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Serological and immunohistochemical biomarkers for discrimination between benign and malignant ovarian tumors

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Abstract

Background: Ovarian tumors are difficult to diagnose because symptoms are nonspecific, occurring in late stages when the tumor mass reaches large proportions, when complications arise or when dissemination occurs in neighboring organs. Research over the past decades has been aimed at clarifying the mechanisms of ovarian oncogenesis, to identify ways of transforming normal cells into a neoplastic cell, as well as discovering of tumor markers used in the detection of neoplastic processes, along with the synthesis of therapeutic substances, which would influence its development. *Aims*: In our study, we aimed to determine the serum concentrations of cancer antigen 125 (CA125), human epididymis protein 4 (HE4) and the risk of ovarian malignancy algorithm (ROMA) in patients with ovarian tumors, as well as assessing their diagnostic performance. Furthermore, another objective of the study was to identify a concordant relation between serological and immunohistochemical (IHC) biomarkers in supporting and aiding the differentiation between benign and malignant tumors, here including the group of borderline tumors. *Patients, Materials and Methods*: We accomplished a study that included a group of 92 patients diagnosed with ovarian tumors (benign and malignant), who were examined and treated between January 2015 and July 2018. The study was conducted at the Clinics of Obstetrics and Gynecology, "Filantropia" Municipal Hospital of Craiova, Romania. The patients were divided into two groups: the group of patients with benign tumors, subdivided into pre-menopausal (51 cases, 55.43%) and post-menopausal (30 cases, 32.6%) patients, and a group of patients who presented with malignant formation (seven cases with malignant tumors, 7.61% and four cases with borderline tumors, 4.34%, respectively). In parallel, we investigated 35 women as control subjects, who did not have a personal history of ovarian tumors. *Results*: In our study, we have observed that for the analyzed parameters, CA125, HE4, and the ROMA index, significantly higher serum concentrations were detected in the malignant tumor group, when these have been compared to the values obtained for the pre-menopausal and for the post-menopausal subgroup, respectively. The IHC results also showed different expression patterns for the different markers studied. Corroboration of the results of the serological biomarkers with the IHC data is necessary and useful for differentiating borderline tumors and for their final integration as benign or malignant ovarian tumors. This can only be done for the cases with surgical resections, thus having tissue available. *Conclusions*: The serum levels of CA125 and HE4, ROMA index and IHC markers for surgical tissue fragments play a very important role in discriminating and reporting borderline ovarian tumors, as well as benign or malignant ovarian forms. Due to the superior sensitivity and specificity of CA125 and HE4, we can consider these markers as an alternative or additional diagnostic criterion to the ROMA index.

Keywords: cancer antigen 125, HE4, ROMA index, IOTA simple rules, immunohistochemical markers.

Introduction

Non-neoplastic lesions of the ovary are a very common diagnostic entity, mainly due to the appearance of an ovarian tumor, often accompanied by abnormal hormonal manifestations, which can mimic ovarian cancer both at the clinical and intraoperative examination and even at the histopathological (HP) examination [1].

The natural history of ovarian cancer is unknown. The onset of the disease is a silent, asymptomatic process and in most cases accidentally diagnosed. There are not known precursor lesions for ovarian cancer and the amount

of the time required for the tumor located in the ovary to diffuse is unknown. So far, regarding the mechanisms of ovarian carcinogenesis, the hypothesis is accepted that the different tumors are all derived from the ovarian surface epithelium (mesothelium) and that any subsequent metaplastic changes can lead to the appearance of different cell types (serous, endometrioid, clear cells, mucinous and transient, such as Brenner cells) [2, 3].

Ovarian tumors are difficult to diagnose because symptoms are nonspecific, occurring in the late stages when the tumor mass reaches large proportions, when complications occur or when disseminated into neighboring organs [4–7].

According to estimates by the *American Cancer Society* (ACS), ovarian cancer and endometrial cancer are the most common gynecological cancers. These malignancies are defined by a relatively high 5-year survival rate (SR) in the first stage, with the reserved prognosis being characteristic for the advanced-stage diagnosed forms. It was also observed that the 5-year SR in the early stages of ovarian cancer corresponds to 92%, while the 5-year overall SR is less than 50%. This can be explained by the fact that out of all malignant ovarian tumors, only 19% are diagnosed before the extra-ovarian spread due to reduced symptoms before progression. There is clearly an urgent need to develop early detection methods for such diseases [1, 8].

Research over the past decades has been aimed at clarifying the mechanisms of ovarian oncogenesis, at identifying ways to transform normal cells into a neoplastic cell, as well as the discovery of tumoral markers used to detect the neoplastic process and the synthesis of therapeutic substances, which would influence its development. Also, the performed studies have revealed different methods for the early detection of ovarian cancer, methods of differentiating benign or functional cystic tumors from ovarian malignancies.

Thus, the *International Ovarian Tumor Analysis* (IOTA) *Group*, using ultrasound (US), has proposed simple rules to differentiate the two entities, which have been accepted (since they were proposed in 2008) as the best way to preoperatively classify ovarian tumors, due to sensitivity, specificity and diagnostic performance [9–13].

Numerous studies have also highlighted the role of serum concentrations of cancer antigen 125 (CA125) [14–19], human epididymis protein 4 (HE4) [20–25] and later the importance of the *risk of ovarian malignancy algorithm* (ROMA) index, in the preoperative evaluation and diagnosis, as well as in assessing neoplastic progression or recurrences [26–37].

Aim

In our study, we aimed to determine the serum levels of CA125 and HE4 and the ROMA index in patients with ovarian tumors, as well as assessing their diagnostic performance. We also intended to evaluate the usefulness of these serum biomarkers together with the IOTA simple rules and the immunohistochemical (IHC) markers for surgical tissue fragments, to discriminating and reporting borderline ovarian tumors, as well as benign or malignant ovarian forms, in the study area of Dolj County, Romania.

Patients, Materials and Methods

Patients and study protocol

We completed a study that included a group of 92 patients diagnosed with ovarian tumors (benign and malignant). They were examined and treated between January 2015 and July 2018. The study was conducted at the Clinics of Obstetrics and Gynecology, "Filantropia" Municipal Hospital of Craiova, Romania. Patients were evaluated through US, serological, HP and IHC testing and then included in a risk group, which led to the establishment of the therapeutic protocol with or without surgery.

The patients were divided into two groups: a group of patients with benign tumors, subdivided into premenopausal (51 cases, 55.43%) and post-menopausal (30 cases, 32.6%) patients, and a group of patients who presented with malignant formation (seven cases with malignant tumors, 7.61% and four cases with borderline tumors, 4.34%, respectively). In parallel, we investigated 35 women as control subjects, who did not have a personal history of ovarian tumors.

Demographic characteristics and pathological personal history data for each subject included in the study were derived from their medical records. For each patient enrolled in the study, we have compiled an initial case assessment, on which we have included cases in this study.

Inclusion and exclusion criteria in the study

Patients between the ages of 19 and 71 years old were selected, who presented with ovarian tumors, clinically and diagnosed by way of US, in the gynecology clinics but also in other specialties (gastroenterology and internal medicine) after having demonstrated non-specific abdominal symptoms. The US aspects, as a criterion for inclusion in the study, were represented by the appearance of suspicious images. During the aforementioned period, the patients enrolled in the study completed a medical record, signed informed consent and were subjected to an investigation protocol that included a set of quantifiable parameters. The clinical and serological evaluation was considered and the subsequent management was adapted for each case.

Samples collection

Samples from all patients included in the study were collected in the morning, during fasting, by venous puncture, with the blood collected in vacutainers without anticoagulant, to obtain the serum. After harvesting, the vacutainers were centrifuged at 3500 rpm for 15 minutes, after which the separated serum was aliquoted into several cryopreservation tubes and then stored at -80°C, until the time that the serological markers were dosed. For the dosing of serum markers, they were removed from the ice, an equal number of cryopreservation tubes were brought to room temperature, thus avoiding freezing–re-freezing cycles that can distort the structure of proteins.

Immunological investigations

The serum marker analysis was performed by an immunoenzymatic technique, enzyme-linked immunosorbent assay (ELISA), quantitative and sandwich method, according to the working protocol provided by the manufacturer. Serum CA125 and HE4 were determined

by using the Fujirebio Diagnostics AB (Göteborg, Sweden) enzyme immunoassay kit (HE4 and CanAg® CA125 EIA) (*https://www.fujirebio-europe.com/sites/default/files/EIA_Kit_Catalogue_September_2017_FDAB-034_r0.pdf*).

ELISA is a competitive solid-phase immunoenzymatic method, based on the sandwich technique, where calibrators, controls, and patient samples are incubated (at the same time) with biotinylated monoclonal antibodies (MoAbs) directed to CA125 and HE4 in Streptavidin-plated wells. During incubation, CA125 and HE4 will be absorbed into the Streptavidin-coated wells by biotinylated MoAbs. The wells are then washed and incubated with the second MoAb labeled with an enzyme in a conjugate [*e.g.*, Horseradish Peroxidase (HRP)], which will bind to the complex formed between the first two reagents, also through a specific immune response. Following another washing step, the substrate is then added to each well, which contains a chromogenic reagent, 3,3',5,5'-Tetramethylbenzidine (TMB). During incubation, an enzymatic reaction takes place, with the enzyme retained in the well acting degradative on the chromogenic substrate, which is colorless. Degradation of the substrate by the enzyme induces oxidation of the chromogen, which subsequently becomes a colored compound (a blue color will appear, the intensity of which is directly proportional to the amount of CA125/HE4 in the sample needed to be analyzed). The stop solution is then added to stop the reaction in all wells and, as a result, the color turns yellow with different intensities. Color intensity is measured by reading the optical densities from the threshold detection value of the kit using a spectrophotometer at a suitable wavelength (450 nm, or optionally at 620 nm and 405 nm).

Values obtained were expressed in U/mL for CA125 (normal values <35 U/mL) and pmol/L for HE4 (normal <140 pmol/L), respectively.

ROMA index – calculation formula

ROMA index [38] is used to estimate the risk of occurrence about ovarian cancer, both in pre-menopausal women, as well as those in the post-menopausal period, at which pelvic tumor masses are discovered. Initially, the predictive index (PI) is calculated separately for pre-menopausal and post-menopausal women, in accordance with the values obtained for CA125 and HE4, using equations (LN – natural logarithm):

(*1*) to pre-menopausal period women:

$$12 + 2.38 \times LN(HE4) + 0.0626 \times LN(CA125)$$

(*2*) to post-menopausal period women:

$$8.09 + 1.04 \times LN(HE4) + 0.732 \times LN(CA125)$$

After calculating the value for PI, according to the category of patients, this value will be entered in the following equation:

$$ROMA\ [\%] = exp(PI) / [1 + exp(PI)] \times 100$$

As an interpretation: in pre-menopausal women, a ROMA index ≥12.5% and in post-menopausal women, a value of the ROMA index ≥14.4%, respectively, are associated with an increased risk of ovarian cancer.

HP and IHC investigations

The collected tissue fragments of the patients taken in the study were sent for the processing and elaboration of the HP diagnosis to the Laboratory of Pathological Anatomy, "Filantropia" Municipal Hospital of Craiova. The tissues were fixed in 10% neutral buffered formalin at room temperature and routinely processed. Microtome-cut sections were stained with Hematoxylin–Eosin (HE) and Masson's trichrome (MT). The HP diagnosis was elaborated, which was next completed by IHC methods. Immunohistochemistry was performed at the University of Medicine and Pharmacy of Craiova, within the Research Center for Microscopic Morphology and Immunology. A panel of seven biomarkers was utilized, namely (Table 1): cytokeratin 7 (CK7), in order to identify primitive epithelial tumors; cytokeratin 20 (CK20) for the differential diagnosis of a metastasis originating in the gastrointestinal tract; estrogen receptor (ER) and progesterone receptor (PR), respectively, the immunolabeling showing the presence of these receptors on the tumor cells; tumor protein 53 (p53), tumor suppressor gene showing cells that have undergone certain functional changes, which they have achieved an antiapoptotic capacity; Ki67 cell proliferation factor – Ki67 index of cell proliferation and mitotic activity; B-cell lymphoma 2 (Bcl-2) marker that highlights anti-apoptotic transformations and leads to pre-neoplastic and neoplastic changes.

After antigen retrieval and non-specific antigen binding sites block with a 3% skimmed milk solution, the slides were incubated overnight, at 4ºC, with the primary antibodies. Next day, the excess solution was washed in phosphate-buffered saline (PBS), and a species-specific HRP-labeled secondary was incubated on the slides for one further hour (Nikirei Histofine, Japan). After washing, the color was developed with the 3,3'-Diaminobenzidine (DAB) substrate.

Table 1 – ***Immunohistochemical panel of antibodies***

Antibody	Manufacturer	Clone	Antigenic exposure	Secondary antibody	Dilution	Labeling
Anti-CK7	Dako	OV-TL 12/30	Citrate, pH 6	Monoclonal mouse anti-human CK7	1:50	Covering epithelium of the reproductive tract
Anti-CK20	Dako	Ks20.8	Citrate, pH 6	Monoclonal mouse anti-human CK20	1:50	Glandular digestive epithelium
Anti-ER	Dako	1D5	EDTA, pH 9	Monoclonal mouse anti-human ERα	1:50	Estrogen receptors
Anti-PR	Dako	PgR 636	EDTA, pH 9	Monoclonal mouse anti-human PR	1:50	Progesterone receptors
Anti-p53	Dako	DO-7	EDTA, pH 9	Monoclonal mouse anti-human p53 protein	1:50	Tumor protein 53
Anti-Ki67	Dako	MIB-1	EDTA, pH 9	Monoclonal mouse anti-human Ki67	1:50	Cell proliferation factor
Anti-Bcl-2	Dako	124	EDTA, pH 9	Monoclonal mouse anti-human Bcl-2 oncoprotein	1:50	Oncoprotein

CK: Cytokeratin; ER: Estrogen receptor; PR: Progesterone receptor; p53: Tumor protein 53; Ki67: Cell proliferation factor; Bcl-2: B-cell lymphoma 2; EDTA: Ethylenediaminetetraacetic acid.

The obtained slides were imaged with the Nikon 600 optical microscope coupled with a Nikon 5 Mp color charge-coupled device (CCD) camera.

US examination

Transabdominal and transvaginal US (TVU) play an important role in assessing ovarian tumor masses. TVU evaluation has steered the detection of morphological parameters (sepsis, papillary excitations, increased echogenicity, presence or absence of ascites) that could provide us with a useful positive predictive value in the diagnostic evaluation of ovarian lesions. Also, the examination was completed by studying tumor vascular characteristics using the Doppler color US. US, especially through the transvaginal approach, is the method of choice in the detection of ovarian tumors. Although the sonographic characteristics of the annexes are not standardized and reproducible, US may point to HP diagnosis, despite it being unable to substitute it by establishing suggestive elements.

The ovarian tumor formations analyzed were characterized according to the IOTA simple rules [9], which grouped these formations into two categories: malignant (*M*) ovarian formations and benign (*B*) ovarian formations. When it comes to interpretation, it is recommended to classify the tumor as malignant based on the following aspects: the presence of at least one of the *M* rules, to which is added the absence of a *B* rule. Instead, identifying the presence of at least one of the *B* rules, to which the absence of *M* rules is added, recommends that the tumor mass be considered benign. If both *M* and *B* rules are identified to be present, then it is considered that the tumor mass cannot be classified into a category. Also, a situation may arise where no rules can be identified, in which case the tumor formation cannot be classified (Table 2).

Table 2 – ***IOTA simple rules and US rules to be able to include ovarian tumor formations in the two categories: benign ovarian tumor and malignant ovarian tumor, respectively***

***B* rules**	***M* rules**
B1 – Unilocular cyst	M1 – Solid but irregular tumors
B2 – Presence of solid components with the largest solid component <7 mm	M2 – Ascites
B3 – The presence of the acoustic shadow	M3 – Highlighting at least four papillary structures
B4 – Detection of a smooth multilocular tumor having the largest diameter <100 mm	M4 – Detection of solid, irregular, multilocular tumors with the largest diameter ≥100 mm
B5 – Avascular	M5 – Intense vascularization

IOTA: *International Ovarian Tumor Analysis*; US: Ultrasound.

Ethical approval

The study was carried out in full compliance with the ethical principles contained in the *Declaration of Human Rights* adopted in Helsinki, in 1975, as revised in 2008. All individual participants voluntarily joined this study and provided written informed consent. To achieve the objectives proposed in this study, we obtained the ethical approval from the University of Medicine and Pharmacy of Craiova – Committee of Ethics, Academic and Scientific Deontology, No. 103/30.09.2013.

Statistical analysis

Data collected from patients' medical records were stored and processed by utilizing the Microsoft Excel and Data Analysis module, while statistical processing was performed using GraphPad Prism 5 Trial Version. In order to compare the means of two or more groups and to determine the significance of the differences between groups, we used nonparametric tests, Mann–Whitney or Kruskal–Wallis test. The correlations between the mean concentrations of the different markers/indices (CA125, HE4, ROMA index), according to the analyzed subgroup (a subgroup of pre-menopausal and post-menopausal patients), as well as the expression of the strong correlation between two parametrical, were performed using the Pearson's correlation coefficient. The difference between the two sample means was considered significant at the 95% significance level ($p \leq 0.05$); all tests were two-sided.

The diagnostic accuracies of the investigated markers were evaluated using the analysis of the receiver operating characteristic (ROC) curves. In order to ascertain the diagnostic performance, we used the area under the ROC curve (AUC), accompanied by the 95% confidence interval (95% CI) and the *p*-value calculated for the statistical difference between the calculated AUC and AUC=0.5 (marker weak discriminator). The threshold values corresponding to the highest performance were determined and for different optimal cut-off values, obtained at each marker, we calculated the indicators which assess the quality of the analyzed markers: sensitivity (Sn), specificity (Sp) and Youden's index (Sn + Sp – 1). The ROC curve analysis was useful for comparing the diagnostic accuracy of the determination of serum concentrations of CA125, HE4, the ROMA index, and IOTA simple rules, following the diagnosis of ovarian tumor formations.

Results

Clinical characteristics of the study patients

In this pilot study, we have included 92 patients with ovarian tumors. The patients were divided into two groups, the group of patients with benign tumor (81 patients, 88.04%), subdivided in subgroup 1, with pre-menopausal patients (51 cases, 55.43%) and subgroup 2, with post-menopausal patients (30 cases, 32.6%), and the group of patients who presented with malignant formation (seven cases with malignant tumors, 7.61% and four cases with borderline tumors, 4.34%, respectively).

The mean age was 35.18 years [standard deviation (SD) 6.83] in the pre-menopausal subgroup, 61.28 years (SD 3.51) in the post-menopausal cases and 646.71 years (SD 7.11), respectively, for the malignant tumor group. The *chi*-square test denotes a statistically significant difference between the mean age of the two-analyzed subgroups (p†: $p<0.0001$), 93.33% of the patients were older than 50 years. We also found statistically significant differences between the mean age of the malignant tumor group and the post-menopausal group (p‡: $p=0.027$, $p<0.05$) (Table 3).

Table 3 – ***Demographic characteristics of the study patients***

Parameters	Benign tumor		Malignant tumor (*n*=11)
	Pre-menopausal (n=51)	*Post-menopausal (n=30)*	
Age [years]			
19–24	17 (33.33%)	–	–
25–34	15 (29.41%)	–	–
35–39	19 (37.26%)	–	1 (9.09%)
40–49	–	2 (6.67%)	–
50–59	–	9 (30%)	4 (36.36%)
60–69	–	17 (56.67%)	5 (45.46%)
70–79	–	2 (6.67%)	1 (9.09%)
Mean±SD	35.18±6.83	61.284±3.51	64.71±7.11
Demographic distribution			
Urban	40 (78.54%)	10 (33.33%)	8 (72.73%)
Rural	11 (21.57%)	20 (66.67%)	3 (27.27%)
Length of lactation [months]	7.67	10.44	12.35
Age of menarche [years]	12.2	14.1	14.1
Oral contraception	34	17	5
HRT	17	10	4
Increased BMI	36	18	7
Associated pathology			
Nutrition disorders	8	6	–
Cardiovascular diseases	14	5	4
Hypothyroidism	5	6	–
Benign mammary pathology	10	9	3

n: No. of cases; SD: Standard deviation; HRT: Hormone replacement therapy; BMI: Body mass index; *Statistically significant *p*-value; *p*†: Statistically significant differences between the mean age of the two analyzed subgroups; *p*‡: Statistically significant differences between the mean age of the malignant tumor group and post-menopausal group.

Analyzing the two subgroups according to the area of residence (rural or urban) parameter, we have noticed that in the pre-menopausal subgroup, urban patients were predominant (40 cases, 78.43%), unlike the post-menopausal subgroup where rural patients predominated (20 cases, 66.67%). Distribution by age group revealed a higher incidence in group 19–24 years, 17 cases (33.33%) and in group 25–34 years, 15 cases (29.41%), suggesting a high incidence of ovarian pathology in the fertile period of the woman (Table 3).

These higher rates of occurrence of ovarian tumor formations, as well as the correlation with these age ranges, can be explained by the more frequent infectious pathology, the early onset of sexual life, the increase of the incidence of sexually transmitted diseases, the relationships with several partners, as well as by the increased incidence of pelvic inflammatory disease, which seems to be involved in late ovarian neoplasms.

Biomarkers values

In our study, we determined that the mean values of CA125, HE4, and ROMA index, ascertained in the group of patients with malignancies, were significantly higher statistically when compared to the values obtained in the benign tumor formations subgroups (Table 4). The mean value of CA125 in the malignant tumor group was 89.75 U/mL (95% CI 72.31–107.19), significantly higher than the value of the post-menopausal subgroup at 24.12 U/mL (95% CI 19.89–28.35) (p<0.0001) and 20.76 U/mL (95% CI 14.91–26.61) (p<0.0001) for the pre-menopausal subgroup, respectively.

HE4 in the malignant tumor group had mean level of 216.12 pmol/L (95% CI 188.53–243.71), which was statistically higher than the value of the post-menopausal subgroup at 85.46 pmol/L (95% CI 67.62–103.3) (p<0.0001) and 47.35 pmol/L (95% CI 36.72–57.98) (p<0.0001), respectively, for pre-menopausal subgroup.

Also, the ROMA index mean value in the malignant tumor group was 38.55% (95% CI 29.13–47.97), in the post-menopausal subgroup was 11.75% (95% CI 10.48–13.02) (p<0.0003) and 6.89% (95% CI 4.68–9.10) in pre-menopausal subgroup, respectively, the differences being statistically significant (p<0.0001).

Table 4 – ***Mean serum concentrations of investigated serological markers (CA125, HE4 and ROMA index) in ovarian tumor groups***

Parameters	Control group	Benign tumor formations groups		Malignant tumor formations group	*p*-value	
		Pre-menopausal subgroup	*Post-menopausal subgroup*		*p*†	*p*‡
Patients (*n*)	35	51	30	11		
CA125 [U/mL] (mean±SD)	14.06±5.23	20.76±5.85	24.12±4.23	89.75±17.44	<0.0001*	<0.0001*
HE4 [pmol/L] (mean±SD)	35.38±9.42	47.35±10.63	85.46±17.84	216.12±27.59	<0.0001*	<0.0001*
ROMA index (mean±SD)	3.25±1.04	6.89±2.21	11.75±1.27	38.55±9.42	<0.0003*	<0.0001*
IOTA index [%]	–	2.09±1.94	2.1±1.73	7.09±5.36	<0.0001*	0.0151*

CA125: Cancer antigen 125; HE4: Human epididymis protein 4; ROMA: Risk of ovarian malignancy algorithm; IOTA: *International Ovarian Tumor Analysis*; *n*: No. of cases; SD: Standard deviation; *Statistically significant *p*-value; *p*†: mean concentrations indicate statistically significant differences in the malignant tumor group and the post-menopausal subgroup; *p*‡: mean concentrations indicate statistically significant differences in the malignant tumor group and the pre-menopausal subgroup.

HP and IHC results

The microscopic features were grouped based on the qualitative analysis in benign serous ovarian (Figure 1) and low malignant potential (borderline) forms together with low and high-grade malignant ovarian tumors (Figure 2).

For all the antibodies used, we analyzed the epithelial, not the stromal, immunolabeling. IHC analysis was quantified at the nuclear and cytoplasmic levels by qualitative reactions, as follows: (–) negative reaction; (+) weakly positive reaction; (++) moderately positive reaction; (+++) intensely positive reaction (Table 5).

Table 5 – ***Reaction to immunohistochemical markers***

Antibody	CK7	CK20	ER	PR	p53	Ki67	Bcl-2
Benign ovarian tumors	+	---	+	+	+	---	+
Borderline ovarian tumors	++	---	++	++	+	+	++
Malignant ovarian tumors	+++	---	+++	+++	+++	++	++

CK: Cytokeratin; ER: Estrogen receptor; PR: Progesterone receptor; p53: Tumor protein 53; Ki67: Cell proliferation factor; Bcl-2: B-cell lymphoma 2; "---": Negative reaction; "+": Weakly positive reaction; "++": Moderately positive reaction; "+++": Intensely positive reaction.

The IHC staining patterns differ when it comes to the markers which were studied. In benign serous ovarian tumors, a moderately positive cytoplasmic reaction can be observed for CK7 (Figure 1C), ER (Figure 1E), Bcl-2 (Figure 1H), a nuclear positive focal reaction for Ki67 (Figure 1G), moderately positive nuclear reaction for p53 (Figure 1D), intensely positive nuclear reaction for PR (Figure 1F), cytoplasmic negative reaction for CK20 (Figure 1I). In malignant ovarian tumors, a moderately positive cytoplasmic reaction can be observed for Ki67 (Figure 2G), intensely positive cytoplasmic reaction for CK7 (Figure 2C), moderately positive nuclear reaction for Bcl-2 (Figure 2H), intensely positive nuclear reaction for p53 (Figure 2D), as well as for hormonal receptors ER (Figure 2E) and PR (Figure 2F), respectively.

Immunostaining for CK20 (Figure 2I) was negative at the cytoplasmic level, which confirmed the non-digestive profile of the ovarian tumors.

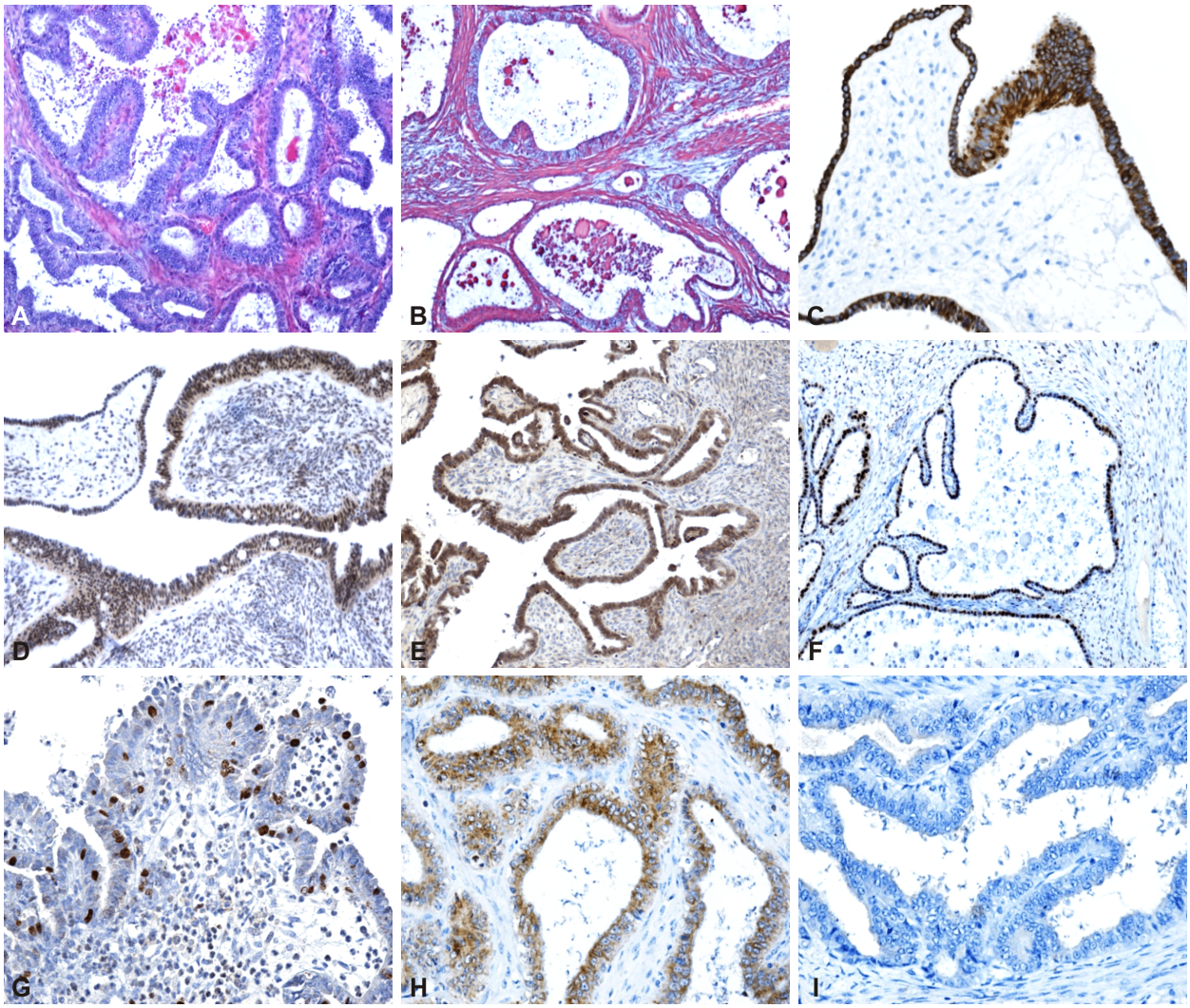

Figure 1 – ***Microscopic features of benign and low-grade borderline serous ovarian tumors: (A) HE staining, ×100; (B) Masson's trichrome staining, ×100; (C) Moderately positive intracytoplasmic and membranous immunostaining for anti-CK7 antibody, ×200; (D) Moderately positive nuclear immunostaining for anti-p53 antibody, especially in borderline type, ×100; (E) Moderately positive nuclear immunostaining for anti-ER antibody, ×100; (F) Intensely positive nuclear immunostaining for anti-PR antibody, ×100; (G) Nuclear positive focal immunostaining of around 10% for anti-Ki67 antibody, ×100; (H) Moderately positive intracytoplasmic immunostaining for anti-Bcl-2 antibody, ×200; (I) Negative membranous and cytoplasmic immunostaining for anti-CK20 antibody, ×200. HE: Hematoxylin–Eosin; CK7: Cytokeratin 7; p53: Tumor protein 53; ER: Estrogen receptor; PR: Progesterone receptor; Ki67: Cell proliferation factor; Bcl-2: B-cell lymphoma 2; CK20: Cytokeratin 20.***

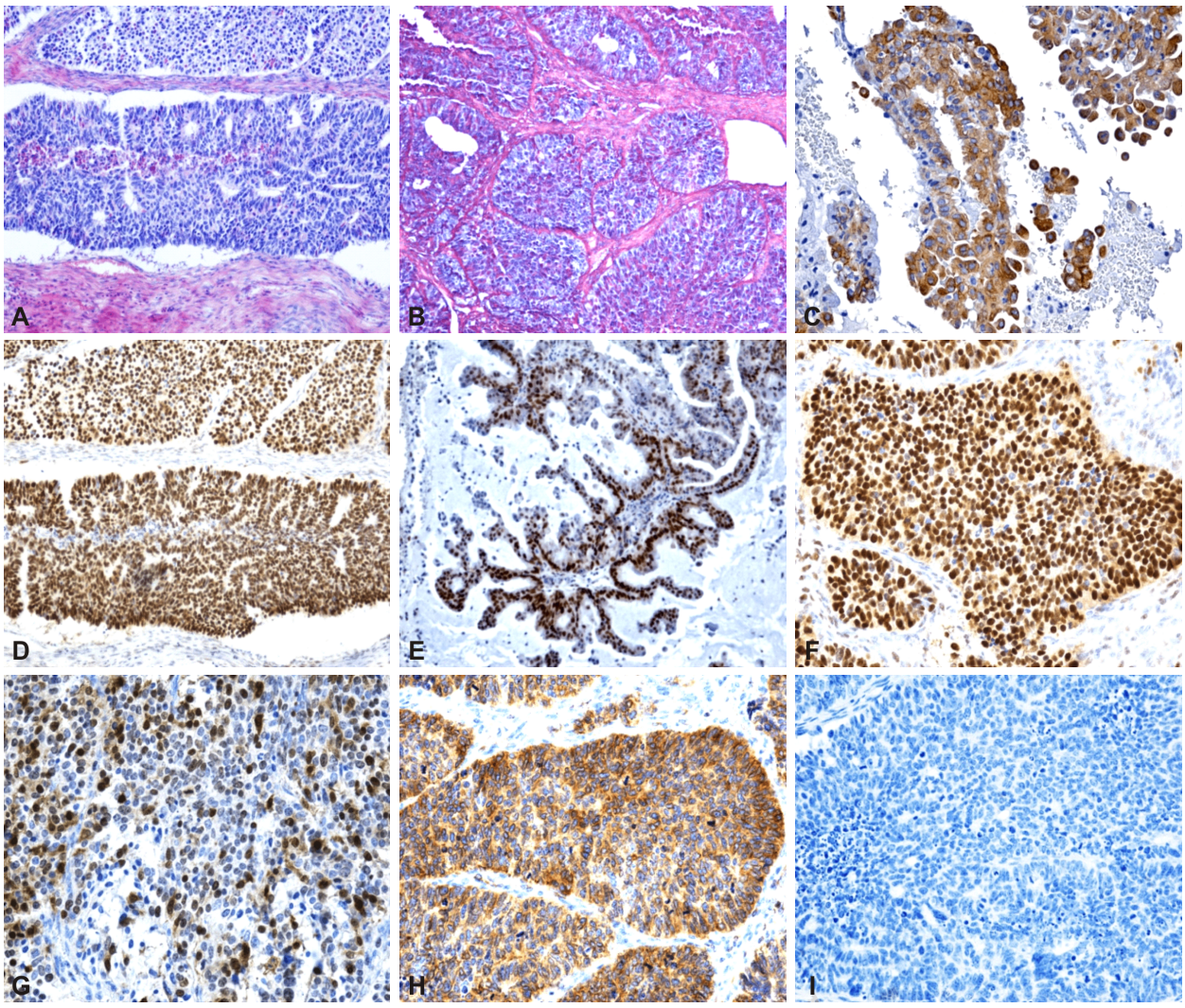

Figure 2 – ***Microscopic features of low- and high-grade serous ovarian tumors: (A) HE staining, ×100; (B) Masson's trichrome staining, ×100; (C) Intensely positive intracytoplasmic and membranous immunostaining for anti-CK7 antibody, ×200; (D) Intensely positive nuclear immunostaining for anti-p53 antibody, ×100; (E) Strongly positive nuclear immunostaining for anti-ER antibody, ×100; (F) Intensely positive nuclear immunostaining for anti-PR antibody, ×200; (G) A 30% Ki67 positivity index – immunostaining for anti-Ki7 antibody, ×100; (H) Moderately positive intracytoplasmic immunostaining for anti-Bcl-2 antibody, ×200; (I) Negative membranous and cytoplasmic immunostaining for anti-CK20 antibody, ×200. HE: Hematoxylin–Eosin; CK7: Cytokeratin 7; p53: Tumor protein 53; ER: Estrogen receptor; PR: Progesterone receptor; Ki67: Cell proliferation factor; Bcl-2: B-cell lymphoma 2; CK20: Cytokeratin 20.***

Correlations between CA125, HE4, ROMA index, IOTA index and the IHC markers

Concentrations of both biomarkers, CA125 and HE4, were not correlated with each other in all of the analyzed subgroups (Table 6). We also found, in the pre-menopausal subgroup, a highly statistically significant correlation between HE4 serum concentrations and ROMA index (strong positive correlation – r=0.737, p=0.0005), in the post-menopausal subgroup a statistically significant correlation between CA125 serum concentrations and IOTA index (positive correlation – r=0.382, p=0.028), and highly statistically significant correlations between CA125 and HE4 serum concentrations (strong positive correlation – r=0.643, p=0.014) and between CA125 serum concentrations and ROMA index (strong positive correlation – r=0.857, p=0.024) in the malignant tumor group.

Diagnostic performance of CA125, HE4, ROMA index and IOTA simple rules

Comparing the ROC curves and AUC, for all patients included in the study, for the four analyzed markers (Table 7), we could see that the highest diagnostic accuracy in distinguishing patients with benign ovarian disorders from ovarian neoplasia can be done using the ROMA algorithm, with the highest detection (100% accuracy to diagnose properly an affected person), followed by CA125 (99.2% accuracy), with better performance than HE4 (98.3% accuracy) but weaker than IOTA index (81% accuracy).

The ROC analysis revealed that the ROMA index indicated the presence of ovarian tumors with an accuracy of 100%, using the percentage of 19.05% as a threshold value, with the power to distinguish between cases presenting ovarian tumor formations (benign and malignant) from the healthy considered cases (95% CI: 1.000–1.000, p<0.0001). In this instance, we obtained the following

probability ratios (likelihood ratio – LR) of the positive and negative results, calculated based on optimal and specific cut-off values: LR(+) = 21.25 and LR(–) = 1.08, where both the sensitivity and specificity have a maximum percentage of 100%; Youden's index was 1.000.

Table 6 – ***Correlations between IOTA index, CA125, HE4, ROMA index***

Parameters	IOTA index [%]	CA125 [U/mL]	HE4 [pmol/L]	ROMA index
Pre-menopausal				
IOTA index [%]		*r*=0.079	*r*=0.003	*r*=0.208
		p=0.578	*p*=0.985	*p*=0.138
CA125 [U/mL]			*r*=0.106	*r*=0.003
			p=0.453	*p*=0.983
HE4 [pmol/L]				*r=0.737*
				*p=0.0005**
Post-menopausal				
IOTA index [%]		*r=0.382*	*r*=0.268	*r*=0.101
		*p=0.028**	*p*=0.132	*p*=0.576
CA125 [U/mL]			*r*=0.053	*r*=-0.105
			p=0.769	*p*=0.560
HE4 [pmol/L]				*r*=-0.165
				p=0.359
Malignant tumor group				
IOTA index [%]		*r*=0.643	*r*=-0.143	*r*=0.071
		p=0.139	*p*=0.783	*p*=0.906
CA125 [U/mL]			*r*=0.250	*r=0.643*
			p=0.595	*p=0.014**
HE4 [pmol/L]				*r=0.857*
				*p=0.024**

IOTA: *International Ovarian Tumor Analysis*; CA125: Cancer antigen 125; HE4: Human epididymis protein 4; ROMA: Risk of ovarian malignancy algorithm; *r*: Pearson's correlation coefficient; *Statistically significant correlations.

In the case of CA125, the calculated threshold value with the power to distinguish between the cases presenting ovarian tumor formations (benign and malignant) from the healthy considered cases was 34.07 U/mL and, with this value, the diagnostic affinity of CA125 was 99.2% (95% CI: 0.974–1.01, $p<0.0001$). The LR of the positive and negative results, calculated based on optimal and specific cut-off values were LR(+) = 42.5 and LR(−) = 1.02, with a maximum sensitivity of 100% and lower specificity than the ROMA index – 98.82%; Youden's index was 0.882.

Furthermore, for HE4, the calculated threshold value with the power to distinguish between the cases presenting ovarian tumor formations (benign and malignant) from the healthy considered cases was 152.6 pmol/L and using this value, the diagnostic affinity was 98.8% (95% CI: 0.965–1.011, $p<0.0001$). The LR of the positive and negative results, calculated based on optimal and specific cut-off values were LR(+) = 28.33 and LR(−) = 1.01, with the same sensitivity encountered in the case of ROMA index and CA125 of 100%, and a specificity lower than that of CA125 – 97.28%; Youden's index was 0.728.

In the subgroup analysis, the diagnostic performance (AUC) was 0.98 for CA125, 1.000 for HE4 and ROMA index and 0.806 for IOTA index, in the pre-menopausal women subgroup, which showed significant statistical differences among the parameters. Also, in the post-menopausal women subgroup, AUC was 1 for CA125 and ROMA index, 0.97 for HE4 and 0.813 for IOTA index, with significant statistical differences among the parameters.

Table 7 – ***Diagnostic accuracy of the investigated markers***

Parameter	AUC accuracy	Cut-off value	*p*-value	Sensitivity [%]	Specificity [%]	Youden's index
Total						
CA125	0.992	34.07	<0.0001	100.00	98.82	0.882
HE4	0.983	152.60	<0.0001	100.00	97.28	0.728
ROMA index	*1.000*	*19.05*	*<0.0001*	*100.00*	*100.00*	*1.000*
IOTA simple rules	0.810	2.395	0.009	85.71	64.71	0.504
Pre-menopausal						
CA125	0.980	33.86	<0.0001	100.00	96.15	0.615
HE4	*1.000*	*130.10*	*<0.0001*	*100.00*	*100.00*	*1.000*
ROMA index	*1.000*	*18.01*	*<0.0001*	*100.00*	*100.00*	*1.000*
IOTA simple rules	0.806	2.395	0.012	85.71	63.28	0.489
Post-menopausal						
CA125	*1.000*	*49.24*	*<0.0001*	*100.00*	*100.00*	*1.000*
HE4	0.970	152.60	<0.0001	100.00	96.37	0.637
ROMA index	*1.000*	*20.23*	*<0.0001*	*100.00*	*100.00*	*1.000*
IOTA simple rules	0.813	2.34	0.007	85.71	66.31	0.520

AUC: Area under the curve; CA125: Cancer antigen 125; HE4: Human epididymis protein 4; ROMA: Risk of ovarian malignancy algorithm; IOTA: *International Ovarian Tumor Analysis*.

Discussions

Screening for ovarian cancer is a challenge for clinicians, being a subject which is still under-explored at both the national level and internationally, although many criteria have been established, the results are still quite controversial, with more studies being needed. An early preoperative diagnosis, by non-invasive methods of benign/malignant nature of the annexes, facilitating the choice of an optimal therapeutic attitude, including conservative attitude, is necessary due to a very important aspect being the high incidence of these formations during the reproductive period [39].

About the pathology seen in gynecological oncology,

due to the antigenic potential of the surface epithelium, there is a multitude of tumor markers, with related studies designated as having high relevance and being the subject of numerous internal and international scientific communications.

A histological screening for ovarian cancer is not possible given the difficult access to ovarian tissue. Thus, a serum test for screening or biomarker platforms would be helpful to help diagnose cancer, detect recurrence and as a means to monitor response to treatment.

When it comes to establishing a panel of biomarkers useful in cancer detection, especially in the early stages of the disease, imaging studies continue to play a critical role in confirming or invalidating these biomarker tests.

In Romania, ovarian cancer is the third cause of death by malignant pathology, being the fifth place in terms of incidence after breast, cervical, colorectal and lung cancer. The global incidence of ovarian cancer is 2% and in Romania, it reaches 4.6% [40], likely due to the lack of an early detection program.

TVU is one of the most commonly used methods for early detection of ovarian cancer. Campbell *et al.* used the first transabdominal US to assess ovarian cancer in asymptomatic patients. In their study, despite obtaining a maximum sensitivity of 100% for a transabdominal US, the specificity was slightly lower (97.7%) and predictive value of 1.5% was noted. Such findings led to the hypothesis that transabdominal US is not sufficiently effective in distinguishing between benign and malignant cystic tumor formations and is, therefore, less suited to assessing ovarian tumors than TVU [41]. Finkler *et al.* [42] and Bourne *et al.* [43] shed light on the efficacy of advanced US along with serum CA125, leading to an increase in the early detection of both ovarian cancers as well as a decreased mortality rate in ovarian cancer.

The *IOTA Group* made an important contribution in developing and validating two models of logistic regression based on US (LR1 and LR2), which estimate the risk of malignancy in the adnexal tumors. In clinical practice, the detected adjacent masses can be classified according to the "simple rules" of IOTA. IOTA index is based on the identification of simple features during conventional US examination and has a high sensitivity. However, the rate of false positives is high. Approximately 40–50% of women with malignant and inconclusive IOTA findings have benign ovarian tumors [44]. Testa *et al.* have reported an analysis of studies in which they found that all IOTA strategies (logistic regression models 1 and 2, LR1, LR2; simple descriptors, SRs; and combinations of the above) proved to be superior when compared to the risk of malignancy index (RMI) for predicting malignancy, having achieved considerable sensitivities between 90–96% and also specificities of 74–79% [45].

Initial data related to CA125 functions was first reported in 1981 by Bast, who found that CA125 acts as an actively secreted transmembrane protein. Serum levels of more than 80% have been reported in women diagnosed with epithelial ovarian neoplasm [46], for which studies have shown that it is the only tumor marker significantly correlated with epithelial ovarian neoplasm [47–49]. Despite many promising features of CA125 for the diagnosis and monitoring of ovarian lesions, there are also several disadvantages. It has been observed that in women with non-malignant gynecological pathology, such as benign ovarian cysts, as well as endometriosis, CA125 serum levels tend to be higher than normal values, agreed to be unanimously accepted at 35 U/mL. These important findings, to which false-positive results were added, as well as reduced specificity, led to the notion of limiting CA125's use as a diagnostic and prognostic marker for ovarian cancer [50].

HE4 is a marker for early detection of ovarian cancer. Normal ovarian tissue has a minimal expression of HE4, whereas overexpression occurs in ovarian cancer patients. When combined with CA125, HE4 raises the level of sensitivity for ovarian cancer detection. HE4 is consistently expressed in ovarian cancer patients and has demonstrated increased sensitivity and specificity above CA125 as a single marker. An elevated serum level for HE4 and CA125 would suggest ovarian cancer, while an elevated CA125 without an increase in HE4 could indicate a benign condition. A high CA125 and a normal HE4 level suggest the presence of another type of cancer (*e.g.*, endometrial). Studies show the benefit of the combined use of CA125 and HE4 as a diagnostic test, useful in discriminating between benign and malignant ovarian tumors [36].

The ROMA algorithm is a simple score that has excellent diagnostic performance for malignant ovarian tumor detection in post-menopausal and less in pre-menopausal patients.

In our study, relating to the analyzed parameters, CA125, HE4 and ROMA algorithm, we have obtained significantly higher serum concentrations in the malignant tumor group when compared with the values obtained for the pre-menopausal and respectively for the post-menopausal subgroup.

Post-data processing, it can be seen that in the pre-menopausal subgroup HE4 serum values correlated strongly, positively and were statistically significant, concerning the ROMA index. By contrast, when evaluating the post-menopausal subgroup, serum values of the ROMA index were statistically significant and positively correlated with those of CA125.

Our results are the first of their kind in the literature in our region, Dolj County, Romania. These results are comparable to those obtained in various other centers for multicentre studies or meta-analyses.

Our study aims to analyze the diagnostic performance of these parameters to differentiate between patients with benign ovarian disorders from patients with ovarian neoplasia. The data we have obtained suggests that both HE4 and CA125 have lower diagnostic accuracy compared to the ROMA algorithm. As such, for patients, overall, it can be stated that the ideal way to differentiate between those with benign ovarian disorders from those with ovarian neoplasia, would be to do so using the ROMA algorithm, with the highest detection (100% accuracy to diagnose properly an affected person), followed by CA125 (99.2% accuracy), with better performance than HE4 (98.3% accuracy) but weaker than IOTA index (81% accuracy).

Analyzing the subgroups, we can say that diagnostic algorithm models can be developed to determine the risk of malignancy, to be more reliable and easier to apply.

Thus, in the pre-menopausal subgroup, it was observed that the model of the diagnostic association between the ROMA index and HE4, in the way of discriminating between patients with benign ovarian disorders from those with ovarian neoplasia, both parameters had 100% sensitivity and specificity. On the other hand, in the post-menopausal subgroup, the association between the ROMA index and CA125 was observed and in these cases, for both parameters, sensitivities, and specificities were also at 100%. Due to the superior sensitivity and specificity of CA125 and HE4, we can consider these markers as a viable alternative or further diagnostic addition to the ROMA algorithm.

Corroboration of the results of the serological biomarkers with the IHC ones is necessary and useful for differentiating benign and malignant ovarian tumors. This can only be done for operating cases. This study used the HP classification of ovarian tumors from 2014 [51].

The IHC profile of the antibodies used corresponds largely to that of the literature. Thus, in the case of benign and low-grade ovarian tumors of our studied cases, CK7 has a weak or moderately positive reaction. In the case of the malignant tumors of the studied cases, the reactivity of CK7 is similar to that described in the literature, being intensely positive [52].

The negative reaction for CK20, both cytoplasmic and nuclear, is common in our study and the results described by other authors [52]. Immunostaining in benign tumors for ER and PR hormonal receptors is moderately or intensely expressed, while other authors have identified their low expression [53, 54]. In the malignant tumors of our study, there is an intensely positive reaction for ER and PR, results that are similar to those found in the literature [53, 55], associated with p53 overexpression and increased tumor proliferation by the Ki67 immunomarking, respectively [56]. Immunolabeling for p53 shows a weakly or moderately positive nuclear reaction for the benign tumors studied and intensely positive for the malignant ones. Other authors indicate a negative or poorly positive nuclear marking in the benign ones [57, 58]. By contrast, for malignancies, the results are similar to our study, according to those published by other authors [56, 59–61]. The IHC results for Ki67 immunoexpression of our study for ovarian (benign and malignant) tumors are similar to the results reported by other authors [56, 62], respectively lower Ki67 index in benign tumors and moderately increased in malignant ones. The results of our study on the expression of Bcl-2 apoptotic markers in benign tumors are weakly positive, while in borderline and malignant tumors, it is moderately positive. The study of the specialized literature mentions high values with diffuse intense staining in high-grade malignancies [61, 63–65].

Conclusions

The serum levels of CA125 and HE4, ROMA index and IHC markers for surgical tissue fragments play a very important role in discriminating and reporting borderline ovarian tumors, as well as benign or malignant ovarian forms. Due to the superior sensitivity and specificity of CA125 and HE4, we can consider these markers as an alternative or additional diagnostic criterion to the ROMA algorithm. Our study, as well as other studies conducted in various centers and areas around the world, has limitations related to its retrospective, descriptive nature and its data collection – a small sample size. Taking into account that the data collected and processed by us was taken over from the "Filantropia" Municipal Hospital, Craiova, Romania, we tend to believe that the results obtained can be underestimated. Further studies are needed to reproduce our findings in different ethnic groups with larger sample size.

Conflict of interests

The authors declare that they have no conflict of interests.

Authors' contribution

Veronica Gheorman and Anda Lorena Dijmărescu equally contributed to the manuscript.

References

[1] American College of Obstetricians and Gynecologists Committee on Gynecologic Practice. Committee Opinion No. 477: the role of the obstetrician–gynecologist in the early detection of epithelial ovarian cancer. Obstet Gynecol, 2011, 117(3):742–746.

[2] Dehari R, Kurman RJ, Logani S, Shih IeM. The development of high-grade serous carcinoma from atypical proliferative (borderline) serous tumors and low-grade micropapillary serous carcinoma: a morphologic and molecular genetic analysis. Am J Surg Pathol, 2007, 31(7):1007–1012.

[3] Kurman RJ, Shih IeM. The origin and pathogenesis of epithelial ovarian cancer: a proposed unifying theory. Am J Surg Pathol, 2010, 34(3):433–443.

[4] Buys SS, Partridge E, Black A, Johnson CC, Lamerato L, Isaacs C, Reding DJ, Greenlee RT, Yokochi LA, Kessel B, Crawford ED, Church TR, Andriole GL, Weissfeld JL, Fouad MN, Chia D, O'Brien B, Ragard LR, Clapp JD, Rathmell JM, Riley TL, Hartge P, Pinsky PF, Zhu CS, Izmirlian G, Kramer BS, Miller AB, Xu JL, Prorok PC, Gohagan JK, Berg CD; PLCO Project Team. Effect of screening on ovarian cancer mortality: the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Randomized Controlled Trial. JAMA, 2011, 305(22):2295–2303.

[5] Carney ME, Lancaster JM, Ford C, Tsodikov A, Wiggins CL. A population-based study of patterns of care for ovarian cancer: who is seen by a gynecologic oncologist and who is not? Gynecol Oncol, 2002, 84(1):36–42.

[6] Goff BA, Lowe KA, Kane JC, Robertson MD, Gaul MA, Andersen MR. Symptom triggered screening for ovarian cancer: a pilot study of feasibility and acceptability. Gynecol Oncol, 2012, 124(2):230–235.

[7] Goff BA, Mandel LS, Melancon CH, Muntz HG. Frequency of symptoms of ovarian cancer in women presenting to primary care clinics. JAMA, 2004, 291(22):2705–2712.

[8] Zhou Y, Irwin ML, Risch HA. Pre- and post-diagnosis body mass index, weight change, and ovarian cancer mortality. Gynecol Oncol, 2011, 120(2):209–213.

[9] Timmerman D, Testa AC, Bourne T, Ameye L, Jurkovic D, Van Holsbeke C, Paladini D, Van Calster B, Vergote I, Van Huffel S, Valentin L. Simple ultrasound-based rules for the diagnosis of ovarian cancer. Ultrasound Obstet Gynecol, 2008, 31(6):681–690.

[10] Garg S, Kaur A, Mohi JK, Sibia PK, Kaur N. Evaluation of IOTA simple ultrasound rules to distinguish benign and malignant ovarian tumours. J Clin Diagn Res, 2017, 11(8): TC06–TC09.

[11] Nunes N, Ambler G, Foo X, Naftalin J, Widschwendter M, Jurkovic D. Use of IOTA simple rules for diagnosis of ovarian cancer: meta-analysis. Ultrasound Obstet Gynecol, 2014, 44(5): 503–514.

[12] Tongsong T, Tinnangwattana D, Vichak-Ururote L, Tontivuthikul P, Charoenratana C, Lerthiranwong T. Comparison of effectiveness in differentiating benign from malignant ovarian

masses between IOTA simple rules and subjective sonographic assessment. Asian Pac J Cancer Prev, 2016, 17(9): 4377–4380.
[13] Tantipalakorn C, Wanapirak C, Khunamornpong S, Sukpan K, Tongsong T. IOTA simple rules in differentiating between benign and malignant ovarian tumors. Asian Pac J Cancer Prev, 2014, 15(13):5123–5126.
[14] Moro F, Pasciuto T, Djokovic D, Di Legge A, Granato V, Moruzzi MC, Mancari R, Zannoni GF, Fischerova D, Franchi D, Scambia G, Testa AC. Role of CA125/CEA ratio and ultrasound parameters in identifying metastases to the ovaries in patients with multilocular and multilocular–solid ovarian masses. Ultrasound Obstet Gynecol, 2019, 53(1):116–123.
[15] Chen F, Shen J, Wang J, Cai P, Huang Y. Clinical analysis of four serum tumor markers in 458 patients with ovarian tumors: diagnostic value of the combined use of HE4, CA125, CA19-9, and CEA in ovarian tumors. Cancer Manag Res, 2018, 10: 1313–1318.
[16] Zheng LE, Qu JY, He F. The diagnosis and pathological value of combined detection of HE4 and CA125 for patients with ovarian cancer. Open Med (Wars), 2016, 11(1):125–132.
[17] Boylan KLM, Geschwind K, Koopmeiners JS, Geller MA, Starr TK, Skubitz APN. A multiplex platform for the identification of ovarian cancer biomarkers. Clin Proteomics, 2017, 14:34.
[18] Lenhard M, Stieber P, Hertlein L, Kirschenhofer A, Fürst S, Mayr D, Nagel D, Hofmann K, Krocker K, Burges A. The diagnostic accuracy of two human epididymis protein 4 (HE4) testing systems in combination with CA125 in the differential diagnosis of ovarian masses. Clin Chem Lab Med, 2011, 49(12):2081–2088.
[19] Kim YM, Whang DH, Park J, Kim SH, Lee SW, Park HA, Ha M, Choi KH. Evaluation of the accuracy of serum human epididymis protein 4 in combination with CA125 for detecting ovarian cancer: a prospective case-control study in a Korean population. Clin Chem Lab Med, 2011, 49(3):527–534.
[20] Ferraro S, Panteghini M. Making new biomarkers a reality: the case of serum human epididymis protein 4. Clin Chem Lab Med, 2019, 57(9):1284–1294.
[21] Simmons AR, Baggerly K, Bast RC Jr. The emerging role of HE4 in the evaluation of epithelial ovarian and endometrial carcinomas. Oncology (Williston Park), 2013, 27(6):548–556.
[22] Xi QP, Pu DH, Lu WN. Research on application value of combined detection of serum CA125, HE4 and TK1 in the diagnosis of ovarian cancer. Eur Rev Med Pharmacol Sci, 2017, 21(20):4536–4541.
[23] Li L, Wan J, Cai G, Yuan L, Liang J, Song J, Wang F, Liu M. Value of serum human epididymis secretory protein 4 as a marker for differential diagnosis of malignant and benign gynecological diseases of patients in southern China. Clin Chim Acta, 2016, 459:170–176.
[24] Chan KK, Chen CA, Nam JH, Ochiai K, Wilailak S, Choon AT, Sabaratnam S, Hebbar S, Sickan J, Schodin BA, Sumpaico WW. The use of HE4 in the prediction of ovarian cancer in Asian women with a pelvic mass. Gynecol Oncol, 2013, 128(2): 239–244.
[25] Hellström I, Raycraft J, Hayden-Ledbetter M, Ledbetter JA, Schummer M, McIntosh M, Drescher C, Urban N, Hellström KE. The HE4 (WFDC2) protein is a biomarker for ovarian carcinoma. Cancer Res, 2003, 63(13):3695–3700.
[26] Wei SU, Li H, Zhang B. The diagnostic value of serum HE4 and CA-125 and ROMA index in ovarian cancer. Biomed Rep, 2016, 5(1):41–44.
[27] Cho HY, Park SH, Park YH, Kim HB, Kang JB, Hong SH, Kyung MS. Comparison of HE4, CA125, and risk of ovarian malignancy algorithm in the prediction of ovarian cancer in Korean women. J Korean Med Sci, 2015, 30(12):1777–1783.
[28] Zhang P, Wang C, Cheng L, Zhang P, Guo L, Liu W, Zhang Z, Huang Y, Ou Q, Wen X, Tian Y. Comparison of HE4, CA125, and ROMA diagnostic accuracy: a prospective and multicenter study for Chinese women with epithelial ovarian cancer. Medicine (Baltimore), 2015, 94(52):e2402.
[29] Huy NVQ, Van Khoa V, Tam LM, Vinh TQ, Tung NS, Thanh CN, Chuang L. Standard and optimal cut-off values of serum CA-125, HE4 and ROMA in preoperative prediction of ovarian cancer in Vietnam. Gynecol Oncol Rep, 2018, 25:110–114.
[30] Gizzo S, Berretta R, Di Gangi S, Guido M, Zanni GC, Franceschetti I, Quaranta M, Plebani M, Nardelli GB, Patrelli TS. Borderline ovarian tumors and diagnostic dilemma of intraoperative diagnosis: could preoperative He4 assay and ROMA score assessment increase the frozen section accuracy? A multicenter case-control study. Biomed Res Int, 2014, 2014:803598.
[31] Goff BA, Agnew K, Neradilek MB, Gray HJ, Liao JB, Urban RR. Combining a symptom index, CA125 and HE4 (triple screen) to detect ovarian cancer in women with a pelvic mass. Gynecol Oncol, 2017, 147(2):291–295.
[32] Oranratanaphan S, Wanishpongpan S, Termrungruanglert W, Triratanachat S. Assessment of diagnostic values among CA-125, RMI, HE4, and ROMA for cancer prediction in women with nonfunctional ovarian cysts. Obstet Gynecol Int, 2018, 2018:7821574.
[33] Romagnolo C, Leon AE, Fabricio ASC, Taborelli M, Polesel J, Del Pup L, Steffan A, Cervo S, Ravaggi A, Zanotti L, Bandiera E, Odicino FE, Scattolo N, Squarcina E, Papadakis C, Maggino T, Gion M. HE4, CA125 and risk of ovarian malignancy algorithm (ROMA) as diagnostic tools for ovarian cancer in patients with a pelvic mass: an Italian multicenter study. Gynecol Oncol, 2016, 141(2):303–311.
[34] Sandri MT, Bottari F, Franchi D, Boveri S, Candiani M, Ronzoni S, Peiretti M, Radice D, Passerini R, Sideri M. Comparison of HE4, CA125 and ROMA algorithm in women with a pelvic mass: correlation with pathological outcome. Gynecol Oncol, 2013, 128(2):233–238.
[35] Zhang L, Chen Y, Wang K. Comparison of CA125, HE4, and ROMA index for ovarian cancer diagnosis. Curr Probl Cancer, 2019, 43(2):135–144.
[36] Montagnana M, Danese E, Ruzzenente O, Bresciani V, Nuzzo T, Gelati M, Salvagno GL, Franchi M, Lippi G, Guidi GC. The ROMA (Risk of Ovarian Malignancy Algorithm) for estimating the risk of epithelial ovarian cancer in women presenting with pelvic mass: is it really useful? Clin Chem Lab Med, 2011, 49(3):521–525.
[37] Cymbaluk-Ploska A, Chudecka-Glaz A, Surowiec A, Pius-Sadowska E, Machalinski B, Menkiszak J. MMP3 in comparison to CA 125, HE4 and the ROMA algorithm in differentiation of ovarian tumors. Asian Pac J Cancer Prev, 2016, 17(5): 2597–2603.
[38] Moore RG, Jabre-Raughley M, Brown AK, Robison KM, Miller MC, Allard WJ, Kurman RJ, Bast RC, Skates SJ. Comparison of a novel multiple marker assay *vs* the risk of malignancy index for the prediction of epithelial ovarian cancer in patients with a pelvic mass. Am J Obstet Gynecol, 2010, 203(3):228.e1–228.e6.
[39] Vaughan S, Coward JI, Bast RC Jr, Berchuck A, Berek JS, Brenton JD, Coukos G, Crum CC, Drapkin R, Etemadmoghadam D, Friedlander M, Gabra H, Kaye SB, Lord CJ, Lengyel E, Levine DA, McNeish IA, Menon U, Mills GB, Nephew KP, Oza AM, Sood AK, Stronach EA, Walczak H, Bowtell DD, Balkwill FR. Rethinking ovarian cancer: recommendations for improving outcomes. Nat Rev Cancer, 2011, 11(10):719–725.
[40] Peltecu G, Trope GC (eds). Gynecologic oncology. Romanian Academy Publishing House, Bucharest, 2010, 3–13, 143–159, 357–462.
[41] Campbell S, Bhan V, Royston P, Whitehead MI, Collins WP. Transabdominal ultrasound screening for early ovarian cancer. BMJ, 1989, 299(6712):1363–1367.
[42] Finkler NJ, Benacerraf B, Lavin PT, Wojciechowski C, Knapp RC. Comparison of serum CA 125: clinical impression, and ultrasound in the preoperative evaluation of ovarian masses. Obstet Gynecol, 1988, 72(4):659–664.
[43] Bourne TH, Campbell S, Reynolds KM, Whitehead MI, Hampson J, Royston P, Crayford TJ, Collins WP. Screening for early familial ovarian cancer with transvaginal ultrasonography and colour blood flow imaging. BMJ, 1993, 306(6884): 1025–1029.
[44] Timmerman D, Ameye L, Fischerova D, Epstein E, Melis GB, Guerriero S, Van Holsbeke C, Savelli L, Fruscio R, Lissoni AA, Testa AC, Veldman J, Vergote I, Van Huffel S, Bourne T, Valentin L. Simple ultrasound rules to distinguish between benign and malignant adnexal masses before surgery: prospective validation by IOTA group. BMJ, 2010, 341:c6839.
[45] Testa A, Kaijser J, Wynants L, Fischerova D, Van Holsbeke C, Franchi D, Savelli L, Epstein E, Czekierdowski A, Guerriero S, Fruscio R, Leone FP, Vergote I, Bourne T, Valentin L, Van

Calster B, Timmerman D. Strategies to diagnose ovarian cancer: new evidence from phase 3 of the multicentre international IOTA study. Br J Cancer, 2014, 111(4):680–688.
[46] Bast RC Jr. CA 125 and the detection of recurrent ovarian cancer: a reasonably accurate biomarker for a difficult disease. Cancer, 2010, 116(12):2850–2853.
[47] Gadducci A, Cosio S, Carpi A, Nicolini A, Genazzani AR. Serum tumor markers in the management of ovarian, endometrial and cervical cancer. Biomed Pharmacother, 2004, 58(1):24–38.
[48] Ginath S, Menczer J, Fintsi Y, Ben-Shem E, Glezerman M, Avinoach I. Tissue and serum CA125 expression in endometrial cancer. Int J Gynecol Cancer, 2002, 12(4):372–375.
[49] Novac L, Niculescu M, Iliescu D, Manolea M, Dijmarescu L, Comanescu A, Tudorache S, Cernea N, Căpitănescu R, Novac M. Chorial villositary vasculogenesis correlated with ultrasound morpho-functional markers of the trophoblast. In: ***. Advance in perinatal medicine. Conference Proceedings of 22nd European Congress of Perinatal Medicine, May 26–29, 2010, Granada, Spain, Monduzzi Editore, 2010, 143–148.
[50] Yurkovetsky Z, Ta'asan S, Skates S, Rand A, Lomakin A, Linkov F, Marrangoni A, Velikokhatnaya L, Winans M, Gorelik E, Maxwell GL, Lu K, Lokshin A. Development of multimarker panel for early detection of endometrial cancer. High diagnostic power of prolactin. Gynecol Oncol, 2007, 107(1):58–65.
[51] Seidman JD, Bell DA, Crum CP, Gilks CB, Kurman RJ, Levine DA, Longacre TA, Pasini B, Riva C, Sherman ME, Shih IM, Singer G, Soslow R, Vang R. Tumours of the ovary: Epithelial tumours – Serous tumours. In: Kurman RJ, Carcangiu ML, Herrington CS, Young RH (eds). World Health Organization (WHO) Classification of tumours of female reproductive organs. 4th edition, vol. 6, WHO Classification of Tumours, International Agency for Research on Cancer (IARC) Press, Lyon, France, 2014, 17–18.
[52] Cathro HP, Stoler MH. Expression of cytokeratins 7 and 20 in ovarian neoplasia. Am J Clin Pathol, 2002, 117(6):944–951.
[53] Escobar J, Klimowicz AC, Dean M, Chu P, Nation JG, Nelson GS, Ghatage P, Kalloger SE, Köbel M. Quantification of ER/PR expression in ovarian low-grade serous carcinoma. Gynecol Oncol, 2013, 128(2):371–376.
[54] Ho SM. Estrogen, progesterone and epithelial ovarian cancer. Reprod Biol Endocrinol, 2003, 1:73.
[55] Lindgren PR, Cajander S, Bäckström T, Gustafsson JA, Mäkelä S, Olofsson JI. Estrogen and progesterone receptors in ovarian epithelial tumors. Mol Cell Endocrinol, 2004, 221(1–2):97–104.
[56] Sylvia MT, Kumar S, Dasar P. The expression of immunohistochemical markers estrogen receptor, progesterone receptor, Her-2-neu, p53 and Ki67 in epithelial ovarian tumors and its correlation with clinicopathologic variables. Indian J Pathol Microbiol, 2012, 55(1):33–37.
[57] Aktaş IY, Buğdayci M, Usubütün A. Expression of p16, p53, CD24, EpCAM and calretinin in serous borderline tumors of the ovary. Turk Patoloji Derg, 2012, 28(3):220–230.
[58] Shao HL, Shen DH, Xue WC, Li Y, Yu YZ. [Clinicopathologic analysis and expression of cyclin D1 and p53 of ovarian borderline tumors and carcinomas]. Zhonghua Fu Chan Ke Za Zhi, 2007, 42(4):227–232.
[59] Köbel M, Reuss A, du Bois A, Kommoss S, Kommoss F, Gao D, Kalloger SE, Huntsman DG, Gilks CB. The biological and clinical value of p53 expression in pelvic high-grade serous carcinomas. J Pathol, 2010, 222(2):191–198.
[60] Marinaş MC, Mogoş DG, Simionescu CE, Stepan A, Tănase F. The study of p53 and p16 immunoexpression in serous borderline and malignant ovarian tumors. Rom J Morphol Embryol, 2012, 53(4):1021–1025.
[61] Mishra SK, Crasta JA. An immunohistochemical comparison of p53 and Bcl-2 as apoptotic and MIB1 as proliferative markers in low-grade and high-grade ovarian serous carcinomas. Int J Gynecol Cancer, 2010, 20(4):537–541.
[62] Shen XX, Yu L, Bi R, Yang WT. [Clinicopathologic study and immunohistochemistry comparison of Pax2, p53 and Ki-67 in low- and high-grade ovarian serous carcinomas]. Zhonghua Bing Li Xue Za Zhi, 2011, 40(8):511–516.
[63] Preda SA, Nechita F, Comanescu MC, Albulescu DM, Tuculina MJ, Docea AD, Burada E, Vasile RC, Mitroi M. Evaluation of bone turnover and DXA markers in premature ovarian failure. Rev Chim (Bucharest), 2019, 70(6):2054–2057.
[64] Dijmarescu AL, Vrabie S, Manolea MM, Novac M, Iliescu D, Tudorache S, Camen I. Preoperative risk assessment of malignancy in ovarian tumors and correlation with histopathological outcome. Conference Proceedings of 6th Congress of the Ultrasound Society in Obstetrics and Gynecology/34th Fetus as a Patient International Congress, May 16–19, 2018, Bucharest, Romania, 167–171.
[65] Stoenescu VE, Niculescu M, Novac L, Manolea MM, Tomescu PI, Dijmărescu AL, Novac MB, Tudorache Ş, Iliescu DG. Immunohistochemical reaction of the glandular epithelium in endometrial hyperplasia compared to endometrial carcinoma. Rom J Morphol Embryol, 2017, 58(3):791–800.

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