Research Article

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CA125, YKL-40, HE-4 and Mesothelin: a new serum biomarker combination in discrimination of benign and malign epithelial ovarian tumor

CA125, YKL-40, HE-4 ve Mezotelin: Benign ve Malign Epitelial Over Tümörünün Ayırımında Yeni Bir Serum Biyobelirteç Kombinasyonu

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Abstract

Objective: Cancer Antigen 125 (CA125) and Risk of Ovarian Malignancy Algorithm (ROMA) score are used for classification of ovarian masses (benign/malign) in preoperative stage. However, their discrimination capacity are considered insufficient, and greatly effected by histological subtype and menopausal status. This study aimed to investigate diagnostic performance of Human epididymis protein 4 (HE4), Y (tyrosine), K (lysine), and L (leucine)-40 (YKL-40), Mesothelin, Rho GDP dissociation inhibitor ß (LyGDI), CA125 or their combinations in discrimination of benign/malign ovarian diseases in preoperative stage.
Materials and methods: The study groups were comprised sera of 31 epithelial ovarian cancer (EOC), 30 benign ovarian tumor patients, and 32 healthy women. The diagnostic performance of the biomarkers were evaluated based on ROC-AUC values and logistic regression analysis incorporating menopausal status and clinical diagnosis of the subjects.
Results: Our data demonstrates that "CA125-HE4-Mesothelin-YKL-40" had the highest sensitivity at 80%, 90%, 95% specificity 96.8%, 93.6%, 93.6%, respectively.
Conclusion: This study provides the first evidence for the combinational uses of "CA125-HE4-Mesothelin-YKL-40" as a panel in distinguishing malign from benign ovarian tumor, not affected by menopausal status unlike ROMA. However, higher patient number may also provide the evaluation of this panel in malign group in terms of tumor stages.

Keywords: Epithelial ovarian cancer; CA125; HE4; Mesothelin; YKL-40.

Öz

Amaç: Preoperatif dönemde over kitlelerinin sınıflandırılmasında (benign/malign) Cancer Antigen 125 (CA125) ve Over Malignansi Risk Algoritması (ROMA) skoru kullanılmaktadır. Ancak, ayırım kapasitelerinin yetersiz olması ve histolojik alt tip ve menopozdan büyük ölçüde etkilendikleri düşünülmektedir. Çalışmamızda, İnsan epididimis proteini 4 (HE4), Y (tirozin), K (lizin) ve L (lösin) -40 (YKL-40), Mezotelin, Rho GDP ayrışma inhibitörü (LyGDI),

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CA125 'in veya kombinasyonlarının preoperatif aşamada benign/malign over hastalıklarının ayırt edilmesindeki tanısal performansını araştırmayı amaçladık.

Gereç ve Yöntemler: Çalışma grupları 31 epitelyal over kanseri (EOC), 30 benign over tümör hastası ve 32 sağlıklı kadından elde edilen serum örneklerinden oluşturuldu. Biyobelirteçlerin tanısal performansı, ROC-AUC değerlerine ve lojistik regresyon analizine dayanarak, bireylerin menopozal durumu ve klinik tanısına göre değerlendirildi.

Sonuçlar: Verilerimiz "CA125-HE4-Mesothelin-YKL-40" kombinasyonunun 80%, 90%, 95% özgüllük değerlerinde, sırasıyla 96,8%, 93,6%, 93,6% değerlerinde olmak üzere en yüksek duyarlılığa sahip olduğunu göstermektedir.

Tartışma: Bu çalışma, "CA125-HE4-Mesothelin-YKL-40" kombinasyonunun bir panel olarak kullanımının, ROMA'dan farklı olarak menopozal durumdan etkilenmeden malign over tümörünü benign over tümöründen ayırt edebilmek amacıyla kullanılabileceğinin ilk kanıtını oluşturmuştur. Bununla birlikte, bu panel aracılığıyla daha yüksek hasta sayısı kullanılarak yapılacak çalışmalar, malign grubun tümör evreleri açısından değerlendirilmesini sağlayabilir.

Anahtar kelimeler: Epitelyal over kanseri; CA125; HE4; Mezotelin; YKL-40.

Introduction

According to 2018 ovarian cancer statistic data, ovarian cancer is 5% of female cancer deaths and epithelial ovarian cancer (EOC) comprises 90% of malign ovarian cancers. Due to nonspecific symptoms, and a lack of sensitive and specific biomarkers, approximately 75–80% of the disease are generally diagnosed at stages II–IV. If EOC can be diagnosed at an early stage, the patient's 5-year survival rates can exceed 90% [1, 2]. Currently, the most frequently used detection methods for EOC are physical examination, symptom assessment, ultrasonography (USG) and measuring of serum cancer antigen 125 (CA125) levels which is the most reliable serum biomarker [3]. CA125 is a glycosylated cell membrane mucin and it is expressed in many epithelial cells and an incidence of high levels of CA125 is generally correlated with cell proliferation. CA125 levels increase in 80% of patients and elevated levels can be found in only 50–60% of early-stage EOC patients. Research revealed that almost 20% of EOC patients presented with a lack of CA125 expression, and that it can also be elevated in certain conditions of non-ovarian cancers (endocervix, endometrium, lung, and lymphoma) and benign gynecological diseases (such as endometriosis and ovarian cysts) [4–6]. In the last decade, several serum biomarkers for EOC diagnosis have been studied, and research has revealed that due to the complexity and heterogeneity of the disease, one biomarker is insufficient to diagnose with high sensitivity and specificity. Several studies and meta-analysis have shown that using a combination of different biomarkers would provide greater potential for the diagnosis of EOC, yielding relative high sensitivity and specificity [7–11].

Recently, biomarker combination with CA125 in a Risk of Ovarian Malignancy Algorithm (ROMA) score was approved for differential diagnosis and malignancy assessment in woman with pelvic mass. However many studies demonstrate that the histological subtype, menopausal status greatly effected levels of ROMA [12–14].

With this knowledge, and the aim of focusing on sensitivity and specificity, we evaluated different potential biomarkers Human epididymis protein 4 (HE4), Rho GDP dissociation inhibitor ß (LyGDI), Tyrosine–Lysine–Leucine-40 (YKL-40), Mesothelin, CA125 and their combinations to determine the optimum means of discriminating benign ovarian tumor from EOC.

HE4 is a type of whey acidic four-disulfide core proteins expressed in the epididymis, female reproductive systems and solid tumor. HE4 and combined usage with CA125 has recently been approved by the Food and Drug Administration (FDA). However, there is still lack of evidence regarding its use as a diagnostic and follow-up biomarker [15, 16].

LyGDI are cytosolic proteins which have a dual role in the regulation of Rho GTPase activity and signaling pathways. It has been shown that LyGDI expression changes in various tumor [17, 18]. This feature may provide an advantage in its implementation as a biomarker and/or combinations in the diagnosis of benign ovarian tumor or EOC.

YKL-40 is a glycoprotein that is released from the cell. Studies have reported that high levels of YKL-40 can be found in primary colorectal cancer, glioblastoma, metastatic breast cancer, and recurrent ovarian cancer. Although many studies agree that YKL-40 is a novel biomarker for the detection of early-stage ovarian cancer, it did not demonstrate superiority over the CA 125 [10, 19].

Mesothelin is a cell surface glycoprotein and researchers showed that mesothelin is expressed in the majority of serous ovarian cancers which contain the greater portion of EOC [20, 21]. The biological role of mesothelin has not been clarified yet. However, experimental results have indicated that mesothelin is a CA125 binding protein which leads to tumor aggregation and metastasis by promoting the attachment of EOC cells to the peritoneum [22, 23].

This study was designed to assess the utilization of HE4, YKL-40, Mesothelin, LyGDI and CA125 biomarkers and/or combinations in the diagnosis of EOC and discrimination of benign ovarian tumor. We believe these

results might be helpful to find a useful combination of serum biomarkers in diagnosis of EOC and distinguishing from benign ovarian tumor according to age, menopausal status, stage and pathological diagnosis of the subjects.

Materials and methods

Patients

Our study groups were comprised of patients who applied to the Dokuz Eylül University and Ege University Faculties of Medicine, Obstetrics and Gynecology Clinics. The study conforms with Ethics of the World Medical Association rules (Declaration of Helsinki) and it was approved by the Dokuz Eylül University local clinical Ethics Committee and written informed consent was received from all subjects. Based on their postoperative clinic-pathological results, the patients were classified into two groups as 31 EOC patients and 30 benign ovarian tumor patients. Thirty-two healthy female volunteers are included as the control group. An existing malignancy other than ovarian cancer, currently undergoing chemotherapy treatment, borderline ovarian cancer and other histological types of ovarian cancer were determined as exclusion criteria. The clinicopathological features of the patients such as menopausal status, histological types and stages were received from the departments of Obstetrics and Gynecology Clinics and Pathology. The disease staging was accomplished based on the International Federation of Obstetrics and Gynecology (FIGO) staging system in EOC [24]. The menopausal status was classified accordingly; the postmenopausal group consisted of subjects with a history of at least 1 year of amenorrhea, and the second group was labeled premenopausal.

Method

Blood samples from the subjects were collected and centrifuged at 3000 rpm for 10 min and serum samples were immediately stored at −80°C until further processing. Hemolytic serum samples were excluded. All experiments were performed in duplicate. The serum CA125 levels were determined using the electrochemiluminescence immunoassay test (ECLIA) according to the instructions from Cobas-e 411 autoanalyzer (Roche Diagnostics, Mannheim, Germany). The within run precision is 1.2–3.3%, total precision is 2.3–4.0%, and measuring range is 2.0–3000 U/mL. The other biomarkers were measured using ELISA kits, a solid-phase immunoassay based upon the direct sandwich technique specific for each one. Analytical performance values of the kits: YKL-40 (Human-YKL40 Quidel, San Diego, CA, USA); the within run intra-assay precision is 5.6–6.0%, inter-assay precision is 6.0–7.0%, and measuring range is 15.6–300 ng/mL, LyGDI (LyGDI-USCN, China); the within run intra-assay precision is CV < 10.0%, inter-assay precision is CV < 12%, and measuring range is 0.625–40 ng/mL, HE4 (HE4-Fujirebio, Göteburg, Sweden); the within run precision is ≤15% total CV and measuring range is 15–900 pM, Mesothelin (Mesothelin-Aviscera Bioscience, Santa Clara, CA, USA); the within run intra-assay precision is 4.0–6.0%, inter-assay precision is 8.0–12.0%, and measuring range is 125–8000 pg/mL. All results that over the measuring range of the kits are diluted with serum physiologic and measured again.

Statistical analysis

Statistical analyses were performed using the SPSS software version 15.0. The normal distribution of the variables (age and values of biomarkers) was assessed using the Kolmogorov Smirnov test. The data comparisons which fit into normal distribution were performed using the One-way ANOVA test and Tukey post-hoc test while variables that did not fit into normal distribution comparisons were carried out by nonparametric Kruskal-Wallis and Mann-Whitney-U tests. The chi-square test was used to compare the groups according to menopausal status. The diagnostic performance of CA125, YKL-40, HE4, Mesothelin and LyGDI were evaluated with receiver operating characteristic and the area under curve (ROC-AUC) with a 95% confidence interval. Sensitivities were calculated at 80%, 90% and 95% specificity values for the all biomarkers. Positive predictive values (PPV) and negative predictive values (NPV) of the biomarkers were determined according to cut-off values. The cut-off values of the biomarkers were determined as values which yielded at least 80% specificity and then determined cut-off values were used for calculation of the sensitivity, specificity, PPV, NPV. The discrimination power of the biomarkers and their combinations were analyzed using the Logistic regression model, which can be used for predicting the relationship between two dependent (benign/malign) or independent variables (menopausal status and levels of biomarkers). For this purpose, ROC curves were generated once again for each biomarker and combination, and the sensitivity of the combinations was determined at certain specificity values (80%, 90% and 95%). The comparisons of ROC-AUC values were performed with DeLong mathematical model and $p < 0.05$ was accepted as statistically significant for all analyses [25].

Table 1: Clinicopathological characteristics of the groups.

Age			
	Healthy group (n = 32)	**Benign group (n = 30)**	**Malign group (n = 31)**
Mean ± Standard deviation	53.4 ± 9.3	41.4 ± 15.9	53.3 ± 9.3
Median	53	37	53
Minimum–maximum	28–69	20–80	28–69
Menopausal status			
Premenopausal (%)		**Postmenopausal (%)**	
Healthy (n = 10)	31.3	Healthy (n = 22)	68.7
Benign (n = 24)	80.0	Benign (n = 6)	20.0
Malign (n = 8)	25.8	Malign (n = 23)	74.2
Pathological diagnosis			
Benign (n = 30)	**(%)**	**Malign (n = 31)**	**(%)**
Cystadenoma (n = 11)	36.7	Serous carcinoma (n = 13)	41.9
Endometrioma (n = 10)	33.3	Clear cell carcinoma (n = 6)	19.4
Mature cystic teratoma (n = 5)	16.7	Mixed carcinoma (n = 6)	19.4
Basic/functional Kist (n = 3)	10	Endometrioid carcinoma (n = 3)	9.6
Ovarian fibrotekoma (n = 1)	3.3	Mucinous carcinoma (n = 2)	6.5
		Malignant mixed (n = 1)	3.2

Results

A total of 93 women, of which 32 were healthy (34.4%), 30 with benign ovarian tumor (32.3%) and 31 with EOC (33.3%) were included in the study groups. The clinicopathological and characteristics of the groups are provided in Table 1. There was no significant difference between healthy and EOC groups in terms of age ($p = 0.999$). However, significant differences were found between malign and benign ovarian tumor groups ($p < 0.05$), healthy and benign groups ($p < 0.001$). The groups were consisted of 42 (45.2%) premenopausal women and 51 (54.8%) postmenopausal women. The ratio of premenopausal status in the benign ovarian tumor group was significantly higher than in the healthy and malign groups ($p < 0.001$). There were no significant differences between healthy and malign groups in terms of menopausal status. Furthermore, the groups had been diagnosed with different histological types of malign and benign diseases. The benign ovarian tumor group was mostly comprised of cystadenoma (36.7%) and endometrioma (33.3%), and the malign group was comprised of serous carcinoma (41.9%), clear cell carcinoma (19.4%) and mixed carcinoma (19.4%). According to the pathological characteristics of the individuals, the detected serum levels of LyGDI, Mesothelin, YKL-40, HE4 and CA125 were compared in all groups. The results showed that all the biomarkers were useful in distinguishing between healthy-malign and benign-malign groups (Figure 1). In addition, no statistical differences were found between LyGDI, Mesothelin and YKL-40 levels in healthy and benign ovarian tumor groups.

The evaluation of the biomarkers according to tumor stages and histological subtypes

The patients were also evaluated and classified based on histopathological subtypes. Pathological stages were categorized as early stage (stage I–II) and late stage (stage III–IV). Seven patients were in stage I (22.6%), 4 patients were in stage II (12.9%), 13 patients were in stage III (41.9%), and 7 patients were in stage IV (22.6%). Data showed that the median value of CA125 and HE4 were significantly higher than in the benign ovarian tumor group for both early-stage and late-stage (CA125 and HE4 $p < 0.001$) (Figure 2). The biomarkers were also compared for the discrimination capacity between benign ovarian tumor and EOC in terms of histological types (serous, mix and clear cell carcinoma) (Table 2).

Evaluation of the biomarkers according to menopausal status

Biomarkers were compared in regard to menopausal status in all groups. The levels of the biomarkers were

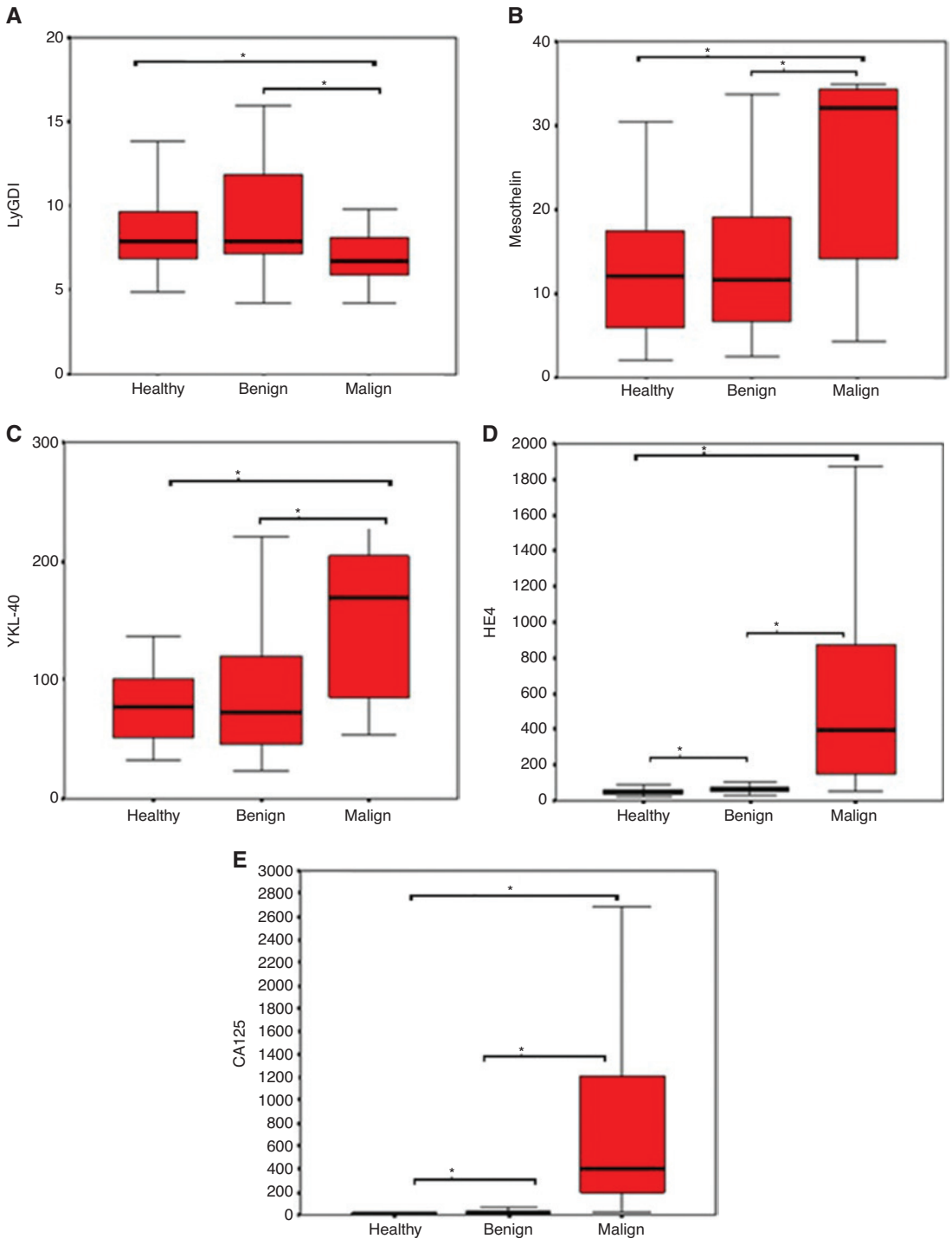


Figure 1: The medians and ranges of LyGDI (A), Mesothelin (B), YKL-40 (C), HE4 (D) and CA125 (E) in healthy, benign and malign groups.

assessed for subjects in each group as premenopausal/postmenopausal status without consideration of their clinical diagnosis (Table 3). There was no significant difference between pre- and postmenopausal women in the healthy group for all biomarkers. However, in the benign ovarian tumor group, Mesothelin and HE4 values were significantly higher in the postmenopausal women, and the CA125 value was significantly lower in the postmenopausal women than the premenopausal women ($p < 0.05$). In addition, LyGDI and YKL-40 did not show a significant difference between pre- and postmenopausal women ($p > 0.05$). In the malign group, only YKL-40 showed a significant difference between pre- and postmenopausal women and the levels were significantly higher in

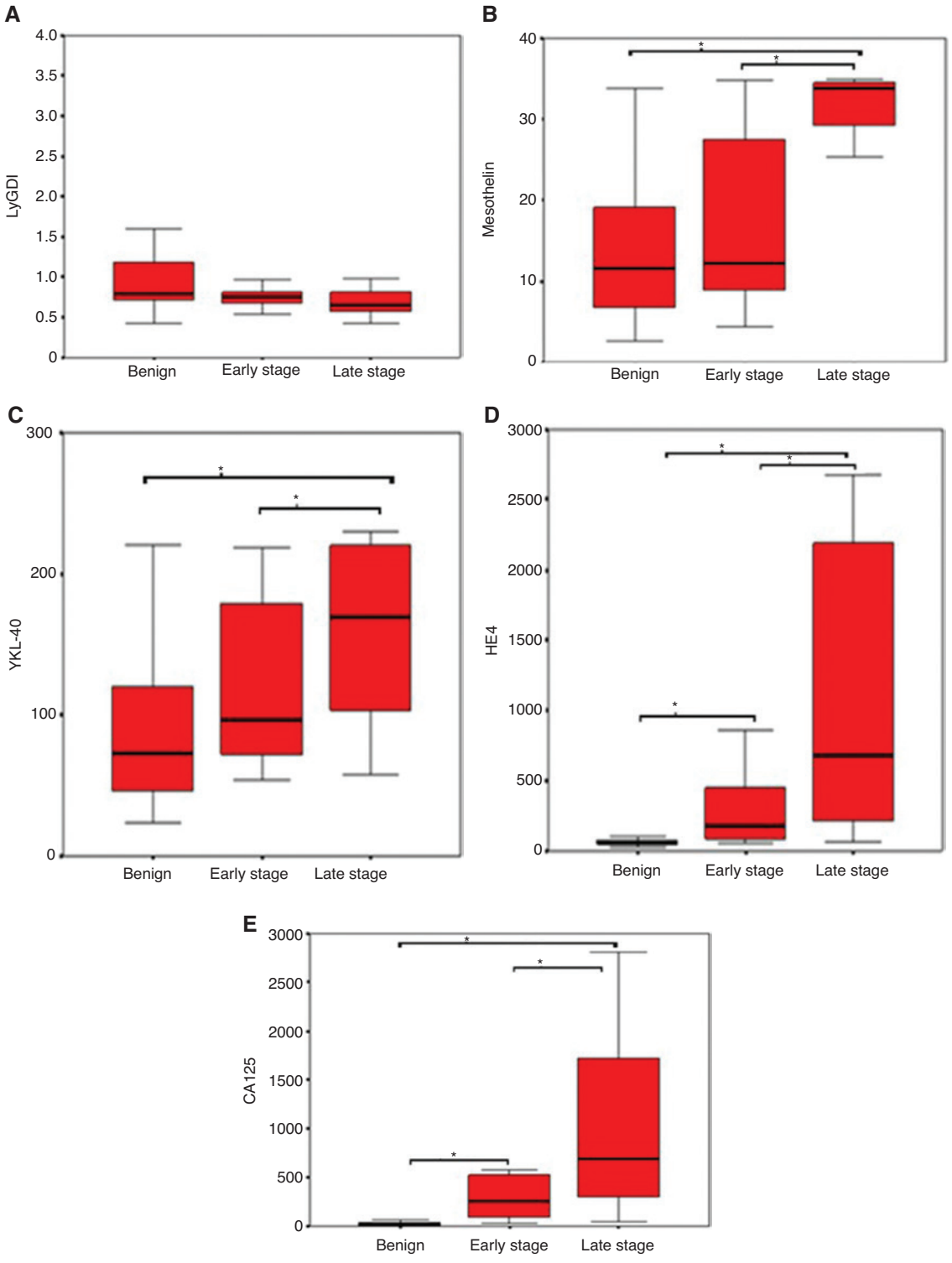


Figure 2: The medians and ranges of LyGDI (A), Mesothelin (B), YKL-40 (C), HE4 (D) and CA125 (E) according to clinical diagnosis.

the postmenopausal women ($p < 0.05$). Comparisons were also conducted based on clinical diagnoses of the groups following the comparison of the biomarkers in terms of menopausal status (Table 4). In the postmenopausal group, significantly higher levels of CA125 were found only in the malign group. However, HE4 and Mesothelin levels were significantly higher in both the benign and malign groups when compared to the healthy group ($p < 0.05$). In addition, YKL-40 values in the malign group were also significantly higher than the healthy group

Table 2: Comparison of the median values of the biomarkers according to histologic subtypes.

	Benign (n=30)	Serous carcinoma (n=13)	Clear cell carcinoma (n=6)	Mixed carcinoma (n=6)	Endometrioid carcinoma (n=3)	Mucinous carcinoma (n=2)	MMMT[a] (n=1)
	Mean±SD (Median)	Mean±SD (Median)	Mean±SD (Median)	Mean±SD (Median)	Mean±SD (Median)	Mean±SD (Median)	Mean±SD (Median)
LyGDI	1.07±0.12	**0.67±0.02**	1.08±0.35	0.65±0.08	0.99±0.25	0.67±0.15	2.93
(ng/mL)	(0.79)	**(0.66)**[b]	(0.73)	(0.59)	(0.80)	(0.67)	
Mesothelin	14.54±1.88	**30.79±1.48**	24.03±4.71	**30.10±4.03**	5.78±1.32	7.36±1.99	25.43
(ng/mL)	(11.60)	**(33.25)**[b]	(27.55)	**(34.29)**[d]	(4.54)	(7.36)	
YKL-40	97.79±11.53	136.3±17.32	**165.38±27.38**	**175.42±20.95**	74.04±10.63	186.11±10.15	227.23
(ng/mL)	(73.17)	(157.85)	**(188.14)**[c]	**(183.31)**[d]	(81.21)	(186.11)	
HE4 (pM)	65.91±4.91	**841.16±225.97**	**672.89±405.76**	**1842.21±828.67**	115.15±42.34	338.42±275.99	249.19
	(61.29)	**(509.25)**[b]	**(188.48)**[c]	**(1546.20)**[d]	(73.20)	(338.42)	
CA125	63.85±28.69	**900.16±348.48**	**979.41±430.07**	**1353.71±396.54**	49.99±10.99	375.15±178.95	1241.0
(U/mL)	(20.74)	**(361.90)**[b]	**(638.65)**[c]	**(1125.50)**[d]	(42.85)	(375.15)	

Bold indicates significant difference between groups by Kruskal-Wallis and Mann-Whitney-U test. $p < 0.05$.

[a]MMMT: Malignant mixed müllerian tumor.

[b]Comparison of serous carcinoma and benign groups by Mann-Whitney-U, $p < 0.05$.

[c]Comparison of clear cell carcinoma and benign groups by Mann-Whitney-U, $p < 0.05$.

[d]Comparison of Mixed carcinoma and benign groups by Mann-Whitney-U, $p < 0.05$.

($p < 0.001$). Statistical results demonstrated that all the biomarkers have the capacity to discriminate healthy from malign groups except for LyGDI ($p < 0.001$). However, statistical significance and discriminating capacities of the biomarkers were found different. For instance; Mesothelin and YKL-40 were able to discriminate the healthy from the malign group ($p < 0.05$) whereas HE4 and CA125 were able to discriminate the benign from the malign group ($p < 0.001$). In the premenopausal group, significant differences were found only for HE4 and CA125. Both of the biomarkers showed a discriminating capacity healthy from benign ($p < 0.05$), healthy from malign ($p < 0.001$) and benign from malign group ($p < 0.001$).

The diagnostic performances of CA125, YKL-40, mesothelin, LyGDI and HE4

The diagnostic performances of the biomarkers in the discrimination of the healthy, benign and malign groups

ROC curves and ROC-AUC values were calculated to assess the discrimination performance of the biomarkers among the healthy-benign, healthy-malign, and benign-malign groups. The ROC curves of LyGDI, Mesothelin, HE4, YKL-40, and CA125 were examined regarding the discrimination capacity between the benign ovarian tumor group and healthy groups (Figure 3), healthy and malign groups (Figure 4) and benign and malign groups (Figure 5). The sensitivity of the biomarkers were assessed at 80%, 90% and 95% specificity levels. The obtained data demonstrated that the sensitivity of CA125 was excellent at the all selected specificity levels (Table 5). ROC analyses data were used to determine the cut-off values of each biomarker with a distinguishing capacity between benign and malign ovarian tumor. The cut-off values of the biomarkers were determined as values which yielded at least 80% specificity from ROC analyses data. The sensitivity, specificity, PPV, NPV values of the biomarkers were calculated for determined cut-off values (Table 6).

A logistic regression model was used to determine the ideal biomarker combination with the highest sensitivity and specificity in discriminating between benign and malign ovarian tumor. With this aim, ROC-AUC curves of the biomarkers and their double, triple and quadruple combinations were calculated once again and their sensitivity was evaluated at selected specificity values (80%, 90%, 95%) (Table 6). Among the individual biomarkers, HE4 demonstrated the highest sensitivity (93.6%) for detecting malignancy at 80% specificity but CA125 (90.3%) was the most sensitive biomarker at 90% and 95% specificity level. However, there was no significant difference of ROC-AUC values between CA125 and HE4. ROC-AUC values of Mesothelin and YKL-40 were significantly lower than CA125 ($p < 0.05$). Among the binary combinations, the combination of "CA125-HE4" and "CA125-YKL-40" showed a higher sensitivity of 93.6% at 80% specificity. Compared with CA125, these combinations provided an increase in sensitivity. Among the triple combinations, "CA125-HE4-Mesothelin" had the highest sensitivity at 80%, 90%, 95% specificity (96.8%, 93.6%, 90.3%, respectively). The addition of Mesothelin or HE4 to

Table 3: Comparison of the biomarkers according to premenopausal and postmenopausal status.

	LyGDI (ng/mL)		Mesothelin (ng/mL)		YKL-40 (ng/mL)		HE4 (pM)		CA125 (U/mL)	
	Mean ± SD (median)	p-Value	Mean ± SD (median)	p-Value	Mean ± SD (median)	p-Value	Mean ± SD (median)	p-Value	Mean ± SD (median)	p-Value
Healthy (n = 32)										
Premenopausal (n = 10)	0.89 ± 0.08 (0.89)	0.290	15.95 ± 2.78 (15.37)	0.167	70.97 ± 10.22 (53.37)	0.290	45.98 ± 3.39 (41.10)	0.263	15.54 ± 2.0 (12.72)	0.489
Postmenopausal (n = 22)	0.80 ± 0.03 (0.76)		12.06 ± 1.68 (10.78)		82.70 ± 6.09 (82.47)		51.73 ± 3.21 (52.22)		13.53 ± 0.98 (12.96)	
Benign (n = 30)										
Premenopausal (n = 24)	0.97 ± 0.11 (0.78)	0.223	**12.95 ± 2.07 (10.15)**	**0.038**	85.04 ± 10.88 (69.36)	0.133	**59.09 ± 3.75 (56.36)**	**0.018**	**76.26 ± 35.55 (27.50)**	**0.029**
Postmenopausal (n = 6)	1.46 ± 0.42 (1.21)		**20.9 ± 3.77 (17.09)**		148.77 ± 32.06 (190.26)		**93.19 ± 15.95 (87.86)**		**14.20 ± 2.28 (11.34)**	
Malign (n = 31)										
Premenopausal (n = 8)	0.72 ± 0.04 (0.68)	0.752	23.73 ± 4.47 (28.93)	0.874	**99.20 ± 15.36 (91.02)**	0.013	568.75 ± 220.98 (397.05)	0.718	1030.67 ± 339.05 (826.30)	0.652
Postmenopausal (n = 23)	0.89 ± 0.13 (0.67)		25.77 ± 2.30 (32.17)		**167.16 ± 12.28 (176.30)**		989 ± 275.68 (292.03)		852.03 ± 229.95 (361.90)	

Bold indicates significant difference statistically.

Table 4: Comparison of the biomarker levels in pre-and postmenopausal groups according to clinical diagnosis.

	Premenopausal				Postmenopausal			
	Healthy (n = 10)	Benign (n = 24)	Malign (n = 8)	p-Value[a]	Healthy (n = 22)	Benign (n = 6)	Malign (n = 23)	p-Value[a]
LyGDI (ng/mL)	0.89 ± 0.08 (0.89)	0.97 ± 0.11 (0.78)	0.72 ± 0.04 (0.68)	0.242	0.80 ± 0.03 (0.76)	1.46 ± 0.42 (1.21)	0.89 ± 0.13 (0.67)	0.065
Mesothelin (ng/mL)	15.95 ± 2.78 (15.37)	12.95 ± 2.07 (10.15)	23.73 ± 4.47 (28.93)	0.075	12.06 ± 1.68 (10.78)	**20.9 ± 3.77 (17.09)**[b]	**25.77 ± 2.30 (32.17)**[c]	**0.001**
YKL-40 (ng/mL)	70.97 ± 10.22 (53.37)	85.04 ± 10.88 (69.36)	99.20 ± 15.36 (91.02)	0.316	82.70 ± 6.09 (82.47)	148.77 ± 32.06 (190.26)	**167.16 ± 12.28 (176.30)**[c]	**< 0.001**
HE4 (pM)	45.98 ± 3.39 (41.10)	**59.09 ± 3.75 (56.36)**[b]	**568.75 ± 220.98 (397.05)**[c,d]	**< 0.001**	51.73 ± 3.21 (52.22)	**93.19 ± 15.95 (87.86)**[b]	**989 ± 275.68 (292.03)**[c,d]	**< 0.001**
CA125 (U/mL)	15.54 ± 2.0 (12.72)	**76.26 ± 35.55 (27.50)**[b]	**1030.67 ± 339.05 (826.30)**[c,d]	**< 0.001**	13.53 ± 0.98 (12.96)	14.20 ± 2.28 (11.34)	**852.03 ± 229.95 (361.90)**[c,d]	**< 0.001**

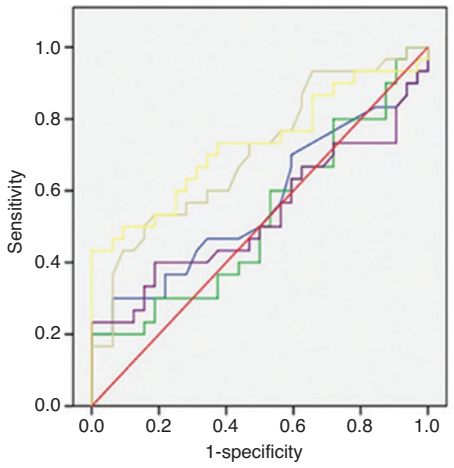
Bold indicates statistical significance.

[a]Kruskal Wallis test was used with significance set at $p < 0.05$.

[b]Comparison between healthy and benign groups significant difference (by Mann-Whitney-U, $p < 0.05$).

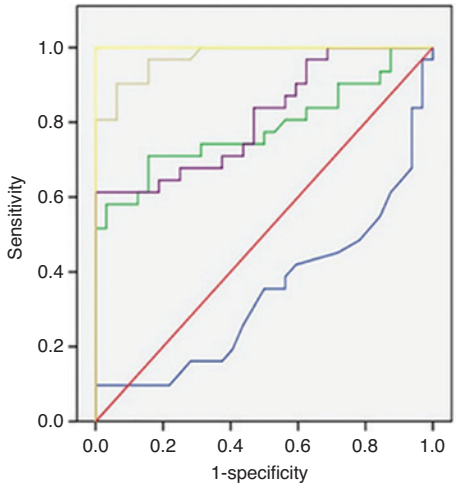
[c]Comparison between healthy and malign groups significant difference (by Mann-Whitney-U, $p < 0.05$).

[d]Comparison between benign and malign groups significant difference (by Mann-Whitney-U, $p < 0.05$).



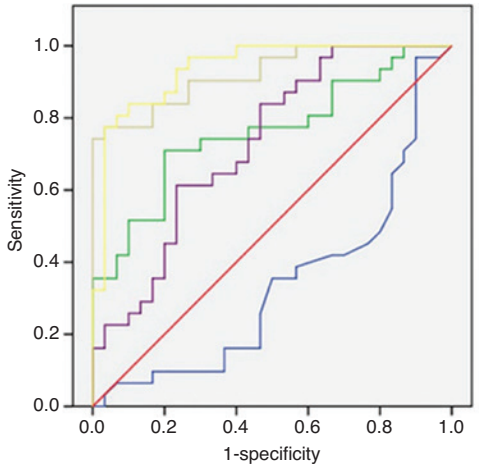
Marker	ROC-AUC	%95 CI	p*	p**
CA125	0.728	0.600–0.833	0.0006	–
LyGDI	0.563	0.416–0.710	0.394	0.0412
Mesothelin	0.524	0.393–0.652	0.7511	0.0458
YKL-40	0.532	0.401–0.660	0.6805	0.0453
HE4	0.705	0.575–0.814	0.0021	0.7938

Figure 3: Receiver-operator characteristics curves for each marker in benign vs healthy group.
*Significance level of the biomarkers vs reference lines, **Significance level of the biomarkers vs CA 125.



Marker	ROC-AUC	%95 CI	p*	p**
CA125	1.000	0.943–1.000	<0.0001	–
LyGDI	0.342	0.204–0.479	0.031	–
Mesothelin	0.785	0.663–0.878	<0.0001	0.0004
YKL-40	0.815	0.697–0.901	<0.0001	0.0006
HE4	0.974	0.899–0.998	<0.0001	0.0865

Figure 4: Receiver-operator characteristics curves for each marker in healthy vs malign group.
*Significance level of the biomarkers vs reference lines, **Significance level of the biomarkers vs CA 125.



Marker	ROC-AUC	%95 CI	p*	p**
CA125	0.937	0.843–0.983	<0.0001	–
LyGDI	0.337	0.198–0.476	0.029	–
Mesothelin	0.759	0.633–0.859	<0.0001	0.0063
YKL-40	0.728	0.599–0.834	0.0004	0.0020
HE4	0.923	0.825–0.975	<0.0001	0.7256

Figure 5: Receiver-operator characteristics curves for each marker in malign vs benign group.
*Significance level of the biomarkers vs reference lines, **Significance level of the biomarkers vs CA 125.

Table 5: Sensitivity of the biomarkers at selected specificity.

Marker	ROC-AUC	Selected specificity		
		80%	90%	95%
		Sensitivity (%)		
CA125	1.000	100	100	100
Mesothelin	0.785	71	58.1	58.1
YKL-40	0.815	64.5	61.3	61.3
HE4	0.974	96.8	90.3	80.7

the "CA125-YKL-40" combination did not alter sensitivity. However, the addition of Mesothelin to the "CA125-HE4" combination increased the sensitivity to 96.8%. A quadruple combination including "CA125-HE4-Mesothelin and YKL-40" yielded a sensitivity of 93.6% at the specificity of 95%. Founded sensitivity and specificity values were higher than all the other biomarkers and combinations.

Discussion

As EOC is a disease that consists of different histological subtypes and as a biomarker CA-125 may not be sufficient to distinguish healthy, benign and malign cases with high sensitivity, a combination of different markers has been suggested to provide greater potential for EOC [9, 26]. Our CA 125 serum levels were similar to other research suggesting the weakness of CA125 in the discrimination of benign ovarian tumor and malign tumor [5, 6].

LyGDI is suggested as a biomarker by some of the researchers for the definition of the malignancy in EOC. Zhen et al. revealed that LyGDI was expressed by EOC cells but not by benign ovarian cells [27]. Stevens et al. also stated that the increased expression of LyGDI in EOC is associated with histological type and grade [28]. Although, our LyGDI results did not indicate a significant difference between the healthy, benign, and malign groups, our data shows compatibility with healthy and benign group's data of Zhen et al. We think that this might be related with the distributions of the histological types of our patient group.

Recent studies have suggested that Mesothelin an attractive biomarker for the diagnosis, prognosis and monitoring target, especially for EOC [29–31]. Madeira et al. performed a systematic review between years of 1990–2015 and meta-analysis to verify the accuracy of mesothelin as a predictor of ovarian cancer. Researchers indicated that serum Mesothelin levels can be considered in the early detection of ovarian carcinoma and postoperative monitoring and they also suggested that it might

Table 6: The diagnostic performance of the biomarkers at determined cut-off values and ROC-AUC values of the biomarkers and combinations.

Marker	Cut-off values	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
CA125	64.3 U/mL	87.1	80	81.8	85.7
Mesothelin	22.97 U/mL	71	80	78.6	72.7
YKL-40	173.25 U/mL	45.2	80	70	58.5
HE4	74.75 pM	83.9	83.3	83.9	83.3

	ROC-AUC (%95 CI)	Selected specificity			p-Value[a]	p-Value[b]
		Sensitivity (%)				
		80%	90%	95%		
CA125	0.943 (0.852–0.986)	87.1	90.3	90.3	**<0.0001**	–
Mesothelin	0.846 (0.731–0.926)	83.9	54.8	38.7	**<0.0001**	**0.048**
YKL-40	0.810 (0.689–0.899)	74.2	35.5	25.8	**<0.0001**	**0.011**
HE4	0.946 (0.857–0.988)	93.6	83.9	77.4	**<0.0001**	0.924
CA125 + HE4	0.958 (0.873–0.993)	93.6	90.3	83.9	**<0.0001**	0.558
CA125 + Mesothelin	0.929 (0.833–0.979)	90.3	90.3	83.9	**<0.0001**	0.297
CA125 + YKL-40	0.941 (0.849–0.985)	93.6	90.3	90.3	**<0.0001**	0.873
CA125 + HE4 + Mesothelin	0.971 (0.892–0.997)	96.8	93.6	90.3	**<0.0001**	0.333
CA125 + HE4 + YKL-40	0.962 (0.879–0.994)	93.6	90.3	83.9	**<0.0001**	0.498
CA125 + YKL-40 + Mesothelin	0.931 (0.836–0.980)	93.6	90.3	87.1	**<0.0001**	0.543
CA125 + HE4 + Mesothelin + YKL-40	0.973 (0.895–0.998)	96.8	93.6	93.6	**<0.0001**	0.301

PPV, positive predictive value; NPV, negative predictive value. [a]Significance level of the biomarkers vs AUC = 0.5, [b]significance level of the biomarkers vs ROC-AUC value of CA125.

be used in combination with CA125 and/or HE4 to reach greater sensitivity for early diagnosis of EOC [20]. Our Mesothelin findings are similar to the studies in which significant differences were found between malign and healthy groups [8, 30]. Ibrahim et al. also stated that the median value of serum Mesothelin was significantly higher in their malign group and it could be considered a significant predictor of early-stage of ovarian cancer. In addition, they proposed that a combination of Mesothelin and CA125 would not provide an advantage over the use of Mesothelin alone [30].

YKL40 which is regarded as an effective biomarker for the diagnosis of ovarian cancer [10]. Our results confirm the other reports in which EOC patients were compared with benign and/or healthy groups and revealed the efficiency of YKL40 in the discrimination between malign and benign groups [32].

HE4 is also considered an important serum biomarker in the early diagnosis of ovarian cancer. Our HE4 data show that HE4 is significantly higher in malign group and there are many studies supporting us [8, 11, 33–35]. The result also revealed that the values of the benign group were significantly higher than the healthy group and these results suggest that HE4 also may be elevated in benign ovarian tumor diseases such as CA125. In addition, the difference of the increases in malign and benign cases, which was 8-fold in our study, may be helpful in the evaluation of EOC and benign ovarian tumor patients. Similarly, fold increases have been reported by different researchers but there are studies that have not found a significant difference between healthy and benign groups [8, 34, 36, 37]. The difference between the studies might be depend on the variation of the histological types in the benign groups. Consequently, our results demonstrated that all of the biomarkers can be used in the discrimination of healthy-malign and benign-malign ovarian tumor and LyGDI, Mesothelin and YKL-40 biomarkers can be used for defining the malign group. Furthermore, the absence of differences between benign and healthy groups in LyGDI, Mesothelin and YKL-40 may provide clinicians with an advantage for diagnosis.

Menopausal status is an important factor affecting the levels of biomarkers which has been revealed by many studies. For instance; Moore et al. showed that CA125 was significantly higher in healthy premenopausal women whereas HE4 was significantly higher in postmenopausal women [38]. Lowe et al. and Johansen et al. also emphasized these effects, especially for Mesothelin and YKL-40 levels [39, 40]. Our results indicate no significant difference between premenopausal and postmenopausal women in the healthy group. However, in the benign group, the median value of Mesothelin and HE4 were higher in postmenopausal women and the median value of CA125 was significantly higher in premenopausal women. Our HE4 and CA125 findings were supports to Moore et al. who emphasized that HE4 and CA125 biomarkers can be used as complementary means [33]. Considering menopausal status, we evaluated the discrimination ability of the biomarkers among the groups (healthy, benign and malign) also.

Considering menopausal status, discrimination ability of the biomarkers among the groups (healthy, benign and malign) revealed that HE4 and CA125 have an ability in the premenopausal period. The most important thing in early diagnosis is to be able to discriminate malign from benign ovarian tumor. Our results in the postmenopausal period indicated that the increases in Mesothelin and YKL-40 which accompanied with elevated values of HE4 and CA125 may provide strong evidence in the discrimination of benign ovarian tumor and malign diseases. Chan et al. also suggested that CA125 and HE4 have the ability to discriminate malign and benign groups both in pre- and postmenopausal periods [14]. Our results may contribute to the literature by indicating the increased diversity of the biomarkers with Mesothelin and YKL-40.

The discrimination capabilities of the biomarkers were also analyzed in early/late stages malignancies. Our HE4 results have displayed a similar pattern with CA125 in the discrimination of the benign group and early stages of EOC. When patients in the late stages were evaluated, not only high median values of HE4 and CA125 (approximately 3 times), but also significantly higher levels of Mesothelin and YKL-40 were observed. In addition, we thought that with the exception of YKL40, the evaluation of the biomarkers together may help to discriminate early/late stages of EOC and benign ovarian tumor. Research indicates that stage related differences of the biomarkers may be related to the synthesis of the biomarkers during the transition periods [5, 41]. Drapkin et al. suggest that HE4 may be used to distinguish early stages of EOC from late stages and the benign group [42]. Different research groups have studied the discrimination sensitivity of both HE4 and CA125. Some of the researchers claimed that, especially in the early stage, the sensitivity of HE4 is higher than CA125. Others studies support the outstanding performance of CA125 [8, 33, 34, 36, 43, 44]. In our study, both biomarkers showed a significant increase related to the severity of disease. Our Mesothelin findings are also agreeable with those of Ibrahim et al. which claimed that Mesothelin was a significant predictor of early stage (stage I/II) ovarian cancer [30]. However, a recent meta-analysis and the study of Abdel-Azeez et al. supporting our results

has been conducted, and concludes that although Mesothelin may not serve alone to discriminate early and late stage in EOC, it might be used in combination with other biomarkers [8, 29]. Our findings showed that YKL-40 and Mesothelin have a capacity to discriminate benign ovarian tumor from the late stage group of EOC. On the other hand, HE4 and CA125 also have the discrimination capacity, regardless of the stage of the disease.

We also evaluated the discrimination capabilities of the biomarkers in different histological types of malignancies. Our results showed that LyGDI, Mesothelin, YKL-40 biomarkers yield different responses for different histological malignancies. Statistical analysis exhibited the significant importance of LyGDI for serous carcinoma, Mesothelin for serous and mixed carcinoma, and YKL-40 for clear cell and mixed carcinoma patients. Our LyGDI results were compatible with the studies of Stevens et al. in which the higher serum levels in serous carcinomas were indicated [28]. Furthermore, our data revealed that CA125 and HE4 can be useful to distinguish clear cell carcinoma, serous and mixed carcinoma and are largely similar to the findings of Yip et al. who also implied that YKL-40 was distinctive for serous and mixed carcinomas [26]. In another study, Høgdall et al. argued that the expression of YKL-40 was mostly abundant in serous carcinoma (n = 84) [45]. Although we found increased YKL-40 levels in serous carcinoma patients, the difference in the benign group was not statistically significant. We believed the different results were due to the limited sample number for serous carcinoma (n = 13).

In the next step, ROC-AUC values were calculated to assess the diagnostic performance of the biomarkers in the discrimination of healthy-benign, healthy-malign, and benign-malign groups. The ROC-AUC analyses demonstrated that the implementation of a combination of CA125 and HE4 biomarkers, regardless of menopausal status, has been more statistically effective in the discrimination of the healthy group from benign ovarian tumor. This may provide advantages to clinicians in diagnosis. Our results were in concordance with results of a study by Azzam et al. in which a combination of CA125 and HE4 were suggested [35].

ROC-AUC analyse results of healthy-malign groups showed that the most successful biomarker was HE4 which has the closest ROC-AUC value to CA125. The other ROC-AUC values implied that LyGDI does not have any distinguishing capacity between healthy and malign groups. However, Mesothelin and YKL-40 also yield closer ROC-AUC values to CA125. Other research focusing on discriminating between healthy and malign groups can be varied due to differences in chosen cut-off values for specificity and sensitivity, categorization and the number of the groups. For instance, contrary to our study, Zhen et al. states that LyGDI has a capacity to distinguish healthy and malign groups [27]. Our ROC-AUC analyses of the biomarkers for benign-malign groups were in concordance with Montagnana et al. in which HE4 did not perform better than CA125 in the discrimination of benign/malign ovarian tumor [37]. However, there are several studies arguing that HE4 outperforms CA125 as a biomarker of ovarian cancer [8, 12, 14, 44]. Our results indicate that the distinguishing capacities of Mesothelin and YKL40 were statistically lower than CA125. Although Dupont et al. and Zou et al. asserted that YKL-40 and CA125 have similar performances, our results confirm the studies that concluded YKL-40 and Mesothelin had a lower ROC-AUC value than that of CA125 respectively [8, 19, 26, 32].

Logistic regression models were used to evaluate the diagnostic performance of each marker and their combinations (double, triple and quadruple) using menopausal effect and biomarker levels as independent variables. The most effective biomarker panel result was obtained from a "CA125-HE4-Mesothelin-YKL-40" combination which had 95% specificity at the highest sensitivity of 93.6%. We advised that a combination including CA125, HE4, Mesothelin, YKL-40 may be useful especially in the discrimination between the benign and malign group. Meanwhile, in comparison to CA125 or other double and triple combinations, this combination improved the sensitivity at 80%, 90%, 95% specificity. Our results confirmed the fact that, the increase in the number of biomarkers may provide better diagnostic performance, especially in the discrimination between benign ovarian tumor and malign diseases. Considering the discrimination capacity of the individual biomarker as true positive or true negative, these results demonstrate that using of quadruple combination may prevent misdiagnosis at preoperative stage.

In summary, the findings of this study suggest that a quadruple biomarker combination including CA125, HE4, Mesothelin, YKL-40 is successful and promising for EOC diagnosis and it is valuable because it is independent of menopausal status which may provide a considerable advantage to clinicians.

This is the first study to investigate different biomarkers and their double, triple and quadruple combinations to distinguish benign ovarian tumor from EOC. As mentioned, since there are many variable related differences (such as groups, biomarkers, menopausal effects, histological types, stage and grade) unfortunately it is quite difficult to make effective one-on-one comparisons of the studies in this field. For this reason, the performance of each study should be assessed within itself. If our study is evaluated in this manner, it is possible to state that a

quadruple combination is more sensitive in the discrimination of benign-malign ovarian tumor than CA125 alone. However, we are aware that the number of study groups should be increased in order to yield a more efficient evaluation of the biomarker combinations corresponding to histological type, stage and menopausal status which might provide a comparison of the results with ROMA or other indexes.

This study provides the first evidence for the combinational use of "CA125, HE4, Mesothelin, YKL-40" as a panel with high specificity and sensitivity in diagnosis or follow-up of ovarian tumor. These findings also show that combining multiple biomarkers may result in better diagnostic performance in distinguishing benign ovarian tumor from EOC.

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