass tube, evaporated to dryness under a stream of nitrogen, redissolved in methanol, and injected into a HPLC/MS-MS (high performance liquid cromatography with tandem mass spectrometry) system.

The detection and identification of LPA species was performed on a triple quadrupole mass spectrometer, using electrospray ionization (ESI) in the negative ionization and multiple reaction monitoring (MRM) mode using molecular ion and two product ions for each of the LPA species. Since the method developed used very specific and selective MRM detection, HPLC separation of the individual LPA species was not necessary. Therefore, all LPA species were allowed to elute from the HPLC system within two minutes on similar retention times.

Calibration curves were prepared by sets of sera fortified with LPA species in the concentration range between 0.1 and 16 µM on each day of the analysis. A relative peak area of each LPA to that of the internal standard was presented according to each LPA concentration. This method was used to calculate the level of LPA in blind serum samples. The method was validated and sent for publication in *J. Cromatograph B* [19].

The following reagents were used: Merck (Darmstadt, Germany), Baker, p.a.: J. T. Baker (Deventer, The Netherlands) and Fluka (Buchs, Switzerland).

The reference standards of LPA species are commercially available in the form of salts: (Avanti Polar Lipids, Inc. Alabaster, AL, USA). Equipment: HPLC System Waters Model 2790 (Milford, MA, USA), mass spectrometer Quattro II (Manchester, UK), computer program: MassLynx 4.0, column: Phenomenex (Torrance, CA, USA) Synergi MAX-RP.

Statistical analysis

Statistical analysis was done using the SPSS v14 (SPSS Inc., Chicago, IL, USA) software. Firstly, the normality of distribution of distinct variables was tested using the Kolmogorov-Smirnov test. Wherever possible, the variables were logarithmically transformed (CA125).

To determine the differences in variables between the study and the control group, the chi-square test was used for attributive variables, Mann-Whitney test for variables with non-normal distribution, and Student's t-test for variables with normal distribution. A univariate correlation among continuous variables was determined using Pearson's or Spearman's correlation, depending on the normality of the distribution. For determination of malignancy, multivariate logistic regression was used. The efficiency of the model was determined by Nagelkerke R Square.

The efficacy of total LPA and its species for screening was determined using the analysis of ROC curves. The area under the curve was used to determine the efficacy of the test. For ROC curve analysis, the MEDCALC v4.16f (MedCalc Software, Mariakerke, Belgium) was used.

Results

General characteristics of study population

The median age in the study group was 48.0 ± 16.7 years, and 42.2 ± 9.4 years in the control group. The control group women were significantly younger than the study group women ($p < 0.001$); 52% of women in the study group were in the reproductive period compared to 79.5% in the control group.

Histological analysis in the study group showed ovarian cancer in 42 specimens, of which 38 were epithelial (31 invasive and 7 borderline), three were stromal and one was a germinal ovarian tumor. The remaining 100 patients had benign ovarian tumors: 27 non-neoplastic cysts (follicular, luteal and paraovarian), 31 endometriomas, 13 inflammatory and 29 neoplastic tumors (teratomas, cystadenomas).

LPA determination

No statistically significant correlation was found between the women's age and the concentration of LPA species (Pearson's correlation). Additionally, the concentrations of the LPA species did not significantly differ between the women in the reproductive and those in the postmenopausal period (Student's t-test).

The levels of total LPA and of LPA species were normally distributed in the control group. In the study group, the levels of total LPA were distributed in a non-normal pattern. There was a significant difference in the levels of total LPA and in the levels of all LPA species between the study and the control group ($p < 0.001$) (Table 1).

Table 1. — *Levels of total LPA and its species in study and control group.*

Total LPA and its species	Control group (No. = 78) Mean ± SD	range	Study group (No. = 142) Mean ± SD	range	p*
C16-0	0.71 ± 0.14	0.24-1.24	2.10 ± 1.78	0.13-13.23	< 0.001
C18-2	0.54 ± 0.15	0.00-0.85	1.38 ± 1.25	0.16-11.50	< 0.001
C18-1	0.46 ± 0.13	0.03-0.66	0.99 ± 0.61	0.13-5.32	< 0.001
C18-0	0.62 ± 0.18	0.21-1.20-	1.13 ± 0.51	0.40-3.19	< 0.001
C20-4	0.65 ± 0.30	0.17-1.57	2.55 ± 1.50	0.27-8.89	< 0.001
LPA	2.98 ± 0.50	1.70-4.33	8.14 ± 4.97	2.18- 41.77	< 0.001

* Statistical significance determined by the Mann-Whitney test.

Comparing the patients with malignant tumors and healthy women, there was a similar statistically significant difference in all LPA concentrations ($p < 0.001$) (Table 2). The concentration of total LPA species did not depend on ovarian cancer stage.

The "cut-off" value for prediction of the ovarian tumor was chosen according to ROC curve analysis; the total LPA level proved to be the most suitable variable. Area under curve (AUC) for total LPA was 0.97, 95% CI (0.94-0.98). The value of 3.9 μM was chosen (Figure 1). Specificity and sensitivity for ovarian tumor presence were 99% and 89%, respectively.

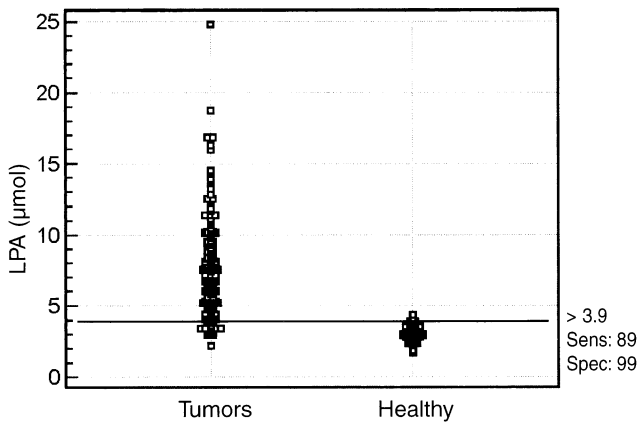

Figure 1. — Cut-off values for ovarian tumors.

LPA and ovarian cancer

By determining a cut-off value of total LPA to be 3,9 μM, the specificity and sensitivity for discriminating between women with cancer from healthy women were 97.4% and 89.5%, respectively. Twelve women included in our study had Stage I ovarian cancer, ten women had Stage II, 11 Stage III and seven Stage IV, and for two women the stage was not determined. In 11 women with Stage I (91.7%) the level of total LPA was above the cut off 3.9 μM, and the CA125 level was increased. In one woman with a borderline serous ovarian cancer Stage Ic, the level of total LPA was 3.82 μM, and the CA125 level was normal.

Comparison of LPA, CA 125 and US score in the differentiation of benign from malignant ovarian tumors

The level of CA125 significantly increased with increasing cancer stage ($p = 0.005$).

US score and the CA 125 level significantly differentiated malignant from benign ovarian tumors ($p < 0.001$) (Table 3), whereas LPA levels did not (Table 2, Figure 2).

For this reason the US scores and CA125 levels only were used in the multivariate analysis. The US score was classified in three groups according to the stage of malignancy. In the group with 2 points there were 5.5% of malignant tumors, in the group with 3 points 39% of malignant tumors and in the group with 4 or more points 65% of all malignant tumors. The three groups and the log values of CA125 were entered in the regression model. The risk of malignancy increased with US score of 4 or more (OR 18.3, 5.1 to 65.2, $p < 0.001$) and log CA 125 (OR 5.7; 2.4 to 13.6, $p < 0.001$). Nagelkerke R Square for the model was 0.58.

LPA and CA 125 in ovarian tumor screening

To assess the value of LPA determination in ovarian tumor screening, the ROC curves of total LPA and CA125 were compared. LPA and CA125 were compared as a test for ovarian tumor presence according to the reproductive status of women. The comparison showed that in the reproductive period total LPA was a significantly better predictor than CA 125 ($p < 0.001$) (Figure 3).

Discussion

A novel method for LPA determination in the serum was developed. We found that total LPA or its species determination is very efficient for predicting ovarian tumor presence, however it does not differentiate benign from malignant ovarian tumors.

The differentiation of benign from malignant ovarian tumors is routinely performed using the serum tumor marker CA125 level and US score. These are rather reliable methods of predicting malignancy in the postmenopausal period, but they are not sufficiently specific in the reproductive period. A method that would increase the specificity of differentiation would therefore greatly improve the quality of treatment. Possible differences in the levels of LPA and its species in women with malignant and in those with benign ovarian tumors were the main motive for a detailed study of LPA and its determination, since we had not found the relevant data in the existing literature.

We decided to determine LPA levels in the serum, part of which was used for CA125 level determination.

The method we used for LPA determination differed from previously described lipid extraction methods. The extraction was completed in one phase and was therefore shorter and more precise, without using semi-quantitative thin layer chromatography. The possibility of a parallel entry of six distinct masses by a tandem spectroscopy analysis contributed greatly to the feasibility of data identification. The evaluation of LPA concentrations was exact and fast. Previously described methods [9, 20] are time-consuming.

Table 2. — *Values of total LPA and its fractions in study and control groups (benign and malignant separately).*

Total LPA and its fractions	Control group (healthy)	Study group (tumors) Benign	Malignant	pH-B*	pH-M*	pB-M*
C16-0 Mean ± SD	0.71 ± 0.14	2.00 ± 1.59	2.34 ± 2.15			
(range)	(0.24 – 1.24)	(0.18 – 10.75)	(0.61 – 13.23)	< 0.001	< 0.001	NS
C18-2 Mean ± SD	0.54 ± 0.15	1.36 ± 1.02	1.43 ± 1.68			
(range)	(0 – 0.85)	(0.16 – 7.50)	(0.47 – 11.50)	< 0.001	< 0.001	NS
C18-1 Mean ± SD	0.46 ± 0.13	0.96 ± 0.54	1.05 ± 0.75			
(range)	(0.03 – 0.67)	(0.13 – 4.11)	(0.27 – 5.32)	< 0.001	< 0.001	NS
C18-0 Mean ± SD	0.62 ± 0.18	1.11± 0.49	0.41 ± 2.83			
(range)	(0.21 – 1.20)	(0.40 – 3.20)	(1.17 – 0.60)	< 0.001	< 0.001	NS
C20-4 Mean ± SD	0.65 ± 0.30	2.59 ± 1.49	2.45 ± 1.53			
(range)	(0.17 – 1.60)	(0.27 – 7.44)	(0.63 – 8.90)	< 0.001	< 0.001	NS
LPA Mean ± SD	2.98 ± 0.50	8.02 ± 4.47	8.44 ± 6.06			
(range)	(1.70 – 4.33)	(2.18 – 31.75)	(1.70 – 41.77)	< 0.001	< 0.001	NS

* Statistical significance determined by the Mann-Whitney test, Bonferroni corrected for multiple comparison; H, B and M stands for "healthy", "benign" and "malignant", respectively.

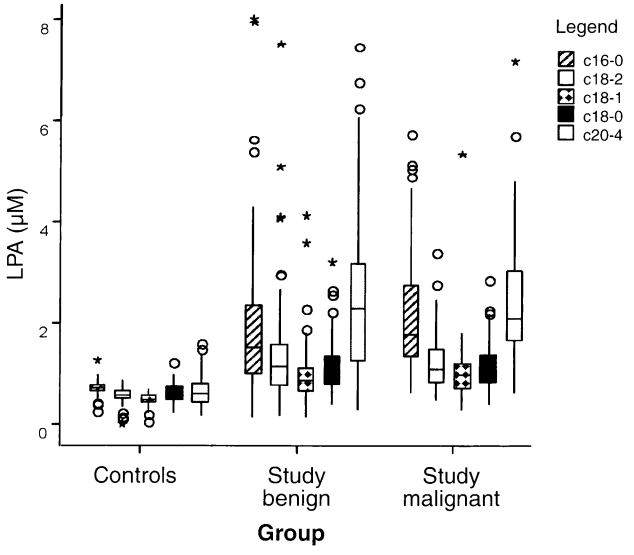

Figure 2. — LPA species levels in study and control groups (benign and malignant tumors separately).

Table 3. — *Values of CA125 and US score for patients in the study group (benign and malignant subgroups).*

	Benign tumors	Malignant tumors	p*
CA125 mean ± SD	105.52 ± 229.60	1193 ± 2129	
(range)	(3.8 – 1452,0)	(1,40 – 8104)	< 0.001
US score mean ± SD	2.3 ± 1.6	4.7 ± 1.6	
(range)	(1 – 7)	(2 – 7)	< 0.001

* Statistical significance determined by the Mann-Whitney test.

We found that the preparation of samples plays a crucial role, since when we first started using the method, only minimal concentrations of LPA were detected and determination of differences among the samples was impossible. After the article by Sutphen [16] and personal communication with Xu, we introduced siliconized test tubes and obtained the first favorable results. The initial failure was due to the binding of lipids to the walls of standard plastic test tubes, which prevented determination of lipids in the sample. Due to the influence of centrifugation time and power on the lipid bilayer formation, and consequently on the concentration, the effects of centrifuging parameters were reassessed. We found that after 30 minutes of centrifugation, the level of LPA decreased by 70%. The methodology we applied was similar to that described by Kim, which, however, employed a biphasic extraction of the sample and did not include HPLC [21]. On the other hand, Baker used butanol as a solvent [22]. From the experience we learned that LPA was poorly soluble in butanol, therefore the extraction of LPA in butanol was negligible.

Despite the fact that patients and healthy volunteers differed in median age and reproductive status, the comparison of both groups proved to be adequate because the levels of LPA and LPA species did not depend on these variables.

According to the literature, LPA in the serum of healthy individuals is bound to albumin in concentrations ranging from 2 to 5 µM. It consists mostly of saturated LPA species (C16:0, C18:0) [10].

In our control group of healthy volunteers all species of LPA were evenly represented. The most abundant was the species C16:0 and the least abundant C18:1. The concentrations and the prevailment of saturated species were in

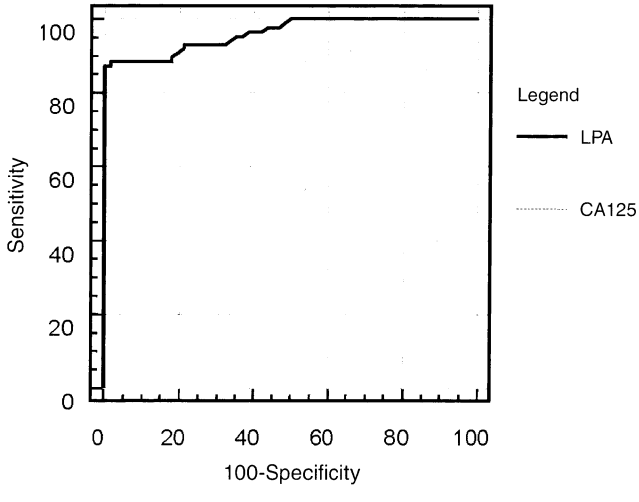

Figure 3. — ROC curve for LPA and CA125 for ovarian tumor prediction in the reproductive period ($p < 0.001$).

agreement with the findings in the literature [10]. There has been no information about the unsaturated species in healthy individuals. Our results showed their presence, although in slightly lower concentrations than those of saturated species. This was expected due to large quantities of unsaturated lysophospholipid precursors found in the animal plasma [23]. Baker *et al.* [12] found unsaturated species in the serum of healthy individuals as well, but Shen and colleagues did not find them in the plasma. The absence of unsaturated species in Shen *et al.'s* study might be due to a low yield of the method [19].

We found that the species ratios in the study group were different from those in the control group. In the study group, the most abundant species was C20:4, followed by C16:0, C18:2, C18:0 and C 8:1. The LPA species were unevenly distributed, therefore their logarithm values were used in further analyses. We assume that the ratios of species are different in different diseases. Moreover, Kim and co-workers found the greatest difference between a group of healthy women and a group of patients with malignant tumors in the content of saturated C16:0 and unsaturated C20:4 species, the two being more abundant in patients with tumors [20].

We found significant differences in LPA concentrations and in species ratios between the study and the control group. The plasma LPA concentrations in cancer patients have been found to be as high as 20 μM. LPA originating in cancer cells is different from LPA originating in thrombocytes, but there is no definite opinion about the content of saturated and unsaturated species [9]. LPA probably enters blood from the peritoneum [24]. LPA is also found in ascites; some have found mostly unsaturated [24], whereas others mostly saturated species [12].

Our comparison of LPA levels in healthy women and patients with malignant tumors confirmed the findings by Xu and co-workers [9], who did not analyze the content of different LPA species, and those by Sutphen and Kim that stressed the importance and the relationship of C16:0 and C20:4 species in women with malignant tumors [16, 21]. In our study, we found great differences in LPA species and total LPA. However, the comparison of LPA levels in borderline and invasive tumors did not show specific differences in the LPA species. Therefore, it can be assumed that malignant cells produce LPA regardless of the stage of malignancy.

The level of total LPA in women with malignant disease was 2.86 times higher than the level of total LPA in healthy women, which is in agreement with previous studies [16]. The non-significant difference in levels of total LPA between the study and the control group in Baker *et al.'s* study [12] could be due to the method, the solvent or the test tubes used, but was most probably due to inappropriate centrifugation.

Using a cut-off value of 3.9 μM for total LPA, a positive test result was obtained in 92.9% of patients with cancer.

Similar results were obtained by a research group that applied the same analysis and correctly classified 41 of the 45 (91.1%) patients with ovarian cancer [16].

The results of this study confirm the findings in the literature that LPA is increased in 90% of Stage I ovarian cancer, which is superior to CA125, found increased in only 50-60% of cases [25, 26]. In our study group, both CA125 and LPA proved to be good predictors. This is most probably due to recruitment of patients in the study group, who had been prescreened before entering our study. Prescreening also explains a high percentage of Stage I patients (26.2%) in the study. The sensitivity of LPA and CA125 for epithelial ovarian cancer was 88.9 and 91.7%, respectively.

We found that LPA level did not depend on cancer stage, which has also been reported in the literature and suggests a possibility of LPA to be an early marker for ovarian cancer [9].

In the literature the data on LPA levels in benign tumors is scarce, and there is none comparing different types of benign tumors. The levels of total LPA and LPA species were significantly higher in the group of patients with benign tumors compared to the control group. Species C20:4 and C16:0 had the highest levels. Proportions of distinct LPA species were similar in benign and malignant tumors, which indicates that unsaturated species are not characteristic of malignant tumors.

Our findings are in disagreement with a previous report by Xu *et al.* [9] where in patients with benign gynecological diseases, LPA levels differed from those in patients with malignant tumors and from those in healthy women. This is probably due to a different sample of patients with benign diseases, which in Xu *et al.'s* study consisted of 17 patients - nine myomas, one endometriosis and seven ovarian tumors with no data on histology. The different LPA levels were therefore most likely due to a different non-ovarian pathology or to a different histological type of benign tumors.

Shen and co-workers [20] analyzed the same group of patients as Xu *et al.* [9] and found that the relative proportions of LPA species in patients with benign gynecological disease did not differ from those in healthy women. It would be interesting to investigate whether different types of benign ovarian tumors differ in the proportion of LPA species, but this was not a subject of this study.

The comparison of total LPA and levels of its species showed no difference between benign and malignant tumors, however, a statistical difference was obtained using CA125 and the US score; 78.5% of malignant tumors and 91.8% of benign tumors were classified correctly.

According to other authors, the malignancy risk based on suspect US scan is 20-39% [5, 27, 28]. The US scoring system we applied proves to be adequate; in our series, 40% of tumors were malignant, probably because unsuspected tumors were excluded from the study.

We found that in women with either benign or malignant ovarian tumors, total LPA and its species were increased. By setting the limiting value of total LPA at 3.9 μM, two healthy women were misclassified among patients, and 16 patients among healthy individuals. LPA was increased in 2.56% of healthy women, whereas other authors [9, 16] reported increased LPA in 3.7-10% of

healthy individuals. Of the patients that were misclassified into the healthy group, two were diagnosed with malignant tumor, two with borderline tumor and 12 with benign tumors. Two patients with malignant tumors (Stage II and III) had high CA125 levels (5700 IU and 2586 IU, respectively). Among the patients with borderline tumors, one patient with Stage Ic tumor had a negative CA125 serum level.

Specificity of CA125 level for ovarian tumor presence was 100%, because in healthy women an increased CA125 level was an exclusion criterion. Sensitivity of CA125 was 69% (50 patients were misclassified into the healthy group). Comparison of LPA and CA125 under the same conditions (100% specificity of LPA at a cut-off value of 4.7 μM), showed that the sensitivity of LPA was 81%, which was markedly higher than that of CA 125.

Specificity of CA125 is particularly poor in women of reproductive age. LPA and all its species have been found to have a statistically better predictive value than CA125. In women in the postmenopausal period, the predictive value of LPA was better than CA125, however the difference was not statistically significant.

Conclusion

We may conclude that LPA is not useful in predicting malignancy of ovarian tumors. However, it is useful in screening for presence of ovarian tumors, especially in women of reproductive age. An increased LPA level would indicate an US examination. This particularly concerns the women at increased risk for ovarian cancer. Detection of early cancer stages improves the prognosis and, provides an option of conservative surgical treatment for younger patients.

To determine the malignancy of ovarian tumors, however, tumor morphology combined with CA125 determination remains the method of choice.

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