Medical University - Pleven

Faculty of Health Care

Department of Clinical Laboratory, Clinical Immunology and Allergology

Dr. Valeria Zdravkova Racheva

Alteration in the levels of some proteins in women with ovarian tumors

ABSTRACT

of a dissertation

to acquire an educational and scientific degree „Doctor"

Doctoral program: „Clinical Laboratory"

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Scientific supervisors: Prof. Dr. Adelaide Ruseva, d.m.

Assoc. Prof. Dr. Pavlina Yordanova-Laleva, d.m.

Reviewers: Prof. Dr. Milena Velizarova, d.m.

Prof. Dr. Tania Deneva, d.m.

Pleven 2023

The dissertation is written on 180 standard pages and illustrated with 79 figures, 65 tables and 5 appendices.

The literary sources used include 160 titles - 4 in Cyrillic and 156 in Latin.

The numbers of the figures and tables in the abstract do not correspond to the numbers of the figures and tables in the dissertation.

The author is a full-time doctoral student at the Department of Clinical Laboratory, Clinical Immunology and Allergology, Faculty of Health Care, Medical University - Pleven and works as an assistant at the Department of Medical Physics, Medical Biophysics, Preclinical and Clinical Sciences, Faculty of Pharmacy at Medical University - Pleven and as Head of the Clinical Laboratory of the Saint Marina UMBAL - Pleven.
The dissertation was discussed, accepted and proposed for public defense at a meeting of the Departmental Council of the Department of "Clinical Laboratory, Clinical Immunology and Allergology" at the MU - Pleven, held on 20.10.2023.

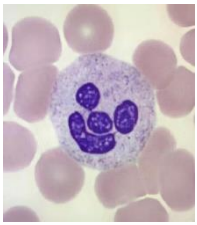
Scientific jury composed of:
Chairman: Assoc.Prof. Dr. Irena Gencheva- Angelova, d.m.

Members: Acad. Dr. Grigor Gorchev, d.m.n.
Prof. Dr. Milena Velizarova, d.m.
Prof. Dr. Tania Deneva, d.m.
Prof. Dr. Iana Bocheva, d.m.

Reserve Members: Assoc. Prof. Dr. Angel Jordanov, d.m.
Prof. Dr. Krasimira Ikonomova, d.m.

The public defense of the dissertation work will take place at. in the hall of the MU – Pleven.

The materials used in the dissertation work are published and available on the website of the Medical University of Pleven: http://www.mu-pleven.bg/index.php/bg/



„The teacher can open the door, but the student must enter himself!“
Zen

With huge thanks to:
- my scientific supervisors - for the inspiration, patience, faith and constructive criticism during my scientific work!
- my family for being my biggest judge and best friend, for supporting my dreams and helping them become reality!

CONTENTS

ABBREVIATIONS USED IN THE TEXT

OC Ovarian carcinoma
FDA Food and Drug Administration
ROMA algorithm for risk of ovarian malignancy
TTR, Prealb - transthyretin, prealbumin
TFR - transferrin
ApoA1LP - ApoA1 lipoprotein
β_2 MG - β_2 microglobulin
CA 125 - carcinoma antigen 125
Mean - average arithmetic value
Me - median
Xmin - minimum value of the sign
Xmax - maximum value of the attribute
SD - standard deviation
95% CI, CI - confidence interval
ROC curve - Receiver Operating Characteristic curve
AUC - Area Under the Curve area under the ROC curve
Sp - specificity (Specificity)
Sn - sensitivity (Sensitivity)
PPV - positive predictive value
NPV - negative predicted value
CG - Control group
α - level of significance
p - guarantee probability
rpb - point-biserial coefficient
r - Pearson's correlation coefficient
J - Youden index
VIF - Multicollinearity Inflation Factor
Exp(B) - odds ratio
S.E. - mean stochastic (standard) error

INTRODUCTION

Ovarian tumors are a heterogeneous group and individual representatives differ from each other in their morphological structure and biological characteristics. They can be seen among women of all age groups.

The majority of ovarian tumors are benign (most commonly ovarian cysts) and can cause problems due to their size, proximity to various organs, and pain or discomfort. The rest of them are malignant and their early diagnosis is of great importance to improve the survival of patients.

The gold standard for distinguishing benign from malignant ovarian tumors is histopathological biopsy. It is an invasive method and obtaining a result from it requires a long period of time.

Ovarian carcinoma (OC) has the highest mortality among gynecological carcinomas in developed countries, and its five-year survival rate has remained low over the past 50 years. The main reasons for this are the lack of early clinical symptoms, diagnosis at an advanced stage as well as ineffective screening programs.

The present work aims to identify serum markers and panels of markers that could aid in the preoperative differentiation of benign from malignant ovarian tumors.

When reviewing the specialized literature on the subject, we identified the following unresolved issues and debatable points:

1. The determination of the concentration of CA125 in serum is widely used, but it is insufficient as a single biomarker for early OK and has the greatest importance for monitoring therapy and detecting disease recurrence.

2. The FDA-approved OVA1 test and ROMA test have advantages and disadvantages. Further testing and analysis of the pros and cons are needed before any definitive conclusions can be drawn regarding these tests.

3. A wide range of serological markers for OC have been investigated. A perfect and reliable biomarker - stable, highly specific and sensitive, inexpensive - is currently

lacking. To date, no method for early diagnosis and screening has been approved. Therefore, the discovery and validation of new biomarkers for this disease gives hope for its early detection.

4. From all studied biomarkers demonstrating association with OC, the biomarkers with the highest informative value should be selected, individually or combined in multi-biomarker panels. It is necessary to meet the following requirements:

● the biomarker must show measurable changes in non-invasive samples obtained from patients with early-stage or preclinical disease.

● the biomarker should be informative for all histological and pathological subtypes of OC

● the biomarker must be sufficiently reliable

5. Plasma protein levels show predictable changes in response to advanced malignancy. The blood proteome contains an invaluable supply of plasma proteins, which reliably selected and appropriately combined represent a good way to diagnose ovarian tumors.

6. An effective screening strategy is needed to detect early-stage disease that has the ability to distinguish between benign tissue and early malignantly transformed counterparts, with adequate diagnostic sensitivity and specificity. This would significantly reduce OC mortality.

The analysis of data from the literature and our own observations in daily clinical practice show the relevance of the problem of modern diagnosis of OC through new markers and marker biopanels.

The possibilities of serum proteins for the diagnosis of ovarian tumors, as well as their application for preoperative differentiation of malignant from benign ovarian tumors, have not been considered in the Bulgarian scientific literature. These unsolved problems in clinical practice gave us the reason to choose the topic of the dissertation work, with the aim of as quickly as possible, ahead of the invasive method - histopathological biopsy, to support the making of correct and targeted

decisions on the diagnosis of ovarian tumor and differential diagnosis of benign and malignant ovarian tumors.

For this purpose, we followed and analyzed the changes in the concentrations of five serum proteins - transthyretin (prealbumin TTR), transferrin (TFR), ApoA1 lipoprotein (ApoA1LP), β_2 microglobulin (β_2 MG) and carcinoembryonic antigen 125 (CA 125) in women with ovarian tumors .

OBJECTIVE AND TASKS

Objective: To follow the change in the concentration of five serum proteins - prealbumin (transthyretin), transferrin, ApoA1lipoprotein, β2microglobulin and CA125 in women with ovarian tumors and to study their informative value and their diagnostic possibilities for proving ovarian tumor, as well as for the preoperative differentiation of ovarian carcinoma from benign ovarian diseases.

Tasks:

1. To analyze and compare the serum concentrations of TTR, TFR, Apo A1 LP, β_2 MG and CA125 in the target group of women with ovarian tumors and healthy controls and to determine whether there is a correlation between the changes in the five proteins and the menopausal status of the patients.
2. To study and analyze the diagnostic reliability and determine the prognostic value of the studied laboratory biomarkers for proving ovarian tumors.
3. To analyze and compare the serum concentrations of TTR, TFR, Apo A1 LP, β_2 MG and CA125 in group A1 (women with ovarian carcinoma) and group A2 (women with ovarian cysts) and to determine whether there is a correlation between the changes in the five proteins and the menopausal status of the patients.
4. To analyze and compare the serum concentrations of TTR, TFR, Apo A1 LP, β_2 MG and CA125 in group A1 (women with ovarian carcinoma) and to determine whether there is a correlation between the changes in the five proteins and the stage of OK- early (I and II) or late (III and IV).
5. To investigate and analyze the diagnostic reliability and determine the prognostic value of the studied laboratory biomarkers for distinguishing ovarian carcinoma from benign ovarian cysts.
6. Based on the obtained results, to propose a diagnostic algorithm (formula) with the studied biomarkers in ovarian tumors, to be applied in the preoperative diagnosis and differentiation of malignant from benign ovarian tumors.

MATERIAL

1. Study design

In nature, the study is prospective with respect to the biomarkers studied, comparative with the use of a control group, diagnostic and non-interventional with respect to the patients studied.

2. Study patients

2.1. Criteria for inclusion and exclusion of the researched persons for shaping the target groups

A. Inclusion criteria - women over 18 years of age, with a documented ovarian tumor planned for surgical intervention and no history of malignancy in the previous 5 years.

B. Exclusion criteria - women under the age of 18 and malignant disease diagnosed in the previous 5 years.

2.2. Criteria for inclusion and exclusion of the studied persons to form the control group

A. Inclusion criteria-women over 18 years of age, with no documented ovarian tumor and no history of malignancy in the previous 5 years.

B. Exclusion criteria - women under the age of 18 and malignant disease diagnosed in the previous 5 years.

3. Forming the target and control groups

During the period January 2020 - November 2020, a total of 180 women with a mean age of 45.94 years (range 21.0-83.0 years) who met the inclusion and exclusion criteria were examined. According to the developed criteria, the subjects were divided into two groups: a target group - women with ovarian tumors and a control group - healthy women without ovarian tumors. A detailed medical history was collected from all patients regarding their current clinical condition, past illnesses, and menopausal status.

Target group (A): 120 women with an average age of 48.95 years (range 21.0-83.0 years), hospitalized in the Gynecological Clinic of the "St. Marina" UMHAT, Pleven in the period 01.2020. - 11.2020 An ovarian tumor was found in these patients during gynecological examination and TVUS. After the surgical intervention and the histopathological biopsy, the final diagnosis was made, which led to the formation of two target subgroups.

First target subgroup (A1): 60 patients with histologically proven ovarian carcinoma, mean age 57.67 years (range 29.0-83.0 years).

From the anamnestic data of the patients, we obtained information about their menopausal status. 40 of them (66.7%) are in menopause, the remaining 20 (33.3%) are in premenopause.

Menopause is defined as the absence of menses for 12 consecutive months or at age ≥ 50 years when the woman is uncertain about her menses.

Premenopause is the period between the first menstruation and the onset of perimenopause. In this period, there are no symptoms of perimenopause or menopause, there is still menstruation (regular or not) and reproductive abilities are preserved.

Second target subgroup (A2): 60 patients with histologically proven ovarian cyst, mean age 40.23 years (range 21.0-70.0 years). According to the anamnesis data, 15 (25%) are in menopause, and the remaining 45 (75%) are in premenopause.

Control group (B): 60 healthy, asymptomatic women, without clinically or paraclinically evident diseases, with an average age of 39.92 years (range, 22.0-68.0 years), who visited „St. Marina" UMHAT for a preventive examination, including gynecological examination and laboratory tests. During the gynecological examination and TVUS, no ovarian tumors were detected. From the anamnestic data, we found that 12 (20%) were in menopause and 48 (80%) were in premenopause.

When working with all participants in the study, the recommendations and guidelines laid down in „Ethical Issues in Patient Safety Research: Interpreting Existing Guidance" of the World Health Organization (WHO) were followed. All procedures

were followed in accordance with the ethical standards of the Human Research Commission (institutional and regional) as well as the 1975 Declaration of Helsinki, revised in 2000. On the basis of Art. 87, Art. 88 and Art. 89 of the "Health Law", all patients have expressed their informed consent in writing for the medical activities that are performed on them. The study was approved by the Committee on Research Ethics at the Medical University - Pleven.

For the purposes of the study, biological material - venous blood - was taken from all patients. Blood was collected under standardized conditions - in the morning, on an empty stomach (after a 12-hour food break) from the cubital vein in a vacuum container gel containing VACUETTE® Greiner Bio separating gel. After a 30-minute stand at room temperature to retrache the clot, the blood was centrifuged for 15 minutes at 3000g to separate serum. Using a variable automatic pipette with a disposable plastic tip, 500 µl of serum was transferred into an Eppendorf tube that was accurately labeled with an ID number. All samples with hemolysis, icterus, or lipemia, which could compromise the obtained results, were removed from analysis. Separated sera were stored at – 20 °C until analysis.

4. Place of scientific research

All laboratory tests were performed in the Clinical Laboratory of the „St. Marina" UMHAT Pleven.

5. Reference intervals and cut off values of the investigated indicators for diagnosis.

Reference / cut off values from the companies producing the reagent kits were used, with which the laboratory determinations of the investigated indicators were carried out.

Table 1. Reference intervals and cut off values according to the companies producing the working reagents (TOSOH - ST AIA-PACK OVCA and MEDICON - PREALBUMIN, APOA1, TRANSFERRIN, B2 MICROGLOBULIN).

Indicator Reference / cut off value	Reference / cut off value
CA 125	< 35 IU/ml
prealbumin / transthyretin	0,2-0,4 g/l
transferrin	2-3,6 g/l
β_2 microglobulin	< 2,4 mg/l
ApoA1 lipoprotein	>1.4 g/l

METHODS

1. Questionnaire method - to gather information about: age, menopausal status, past and present illnesses.

2. Clinical methods - history and physical examination - gynecological status

3. Laboratory methods for determining the investigated plasma proteins

When conducting all laboratory tests, the requirements of the Medical Standard for Clinical Laboratory and the International Standard for Quality and Competence of Medical Laboratories ISO 15189 are met.

3.1. Determination of prealbumin (transthyretin)

To determine the concentration of prealbumin (transthyretin), an in vitro test PREALBUMIN (MEDICON) for the quantitative determination of prealbumin in human serum with an automatic biochemical analyzer BECKMAN COULTER AU 480 was used.

a/ Principle of the method

An immunoturbidimetric method is used. When the sample is mixed with the appropriate buffer (R1) and antiserum solution (R2), the prealbumin reacts specifically with the anti-human prealbumin antibodies, resulting in the formation of insoluble aggregates. The absorbance of these aggregates at 340 nm is proportional to the concentration of prealbumin in the sample.

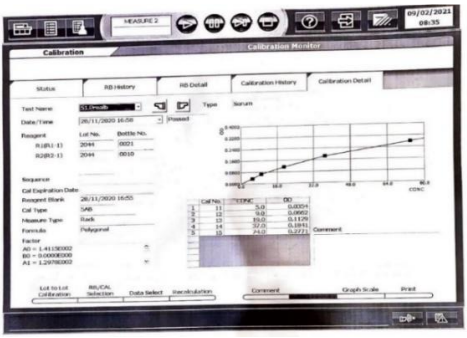


Figure 1. Transthyretin calibration curve.

3.2. Determination of transferrin

To determine the concentration of transferrin, an in vitro test TRANSFERRIN (MEDICON) for quantitative determination of transferrin in human serum was used with an automatic biochemical analyzer BECKMAN COULTER AU 480.

a/ Principle of the method

The immunoturbidimetric method is applied. When the sample is mixed with the appropriate buffer (R1) and antiserum solution (R2), transferrin selectively reacts with anti-human transferrin antibodies, leading to the formation of insoluble aggregates. The absorbance of these aggregates at 520 nm is proportional to the concentration of transferrin in the sample. The reaction is two-point kinetic.

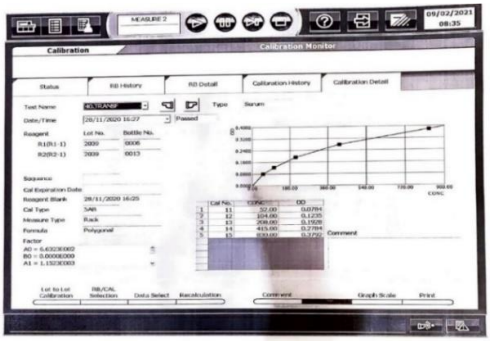


Figure 2. Transferrin calibration curve.

3.3. Determination of ApoA1lipoprotein

To determine the concentration of ApoA1lipoprotein, an in vitro test APO A1 (MEDICON) for the quantitative determination of ApoA1lipoprotein in human serum with an automatic biochemical analyzer BECKMAN COULTER AU 480 was used.

a/ Principle of the method

The immunoturbidimetric method is applied. Mixing of anti-Apo A1 antibodies leads to the formation of antigen-antibody complexes, which leads to an increase in the turbidity of the solution. This is associated with an increase in absorbance at 540 nm.

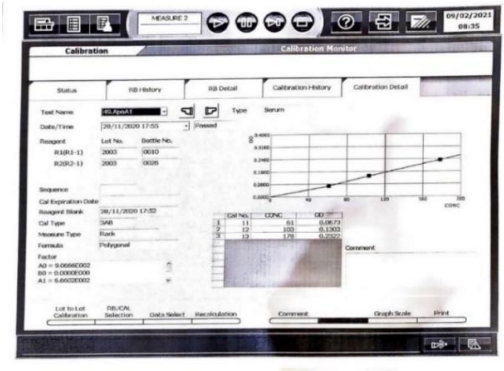


Figure 3. Calibration curve of Apo A1 lipoprotein.

3.4. Determination of β2 microglobulin

To determine the concentration of β2 microglobulin, an in vitro test B2 MICROGLOBULIN (MEDICON) for the quantitative determination of β2 microglobulin in human serum with an automatic biochemical analyzer BECKMAN COULTER AU 480 was used.

a/ Principle of the method

The immunoturbidimetric method is applied. When a sample is mixed with buffer R1 and latex solution R2, β2 microglobulin reacts specifically with anti-human β2 microglobulin antibodies coated with latex particles and forms insoluble aggregates. The absorbance of these aggregates is proportional to the concentration of β2 microglobulin in the sample.

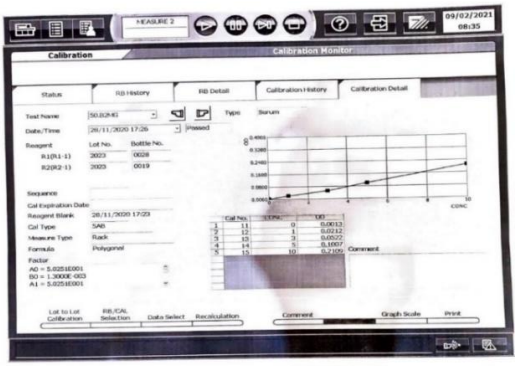


Figure 4. Calibration curve of β2 microglobulin.

3.5. Determination of carcinoembryonic antigen CA 125

The ST AIA-PACK OVCA (TOSOH) in vitro assay for the quantification of CA125 in human serum or heparinized plasma with the TOSOH AIA 360 automated immunoassay platform was used to determine CA125 concentration.

a/ Principle of the method

Enzyme immunoassay is applied. CA 125 present in the test sample binds to monoclonal antibodies immobilized on magnetic beads and to enzyme-labeled monoclonal antibodies in the reagent. Magnetic beads are washed to remove any unbound enzyme-labeled monoclonal antibodies and then incubated with a fluorogenic substrate, 4-methylumbelliferyl phosphate (4MUP). The amount of enzyme-labeled monoclonal antibodies that bind to the beads is directly proportional to the concentration of CA125 in the tested sample.

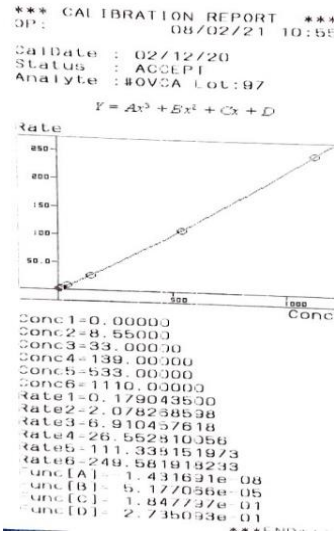


Figure 5. Calibration curve of CA125.

4. Statistical methods

The statistical studies were carried out using methods that are consistent with the nature of the considered processes and the nature of the data for them. Survey data were processed with STATGRAPHICS statistical software packages; SPSS and EXCEL for Windows. The results are described through tables, graphs and numerical indicators for structure, frequency, mean values, correlation coefficients, etc. The critical level of significance used is $\alpha = 0.05$ and the null hypothesis is rejected when the p-value is less than α, i.e. $p < 0.05$.

The following methods were applied:

1. Descriptive analysis – the frequency distribution of the considered signs, broken down by research groups, is presented in tabular form.

2. Non-parametric test of Kolmogorov-Smirnov and Shapiro-Wilk - to check the shape of the frequency distribution against the shape of the normal distribution.

3. Analysis of Variance – to assess the characteristics of central tendency and statistical dispersion. The central tendency is described by the arithmetic mean value

(Mean) when the shape of the distribution resembles a normal curve or by the median (Me) when the distribution is not normal.

4. Graphical analysis – for visualization of the obtained results.

5. Mann-Whitney non-parametric test - for testing hypotheses of difference between two independent samples.

6. Correlation analysis - to establish correlations between quantitative signs and assess the strength of the correlation dependence, through the Pearson correlation coefficient (r). We calculated a point biserial correlation coefficient to establish relationships between a dichotomous (qualitative) and a quantitative variable.

7. Regression analysis - logistic regression - for modeling relationships. We used logistic regression analysis to describe relationships between a dependent variable and one or more independent variables. Thus, the individual action of each factor is established. Logistic regression analysis was used to determine a model by which to predict the probability of a patient falling into the disease group or the disease-free group.

8. Criteria for validation of screening tests and the diagnostic ability of the indicated indicators - through ROC analysis (Receiver Operating Characteristic). The assessment of specificity and sensitivity and determination of threshold values in diagnostic tests is carried out by constructing ROC curves and calculating the area under the curve AUC (area under the curve).

An AUC value of 1.0 means a perfect biomarker.

The following criteria are used to assess the validity of the screening test:

- sensitivity
- specificity
-positive predictive value
-negative predictive value

Table 2. Assessing the diagnostic reliability of laboratory tests.

Diagnosis	Test results		Everything
	Positive	Negative	
With disease	a true positives	b false negatives	a+b
No disease	c false positives	d true negative	c+d
All	a+c	b+d	N

Diagnostic sensitivity is the ability of the test to detect individuals with a disease. It is measured by the probability of a positive test in the screened sick persons. It is defined by the proportion of positive cases from the test (a) in the group of actually sick people (a+b) to the group itself, i.e. with the ratio a/(a+b) . A test with 100% sensitivity correctly identifies all patients with the disease.

Diagnostic specificity characterizes the test's ability to detect healthy individuals. It is measured by the probability of a negative test in the screened healthy individuals. It is defined by the ratio of negative cases according to the test (d) among the group of healthy (c+d), i.e. with the ratio d/(c+d). A test with 100% specificity correctly identifies individuals without data on the disease for which it is intended.

The positive predictive value (Positive predictive value) of the test is measured by the probability of the presence of a disease in individuals with a positive test. It is defined by the ratio of positive cases from the test (a) to all positive cases in the group (a+c), i.e. with the ratio a/(a+c) .

The negative predictive value (Negative predictive value) of the test is measured by the probability of the absence of disease in individuals with a negative test. It is defined by the proportion of negative cases according to the test (d) to all negative cases in the group (b+d), i.e. with the ratio d/(b+d) .

PPV and NPV depend on the prevalence of the disease among the subjects.

RESULTS

Our study was planned to investigate the possibilities of some plasma proteins for the diagnosis of ovarian tumor and especially whether these proteins can be applied in the preoperative differentiation of ovarian tumors. To fulfill our stated aim and objectives, we used IBM SPSS Statistics v.26 and MS Excel to process the survey data. Thus, we were able to prove our concepts, analyze the obtained dependencies, establish the diagnostic sensitivity and specificity of the measured biomarkers and prove their predictive value for the diagnosis of ovarian tumor and for the preoperative differential diagnosis of ovarian tumor.

The study included 180 patients, in whom we determined the plasma concentrations of the five proteins that are the subject of our study - prealbumin (transthyretin, TTR), transferrin (TFR), ApoA1lipoprotein (Apo A1LP), CA125 and β_2 microglobulin (β_2MG).

We used data from the medical history and additional information from the hospital information system documentation and obtained data on the menopausal status of the patients and the histopathological diagnosis of the women with a proven ovarian tumor. This helped us in shaping the study groups.

Using the methods of descriptive statistics, we calculated the main summary characteristics: arithmetic mean (Mean), median (Me), minimum (x_min) and maximum (x_max) value of the feature, as well as indicators of dispersion - standard deviation (SD) and confidence interval 95% CI (CI).

Table 3. Summary characteristics of the groups according to the age indicator.

Indicators	**Groups**			
	Target (A)			**Control (B)**
	OC (A1)	**Ovarian cysts (A2)**	**Total**	
N	60	60	120	60
Mean ± SD	57.67 ± 12.85	40.23 ± 12.24	48.95 ±	39.92 ±
95% CI	54.35 - 60.99	37.07 - 43.39	46.19 - 51.71	37.02 - 42.82
Me	59	39.5	48	40
Min	29	21	21	22
Max	83	70	83	68

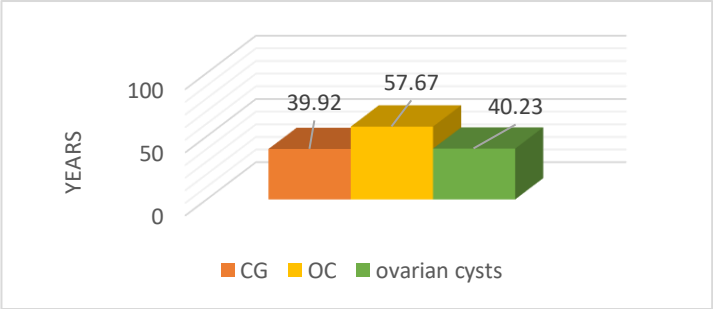


Figure 6. Distribution of work groups by age.

A comparative analysis revealed the presence of statistically significant differences ($p < 0.05$) with respect to age in the following pairs of patient groups - Table 4.

Table 4. Level of significance regarding the age factor in the different compared groups of patients.

Compared patient groups	Mann-Whitney U	Hz	Sig.
Group A - Group B	2396.000	3.652	**0.000**
Subgroup A1 - Subgroup A2	616.000	6.212	**0.000**
Subgroup A1 - Group B	579.000	6.406	**0.000**
Subgroup A2 - Group B	1783.000	-0.087	0.928

We measured the concentrations of the five serum proteins in all 180 patients in our sample. We used correlation analysis to establish and evaluate univariate correlations of menopausal status with various quantitative variables within the entire group of 180 patients and the groups we created.

Table 5. Correlation of menopausal status with the five proteins (n=180).

Proteins	Pearson r	N	Sig. (2-tailed)
Prealbumin	- 0.269	180	**0.003**
ApoA1LP	- 0.126	180	0.091
CA125	0.374	180	**0.000**
TFR	- 0.330	180	**0.000**
β2MG	0.428	180	**0.000**

Because we will report changes in serum concentrations in women with ovarian tumors, we believe it is necessary to establish in group B patients whether changes occur in the investigated proteins, whether they are related to the age of the patients and their menopausal status. We have used the arithmetic mean (Mean) when the distribution shape resembles a normal curve or the median (Me) when the distribution is not normal.

Table 6. Summary characteristics for the five plasma proteins in group B (n=60).

Proteins	Characteristics				
	Mean ± SD	95% CI	Me	Min	Max
Prealbumin	0.27 ± 0.04	0.26 - 0.28	0.28	0.18	0.36
TFR	2.67 ± 0.44	2.56 - 2.78	2.70	1.55	3.48
ApoA1LP	1.80 ± 0.23	1.74 - 1.86	1.76	1.3	2.31
CA125	14.92 ± 9.22	12.54 -	12.95	3	51.6
β2MG	1.38 ± 0.76	1.18 - 1.58	1.04	0.55	4.1

Using correlation analysis, we found that there was no statistically significant correlation between age and the concentrations of the five proteins in the group of healthy women (Table 6).

Table 7. Correlation of age with the five proteins in group B (n=60).

Proteins	Pearson r	N	Sig. (2-tailed)
Prealbumin	0.010	60	0.940
ApoA1LP	- 0.060	60	0.650
CA125	- 0.126	60	0.337
TFR	0.162	60	0.216
β2MG	0.110	60	0.400

We found that there is no statistically significant correlation between the menopausal status and the serum concentrations of the investigated biomarkers in group B patients (Table 7).

Table 8. Correlation of menopausal status with the five proteins in group B (n=60).

Proteins	Pearson r	N	Sig. (2-tailed)
Prealbumin	- 0.07	60	0.587
ApoA1LP	0.06	60	0.620
CA125	- 0.14	60	0.280
TFR	0.62	60	0.637
β2MG	0.23	60	0.080

First stage of the study

What changes occur in the concentrations of the five serum proteins in women with ovarian tumors?

The object of the first stage of our study were the two groups: Group A - women with ovarian tumors (n=120) and Group B - the control group (CG n=60) patients.

Table 9. Summary characteristics for the five proteins in the group of women with ovarian tumors - group A (n=120).

Proteins	Characteristics				
	Mean ± SD	95% CI	Me	Min	Max
Prealbumin	0.21 ± 0.07	0.20 - 0.22	0.22	0.04	0.33
TFR	2.42 ± 0.70	2.29 - 2.55	2.53	0.49	3.90
ApoA1LP	1.76 ± 0.27	1.71 - 1.81	1.74	1.08	2.62
CA125	343.07 ± 488.65	254.74 -	72.8	2.1	1892.00
β2MG	2.17 ± 1.58	1.88 - 2.46	1.86	0.65	10.46

The mean values of the five biomarkers in the two observed groups are presented in Figures 7 and 8.

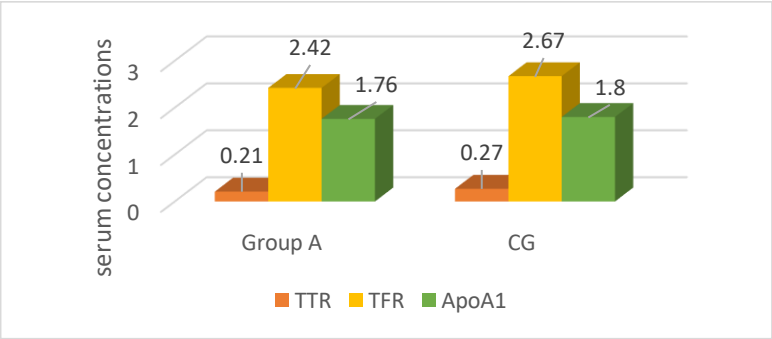


Figure 7. Mean values of prealbumin, transferrin and ApoA1LP in group A and CG

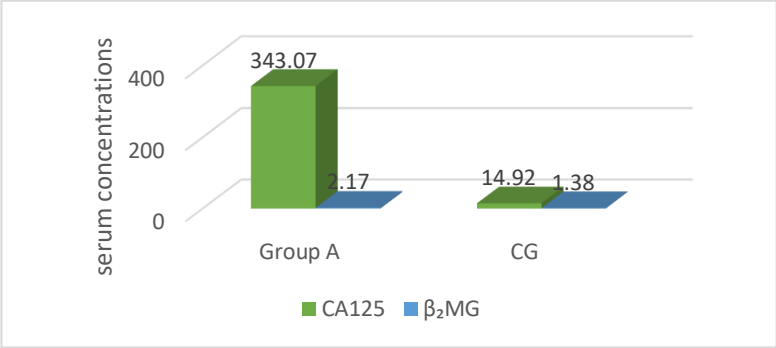


Figure 8. Average values of CA125 and β₂MG in group A and CG

Applied comparative analysis found statistically significant differences for the two groups in Prealbumin, CA125, TFR and β_2 MG values. In the values of ApoA1LP, the differences for the two groups were statistically insignificant.

Table 10. Level of statistical significance for the five proteins in group A (n=120) and group B (n=60).

Proteins	Group A	Group B (CG)	Mann-Whitney U	z	p
Prealbumin	0.21	0.27	1575.000	- 6.140	**0.000**
TFR	2.42	2.67	2829.500	- 2.337	**0.019**
ApoA1LP	1.76	1.80	3281.500	- 0.965	0.337
CA125	343.07	14.92	1337.500	6.860	**0.000**
β_2 MG	2.17	1.38	2204.000	4.230	**0.000**

In the group of women with ovarian tumors, through correlation analysis and calculation of correlation coefficient (r), we proved several dependencies between individual serum proteins. In the case of establishing a relationship between two quantitative variables, a Pearson's correlation coefficient was calculated.

We found a significant negative correlation with statistical significance between:

• prealbumin and β_2 microglobulin;

• transferrin and β_2 microglobulin

Significant positive correlation statistically significant between:

• prealbumin and ApoA1 lipoprotein.

• prealbumin and transferrin

• CA125 and β_2 microglobulin

A moderate correlation with a negative sign and statistical significance between:

• prealbumin and CA125

• transferrin and CA125

• ApoA1lipoprotein and β_2 microglobulin.

We found a moderate correlation with a positive sign and statistical significance between:

• transferrin and ApoA1lipoprotein

Between CA125 and ApoA1lipoprotein, we found a weak correlation with a negative sign, which is statistically insignificant.

How does menopause affect the changes occurring in the concentrations of the five group A serum proteins?

To answer this question, we divided the group A patients with ovarian tumors into: women with ovarian tumors in menopause (n=55) and women with ovarian tumors in premenopausal (n= 65).

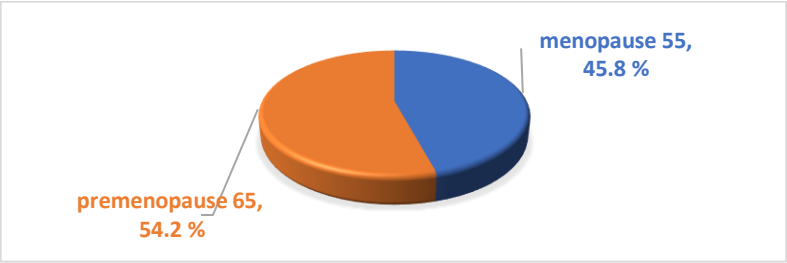


Figure 9. Distribution of women with ovarian tumors according to menopausal status

We determined the general statistical characteristics of the five proteins in the group of menopausal women.

Table 11. Summary characteristics for the five serum proteins in the group of menopausal women (n=55).

Proteins	Characteristics				
	Mean ± SD	95% CI	Me	Min	Max
Prealbumin	0.19 ± 0.08	0.17 - 0.21	0.20	0.04	0.33
TFR	2.12 ± 0.68	1.94 - 2.30	2.15	0.49	3.41
ApoA1	1.71 ± 0.26	1.64 - 1.78	1.72	1.08	2.44
CA125	535.13 ± 568.20	381.52 - 688.74	275.70	4.00	1892.00
β2MG	2.91 ± 1.91	2.39 - 3.43	2.74	0.88	10.42

We determined the common statistical characteristics of the five proteins also in premenopausal patients in the group of women with ovarian tumors.

Table 12. Summary characteristics for the five proteins in the group of premenopausal women (n=65).

Proteins	Characteristics				
	Mean ± SD	95% CI	Me	Min	Max
Prealbumin	0.22 ± 0.06	0.21 - 0.23	0.22	0.06	0.31
TFR	2.67 ± 0.61	2.52 - 2.82	2.72	1.07	3.90
ApoA1	1.80 ± 0.26	1.74 - 1.86	1.78	1.23	2.62
CA125	180.55 ± 331.70	98.36 - 262.74	42.30	2.10	1365.00
β2MG	1.55 ± 0.81	1.35 - 1.75	1.21	0.65	4.02

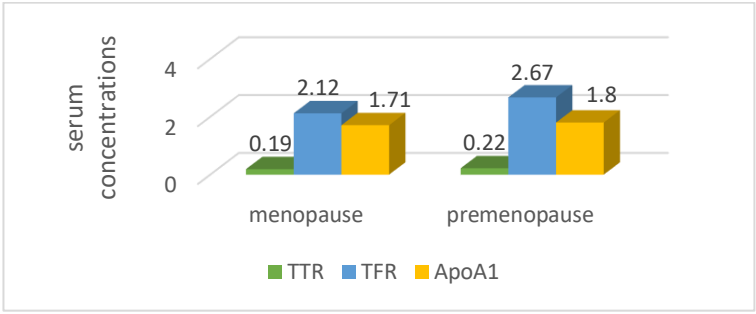


Figure 10. Mean values of prealbumin, transferrin and ApoA1LP in group A by menopausal status.

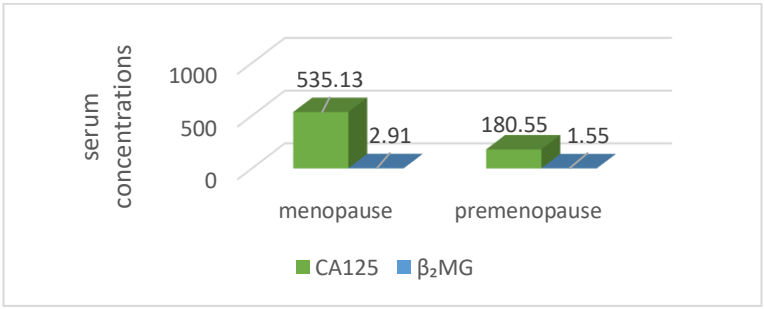


Figure 11. Mean values of CA125 and β₂MG in group A by menopausal status.

We compared the mean values of the measured proteins in menopausal and premenopausal women and applied a correlation analysis calculating the Point Biserial Correlation Coefficient (r_{pb}) and found:

- significant positive correlation with statistical significance between menopausal status and β₂ MG in the group of women with ovarian tumors
- moderate positive correlation with statistical significance between menopausal status and CA125 in the group of women with ovarian tumors
- moderate negative correlation with statistical significance between menopausal status and TFR in the group of women with ovarian tumors
- moderate negative correlation with statistical significance between menopausal status and TTR in the group of women with ovarian tumors

There was a negative weak correlation between menopausal status and ApoA1LP, which was statistically insignificant, according to our accepted level of statistical significance of $p < 0.05$.

Table 13. Correlations between menopausal status and the five proteins in group A.

Proteins	r_{pb}	N	Sig. (2-tailed)
Prealbumin	- 0.21	120	**0.023**
ApoA1LP	- 0.18	120	0.053
CA125	0.36	120	**0.000**
TFR	- 0.39	120	**0.000**
β2MG	0.43	120	**0.000**

Between the concentrations of most of the serum proteins in menopausal patients, various correlation dependences were observed, which were statistically significant.

There is a significant positive correlation between:

• ApoA1LP and prealbumin

• transferrin and prealbumin

Significant negative correlation between:

• prealbumin and β_2 MG

• transferrin and β_2 MG

A moderate positive correlation between:

• β_2 MG and CA125

Moderate negative correlation between:

• prealbumin and CA125

• ApoA1LP and β_2 MG

There were weak correlations between the other proteins in postmenopausal women with ovarian tumors that were statistically insignificant.

In premenopausal women with ovarian tumors, different correlations were also observed in the levels of most of the plasma biomarkers, which were statistically significant.

There is a significant positive statistically significant correlation between:

• transferrin and ApoA1LP

• CA125 and β_2 MG

• prealbumin and ApoD1LP

Moderate strength positive correlation statistically significant between

• prealbumin and transferrin

Negative moderate correlation dependence with statistical significance between

• prealbumin and CA125

There were weak correlations between the other proteins in premenopausal women with ovarian tumors that were statistically insignificant.

What is the diagnostic sensitivity and specificity of the investigated biomarkers in the diagnosis of ovarian tumor?

For this purpose, we used ROC (Receiver Operating Characteristic) curve analysis, with the help of which the diagnostic qualities of the studied indicators are evaluated, based on the sensitivity and specificity of the tests. The diagnostic specificity and sensitivity, as well as the positive and negative predictive value for each parameter are shown in Table 14. The pairs of corresponding numbers for sensitivity and specificity of the corresponding parameter are shown as points on a rectangular coordinate system. Joining them produces a line called the ROC curve that illustrates the relationship between sensitivity and specificity. The area under it AUC (Area Under the Curve) shows to what extent the patients in the sample can be classified into one of two groups according to the values of the corresponding plasma protein: sick (women with ovarian tumors) and healthy (women without ovarian tumors). This is evident from table 16. The closer the AUC coefficient is to 1, the better the diagnostic ability of the respective test.

We used the limit values (cut off) and reference limits for the investigated parameters according to the recommendations of the manufacturers of the individual kit reagents, but we obtained results that, in our opinion, could be improved.

Table 14. Cut off values and reference intervals according to the manufacturers of the reagent kits of the investigated quantitative indicators for proving the ovarian tumor patients and values of the criteria for the validation of screening tests

Indicator	**Cut off / reference intervals**	**Sn(%)**	**Sp(%)**	**PPV(%)**	**NPV(%)**
CA125	≥ 35 IU/mL	63.3	96.7	97.4	56.9
prealbumin	0.20-0.40g/L	44.2	90	89.8	44.6
TFR	2.0-3.6g/L	33.3	90	87	40.3
ApoA1LP	≤ 1.4 g/L	10.8	95	81.3	34.8
β_2 MG	≥ 2.4 mg/L	37.5	91.7	90	42.3

For this reason, we also determined cut off values that would best distinguish women with ovarian tumors from women without ovarian tumors.

Table 15. Cut off values of the studied quantitative indicators for distinguishing patients with ovarian tumor and values of the criteria for validation of screening tests.

Indicator	**Cut off**	**Sn(%)**	**Sp(%)**	**PPV(%)**	**NPV(%)**
CA125	**≥ 31.5 IU/mL**	65.0	96.7	97.5	58.0
prealbumin	**≤ 0.25 g/L**	68.3	78.3	86.3	55.3
TFR	**≤ 1.98g/L**	27.5	96.7	94.3	40.0
ApoA1LP	**≤ 1.94g/L**	77.5	35.0	70.5	43.8
β_2 MG	**≥ 2.06 mg/L**	46.7	85.0	85.9	44.0

Table 16. AUC (Area Under the Curve) - coefficients regarding the risk of ovarian tumor.

Test	AUC	Std. Error	p	95% Confidence Interval	
				Lower Bound	Upper Bound
Prealbumin	**0.781**	0.034	**0.000**	0.714	0.849
ApoA1LP	0.544	0.046	0.334	0.455	0.634
CA125	**0.814**	0.031	**0.000**	0.754	0.874
TFR	0.607	0.042	**0.019**	0.525	0.689
β2MG	**0.694**	0.041	**0.000**	0.614	0.774

From Table 16, it is evident that the p values of Prealbumin, CA125, TFR and β_2 MG are less than the 0.05 significance level (respectively 0.000 ; 0.000 ; 0.019 ; 0.000), therefore they can be reliably used to differentiate ovarian tumor . The diagnostic abilities of Prealbumin, CA125 and β_2 MG for the presence of an ovarian tumor are relatively good - > 69%, as their AUC coefficients are high - for CA125 (AUC=0.814; 95% CI 0.754÷0.874), Prealbumin (AUC=0.781 ; 95% CI 0.714÷0.849) and β_2 MG (AUC=0.694; 95% CI 0.614÷0.774). Their threshold values are ≤ 31.5 IU/ml for CA125, which determines Sn 65% and Sp 96.7%; for β_2 MG ≤ 2.06 mg/l, which determines Sn 46.7 % and Sp 85 %; for Prealb ≤ 0.25 g/l, which determines Sn 68.3 % and Sp 78.3 %.

A Youden index (J) was also calculated for each indicator. This index was proposed in 1950. as a way of summarizing the performance of a diagnostic test. It is a measure of a diagnostic test's ability to balance sensitivity (detection of disease) and specificity (absence of disease). It is calculated as follows:

Sensitivity (%) + Specificity (%) – 1 = Youden Index

The higher and closer to 1 the value of the Youden index, the greater the ability of the given test to be applied for diagnostic purposes.

Figures 12 - 16 present the ROC curves of the five investigated proteins.

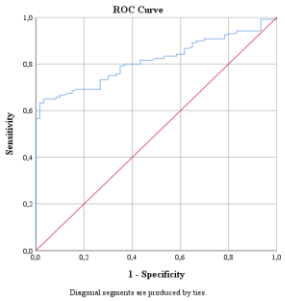


Figure 12. ROC curve for CA125.

J index = 0.617, Sensitivity = 0.650, Specificity = 0.967

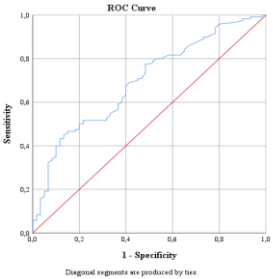


Figure 13. ROC curve for β_2MG.

J index = 0.317, Sensitivity = 0.467, Specificity = 0.850

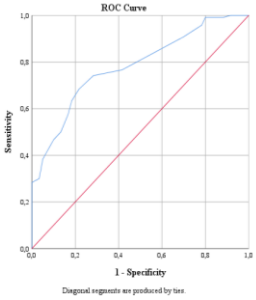


Figure 14. ROC curve for Prealbumin.

J index = 0.467, Sensitivity = 0.683, Specificity = 0.783

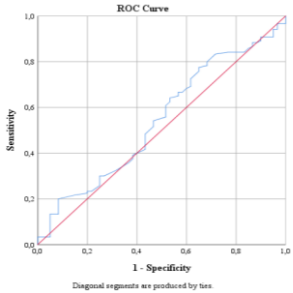


Figure 15. ROC curve for ApoA1LP.

J index = 0.125, Sensitivity = 0.775, Specificity = 0.350

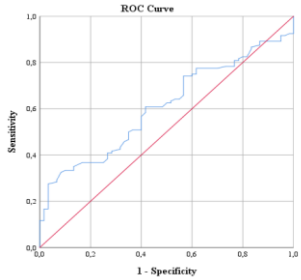


Figure 16. ROC curve for TFR.

J index = 0.242, Sensitivity = 0.275, Specificity = 0.967

Second stage of the study

What changes occur in the concentrations of the five serum proteins in the formed subgroups of patients: subgroup A1 and subgroup A2?

The object of the second stage of our study were the two target subgroups: A1 - women with proven OC and A2 - women with proven ovarian cysts.

The results in Figures 17 and 18 show that OC is in the highest percentage among women in the age range of 51-70 years (N=29; 48.3%), followed by the group in the age range of 31-50 years (N=20; 33.3 %). Female patients in the age range 71-90 years (n=10; 16.7%) and 19-30 years have the smallest relative share (n=1; 1.7%).

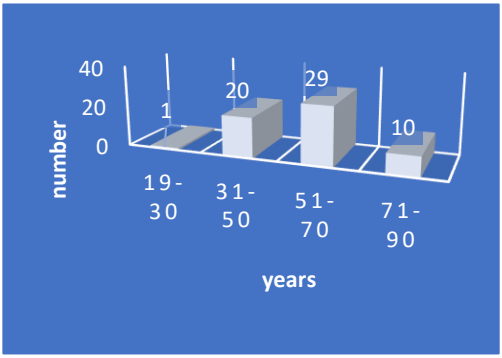


Figure 17. Distribution by years in subgroup A1.

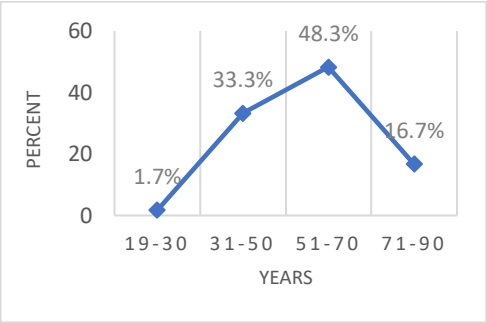


Figure 18. Percentage distribution by age in subgroup A1.

From the data in the hospital information system, we collected information on the histological variant of OC, the stage of the carcinoma according to FIGO, and on its degree of differentiation. From the data in Figure 19, it is clear that serous epithelial ovarian carcinoma predominates as histological variant 53 (88.3%). Mucinous, clear cell and endometrioid are represented quite poorly in our sample of patients, respectively 2 (3.33%); 3 (5%); 2 (3.33%).

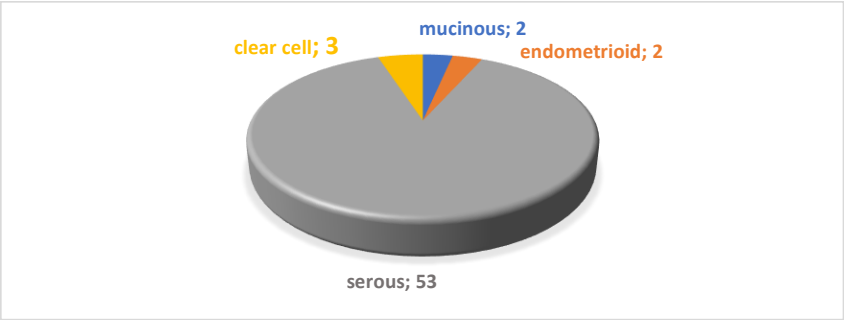


Figure 19. Distribution of OC by histological variant.

The results in figure 20 show that a greater percentage are women with a high degree of differentiation of serous epithelial OC High Grade 47 (88.7 %), and women with a low degree of differentiation of OC Low Grade are only 6 (11.3%).

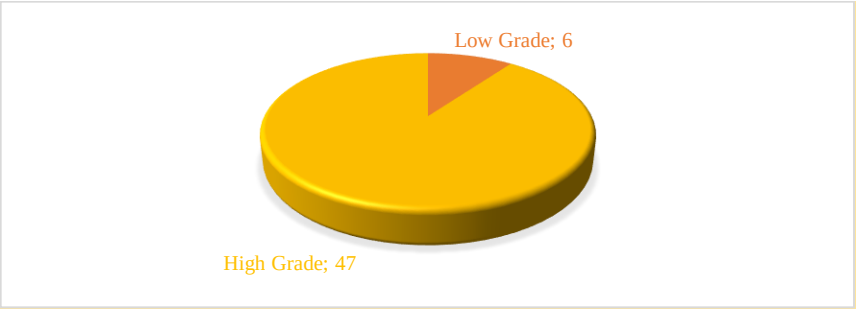


Figure 20. Distribution of epithelial serous OC by degree of differentiation.

Regarding stages according to FIGO, the distribution in the OC group was as follows: 38 (63.3 %) were patients with early stage OC, including stage I and II, 22 (36.7 %) were patients with late stage OC- stage III (Figure 21). Among the women studied, none were in stage IV.

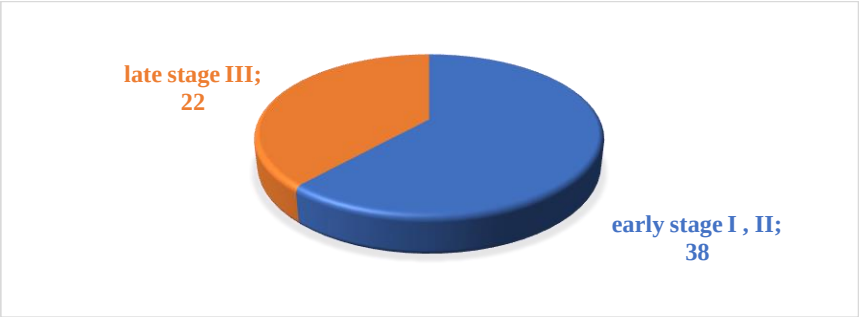


Figure 21. Distribution of OC by stage.

The results in Figures 22 and 23 show that among women with ovarian cysts, the highest percentage was those aged 31-50 years (n=30; 50.0%). Female patients in the age range of 19-30 years (n=16; 26.7%) and 51-70 (n=14; 23.3%) years have almost the same presentation in our studied sample.

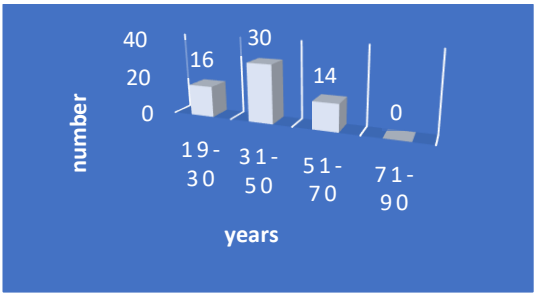


Figure 22. Age distribution in subgroup A2.

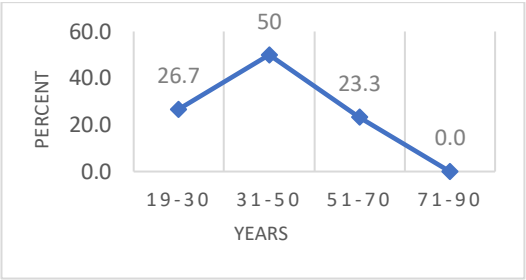


Figure 23. Percentage distribution by age in subgroup A2.

From the hospital information system, we obtained information about the type of ovarian cysts according to the histopathological biopsy performed. The highest percentage are endometrioid cysts (n= 25; 41.7%) and serous cystadenomas (n= 20; 33.3%). Dermoid and follicular cysts are presented with a smaller relative share, respectively 5 (8.3 %) and 10 (16.7 %).

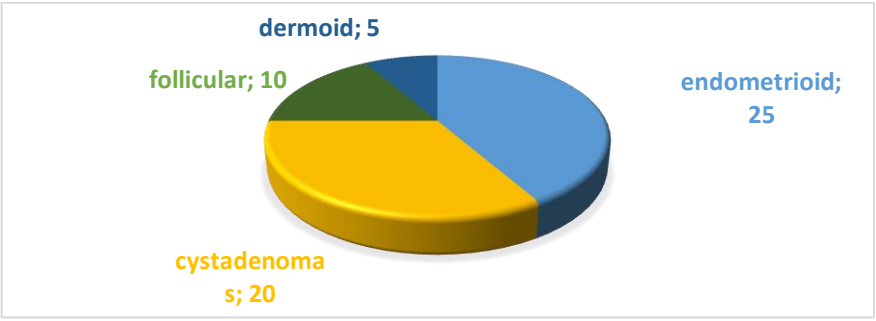


Figure 24. Distribution of ovarian cysts by histological variant.

We used benchmarking and calculated the necessary metrics. The obtained data on the statistical characteristics of the five biomarkers are presented in Table 17 and Table 18.

Table 17. Summary statistical characteristics of the five proteins in the group of women with OC.

Proteins	Characteristics				
	Mean ± SD	95% CI	Me	Min	Max
Prealbumin	0.18 ± 0.08	0.16-0.20	0.19	0.04	0.33
TFR	2.15 ± 0.70	1.97-2.33	2.0	0.49	3.41
ApoA1LP	1.69 ± 0.28	1.62-1.76	1.68	1.08	2.36
CA125	640.70 ± 543.79	500.22-781.18	422.70	4.2	1892
β2MG	2.85 ± 1.85	2.37-3.33	2.49	0.65	10.46

Table 18. Summary statistical characteristics of the five proteins in the group of women with ovarian cysts.

Proteins	Characteristics				
	Mean ± SD	95% CI	Me	Min	Max
Prealbumin	0.24 ± 0.04	0.23-0.25	0.23	0.14	0.31
TFR	2.70 ± 0.58	2.55-2.85	2.71	1.07	3.9
ApoA1LP	1.84 ± 0.22	1.78-1.90	1.79	1.38	2.62
CA125	45.43 ± 68.50	27.73-63.13	20.3	2.1	450.5
β2MG	1.49 ± 0.80	1.28-1.70	1.13	0.74	3.21

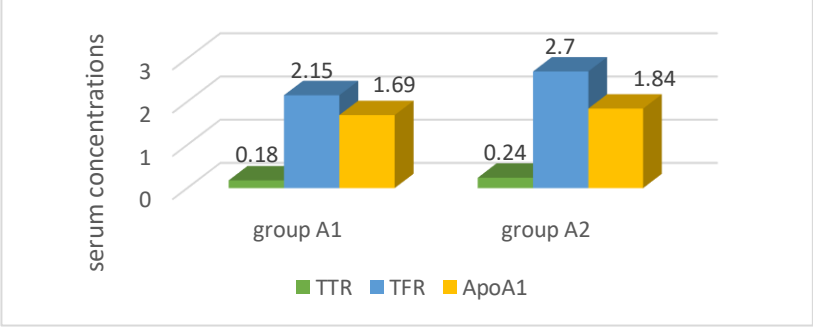


Figure 25. Mean values of prealbumin, transferrin and ApoA1LP for group A1 and A2.

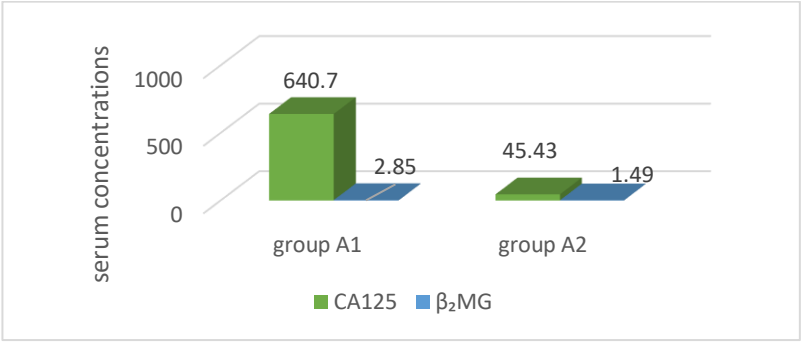


Figure 26. Average values of CA125 and β₂ microglobulin in women for group A1 and A2.

Table 19. Level of statistical significance for the five serum proteins in group A1 and A2.

Proteins	Group A1	Group A2	Mann-Whitney U	z	p
Prealbumin	0.18	0.24	981.5	- 4.293	**0.000**
TFR	2.15	2.70	1044	- 3.965	**0.000**
ApoA1LP	1.69	1.84	1202.5	- 3.133	**0.0017**
CA125	640.70	45.43	404	7.324	**0.000**
β₂ MG	2.85	1.49	741.5	5.553	**0.000**

The comparative analysis found the presence of statistically significant differences regarding the five studied biomarkers in the two studied subgroups.

How does menopause affect the changes occurring in the concentrations of the five serum proteins in the two studied subgroups?

To answer this question, we divided women with OC and those with ovarian cysts according to their menopausal status.

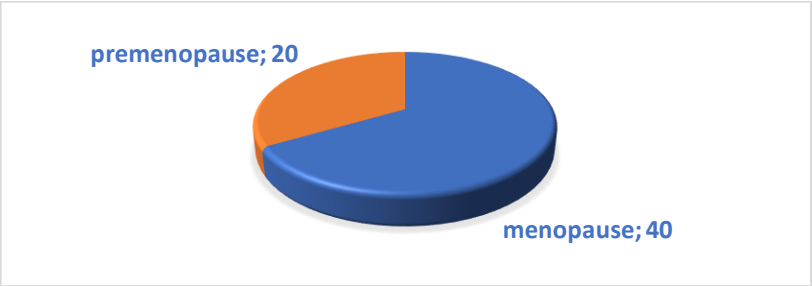


Figure 27. Distribution of OC according to menopausal status.

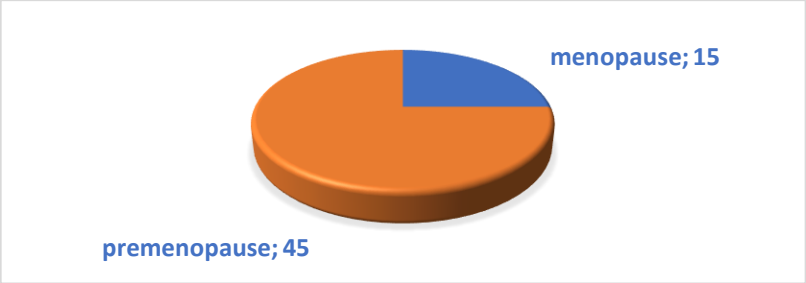


Figure 28. Distribution of ovarian cysts according to menopausal status.

In group A1, there are 40 (66.7%) menopausal women and 20 (33.3%) premenopausal women. Using correlation analysis we found a statistically significant moderate positive correlation between menopausal status and β_2 MG and a statistically significant moderate negative correlation between menopausal status and TFR.

Table 20. Correlations between menopausal status and the investigated proteins in group A1.

Proteins	r_{pb}	N	Sig. (2-tailed)
Prealbumin	- 0.146	60	0.264
ApoA1LP	- 0.133	60	0.313
CA125	0.205	60	0.115
TFR	- 0.323	60	**0.012**
β2MG	0.318	60	**0.013**

Table 21. Summary statistical characteristics of the five proteins in the group of menopausal women with OC (n = 40).

Proteins	Characteristics				
	Mean ± SD	95% CI	Me	Min	Max
Prealbumin	0.17 ± 0.08	0.14-0.20	0.17	0.04	0.33
TFR	1.99 ± 0.71	1.76-2.22	1.98	0.49	3.41
ApoA1LP	1.66 ± 0.26	1.58-1.74	1.67	1.08	2.33
CA125	719.69± 560.85	540.32-899.06	746.05	8.9	1892
β2MG	3.27 ± 2.06	2.61-3.93	2.84	0.97	10.46

Table 22. Summary statistical characteristics of the five proteins in the group of premenopausal women with OC (n = 20).

Proteins	Characteristics				
	Mean ± SD	95% CI	Me	Min	Max
Prealbumin	0.19 ± 0.07	0.16-0.22	0.20	0.06	0.31
TFR	2.46 ± 0.55	2.20-2.72	2.45	1.49	3.13
ApoA1LP	1.74 ± 0.32	1.59-1.89	1.73	1.23	2.36
CA125	482.72 ± 469.64	262.92-702.52	302.65	4.2	1365
β2MG	2.02 ± 0.87	1.61-2.43	2.01	0.65	4.02

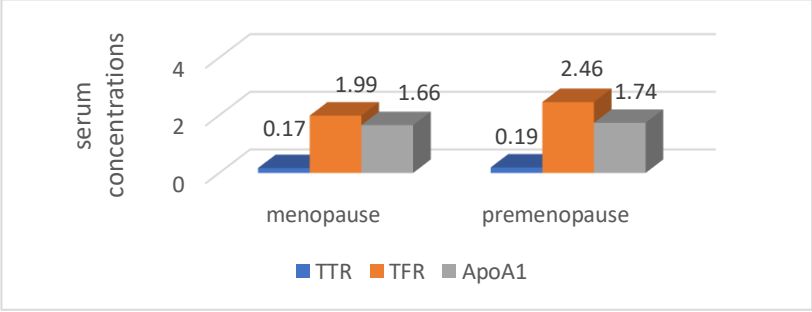


Figure 29. Mean values of prealbumin, transferrin and ApoA1LP according to menopausal status in group A1.

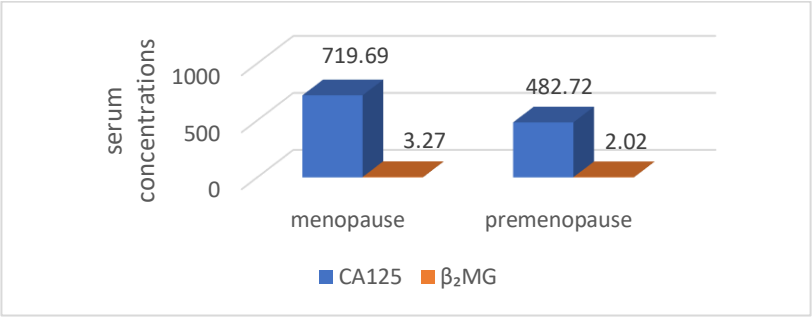


Figure 30. Mean values of CA125 and β₂ MG according to menopausal status in group A1.

Comparing the mean values of the five proteins in the two observed groups, we found statistically significant differences in TFR and β₂ MG ($p < 0.05$). For the other three proteins - Prealbumin, ApoA1LP and CA125, we did not find statistically significant differences ($p > 0.05$).

Table 23. Level of statistical significance for the five plasma proteins according to menopausal status in group A1.

Proteins	Group A1 menopause	Group A1 premenopause	Mann-Whitney U	z	p
Prealbumin	0.17	0.19	316.5	- 1.300	0.194
TFR	1.99	2.46	247.5	- 2.384	**0.017**
ApoA1LP	1.66	1.74	342.5	- 0.894	0.373
CA125	719.69	482.72	297.5	1.599	0.110
β₂ MG	3.27	2.02	221	2.799	**0.005**

In group A2, there were 15 (25.0%) menopausal women and 45 (75.0%) premenopausal women. We found a statistically significant moderate positive correlation between menopausal status and β₂ microglobulin.

Table 24. Correlations between menopausal status and the investigated proteins in group A2.

Proteins	r_{pb}	N	Sig. (2-tailed)
Prealbumin	0.165	60	0.209
ApoA1LP	0.018	60	0.894
CA125	- 0.021	60	0.874
TFR	- 0.200	60	0.125
β2MG	0.320	60	**0.013**

Table 25. Summary statistical characteristics of the five proteins in the group of menopausal women with ovarian cysts (n = 15).

Proteins	Characteristics				
	Mean ± SD	95% CI	Me	Min	Max
Prealbumin	0.25 ± 0.04	0.23-0.27	0.23	0.19	0.31
TFR	2.49 ± 0.40	2.27-2.71	2.47	1.87	3.24
ApoA1LP	1.84 ± 0.21	1.72-1.96	1.75	1.62	2.44
CA125	42.96 ± 109.18	17.50-103.42	14.41	4.0	450.5
β2MG	1.94 ± 0.94	1.42-2.46	1.41	0.88	3.21

Table 26. Summary statistical characteristics of the five proteins in the group of premenopausal women with ovarian cysts (n = 45).

Proteins	Characteristics				
	Mean ± SD	95% CI	Me	Min	Max
Prealbumin	0.23 ± 0.05	0.22-0.25	0.23	0.14	0.31
TFR	2.76 ± 0.62	2.57-2.95	2.76	1.07	3.90
ApoA1LP	1.83 ± 0.23	1.76-1.90	1.79	1.38	2.62
CA125	46.26 ± 47.75	31.91-60.61	28	2.1	246.9
β2MG	1.35 ± 0.69	1.14-1.56	1.1	0.74	3.09

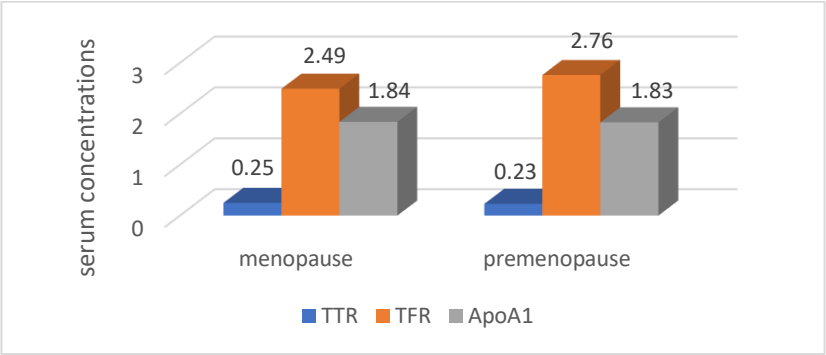


Figure 31. Mean values of prealbumin, transferrin and ApoA1LP according to menopausal status in group A2

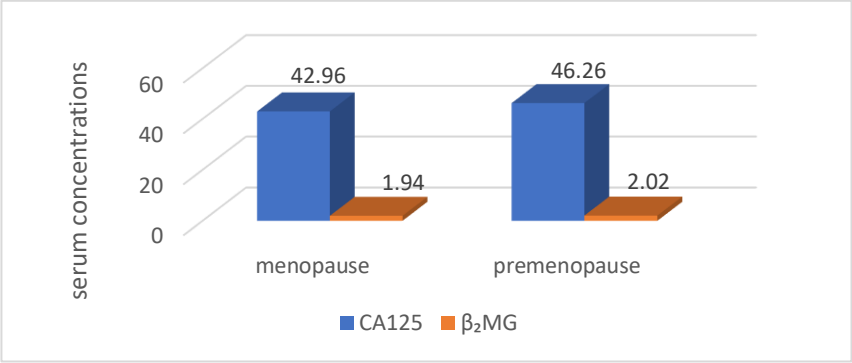


Figure 32. Mean values of CA125 and β_2 microglobulin according to menopausal status in group A2.

Comparing the mean values of the five proteins in the two observed groups, we found the presence of statistically significant differences in β_2 MG and CA125 ($p < 0.05$). For the other three proteins - Prealbumin, ApoA1LP and TFR, we did not find statistically significant differences ($p > 0.05$).

Table 27. Level of statistical significance for the five serum proteins according to menopausal status in group A2.

Proteins	Group A2 menopause	Group A2 premenopause	Mann-Whitney U	z	p
Prealbumin	0.25	0.23	316.5	-1.161	0.246
TFR	2.46	2.76	247.5	1.852	0.064
ApoA1LP	1.84	1.83	342.5	0.077	0.936
CA125	42.96	46.26	297.5	2.612	**0.009**
β_2 MG	1.94	1.35	221	-2.194	**0.029**

What is the diagnostic sensitivity and diagnostic specificity of each protein for differentiating ovarian carcinoma from ovarian cyst?

To check the diagnostic sensitivity and specificity of biomarkers for differentiating patients with OC from patients with ovarian cysts, we used ROC curve analysis and determined cut off values.

Table 28. Threshold values of the investigated quantitative indicators for distinguishing patients with OC and values of the criteria for validation of screening tests.

Indicator	Cut off	Sn(%)	Sp(%)	PPV(%)	NPV(%)
CA125	**≥ 99.05 IU/mL**	**81.7**	**90**	**89**	**83**
prealbumin	**≤ 0.205 g/L**	**73.3**	**66.7**	**71.4**	**68.8**
TFR	**≤ 2.37 g/L**	**78.3**	**61.7**	**74**	**67.1**
ApoA1LP	**≤ 1.615 g/L**	**86.7**	**45**	**77.1**	**61.2**
β_2 MG	**≥ 1.295 mg/L**	**85**	**70**	**74**	**82.4**

Table 29. AUC (Area Under the Curve) - coefficients regarding the presence of OC.

Area Under the Curve					
Test	AUC	Std. Error	p	95% Confidence Interval	
				Lower Bound	Upper Bound
Prealbumin	**0.727**	0.047	0.000	0.635	0.820
Apo A1 LP	**0.666**	0.050	0.002	0.568	0.764
CA125	**0.888**	0.034	0.000	0.822	0.954
TFR	**0.710**	0.047	0.000	0.617	0.803
β2MG	**0.794**	0.041	0.000	0.713	0.875

It is evident from Table 29 that the p values of the five serum proteins are less than the 0.05 significance level, therefore they can be reliably used to differentiate ovarian carcinoma from ovarian cyst. Their diagnostic abilities for distinguishing ovarian carcinoma from ovarian cyst are relatively good - they exceed 66%, because their AUC coefficients are high. Their threshold values define a good Sn and Sp table 28. Youden's index (J) was calculated for each indicator based on Sensitivity and Specificity.

Figures 33 - 37 show the ROC curves for the five proteins in discriminating between OC and ovarian cyst.

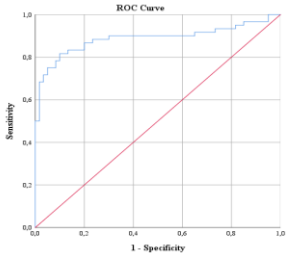


Figure 33. ROC curve for CA125.

J index = 0.717, Sensitivity = 0.817, Specificity = 0.900;

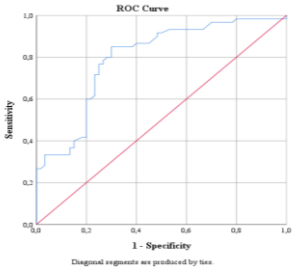


Figure 34. ROC curve for β₂MG.

J index= 0.550, Sensitivity = 0.850, Specificity = 0.700;

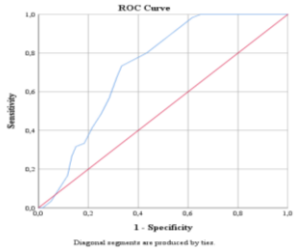


Figure 35. ROC curve for Prealbumin.

J index = 0.400, Sensitivity = 0.733, Specificity = 0.667.

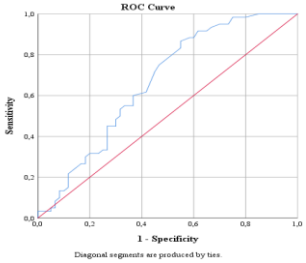


Figure 36. ROC curve for Apo A1LP.

J index= 0.317, Sensitivity = 0.867, Specificity = 0.450

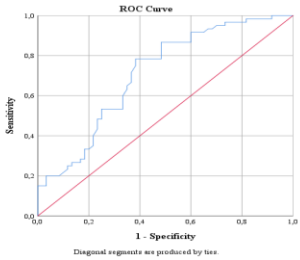


Figure 37. ROC curve for TFR.

J index= 0.400, Sensitivity = 0.783, Specificity = 0.617.

What is the change of the five proteins depending on the stage of OC – early or late? To answer this question, we divided women with OC into two groups: women with early-stage OC, including stage I and II, and women with late-stage OC, including stage III. There were no patients in stage IV OC in our sample. The distribution of patients is presented in Figure 21.

Table 30. Summary statistical characteristics of the five proteins in the group of women with early stage OC (n=38).

Proteins	Characteristics				
	Mean ± SD	95% CI	Me	Min	Max
Prealbumin	0.18 ± 0.08	0.15-0.21	0.19	0.14	0.31
TFR	2.08 ± 0.77	1.83-2.33	1.96	1.07	3.9
ApoA1LP	1.70 ± 0.29	1.61-1.80	1.71	1.38	2.62
CA125	587,98 ± 506,61	368.59-787.37	361.25	2.1	450.5
β2MG	2,88 ± 2,15	2.17-3.59	2.36	0.74	3.21

Table 31. Summary statistical characteristics of the five proteins in the group of women with late stage OC (n=22)

Proteins	Characteristics				
	Mean ± SD	95% CI	Me	Min	Max
Prealbumin	0.17 ± 0.07	0.14-0.20	0.17	0.14	0.31
TFR	2.26 ± 0.54	2.02-2.50	2.32	1.07	3.9
ApoA1LP	1.67 ± 0.27	1.55-1.79	1.60	1.38	2.62
CA125	731.75 ± 591.67	469.42-994.08	666.30	2.1	450.5
β2MG	2.81 ± 1.14	2.30-3.32	2.95	0.74	3.21

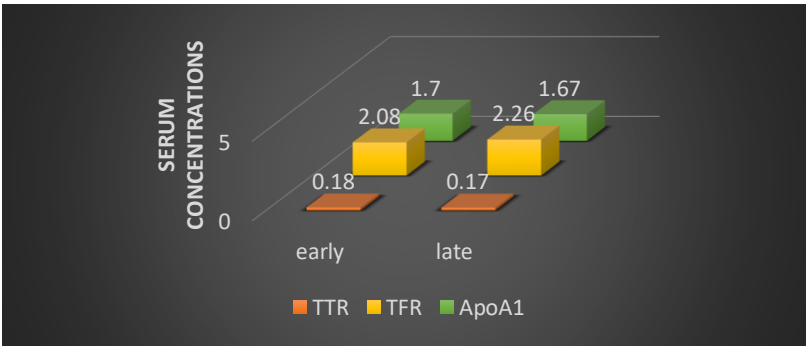


Figure 38. Mean values of prealbumin, transferrin and ApoA1LP in early and late stage OC.

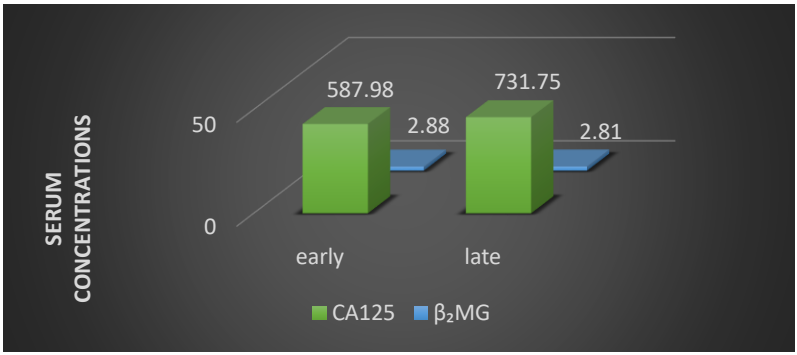


Figure 39. Mean values of CA125 and β_2 MG in early and late stage OC.

Using comparative analysis, we calculated the level of statistical significance for the five proteins according to the stage of OC and found that there was no statistically significant difference in the levels of all five investigated markers in individuals with late and early stage OC.

Using correlation analysis, we did not establish a correlation between stage and the five studied biomarkers.

Third stage of the study

Modeling ovarian tumor associations by logistic regression.

This program was developed specifically for our study and with it we aim to show more precisely the relationships between the presence of ovarian tumor, age and the five laboratory biomarkers.

- **Our research question is:**

"What is the weight of the six factors: age, Prealbumin, ApoA1LP, CA125, TFR and β2MG in determining the risk of ovarian tumor formation?"

Table 32. Logistic model of the relationship between the presence of an ovarian tumor and the six quantitative factors.

	B	S.E.	Wald	Sig.	Exp(B)	95% CI for Exp(B)	
						Lower	Upper
Years	0.029	0.018	2.493	0.114	1.029	0.993	1.067
Prealb β_1	-0.019	0.005	12.573	**<0.001**	0.982	0.972	0.992
Apo A1 LP	1.487	0.936	2.522	0.112	4.424	0.706	27.727
CA125 β_2	0.046	0.014	10.778	**0.001**	1.047	1.019	1.076
TFR	0.334	0.428	0.609	0.435	1.397	0.604	3.233
β2MG	0.248	0.263	0.894	0.345	1.282	0.766	2.145
Constant β_0	-1.309	2.150	0.371	0.543	0.270		

We denote by Y the presence of an ovarian tumor - the alternative dependent variable. The two values are 1 (the patient has an ovarian tumor) and 0 (the patient does not have an ovarian tumor), and the probabilities of their occurrence are p and (1-p), respectively. Let X be a given indicator - age or clinical-laboratory indicator, i.e. independent variable.

The parameter estimates are given in the Exp(B) column of Table 35. Each of these estimates, which is associated with a quantitative factor variable, indicates how many times (or by what percentage) the chance of Y will change as the corresponding factor variable increases by unit at a controlled (constant) level of other factors.

Estimated probabilities are less than the significance level α. This confirms the statistical significance for ovarian tumor development of two parameters - prealbumin $p < 0.001$ and CA125 $p = 0.001$.

Based on the results, the linear form of the estimated logistic model will be:

$ln\frac{\hat{p}}{1-\hat{p}} = -1.309 - 0.019X_1 + 0.046X_2$ where: **X_1 - Prealbumin**

X_2 - CA125

The type of model estimated, expressed in terms of the probability of occurrence of the outcome event Y, will have the following form:

$$\hat{p} = \frac{1}{1 + e^{-(-1.309 - 0.019X_1 + 0.046X_2)}}$$

The chance of having an ovarian tumor:

• will increase 0.982 times, respectively by 1.8%, when Prealb is reduced by 0.01 g/L;

• will increase 1.047 times, respectively by 4.7%, when increasing CA125 by 1 IU/mL.

The regression coefficients B for the remaining quantitative variables are statistically insignificant. Therefore, we do not have sufficient confidence to interpret their corresponding odds ratios Exp(B).

The parameter estimates in this kind of model cannot be interpreted directly. They are only used to calculate the probability of Y occurring at different values of the factor variables.

Multiple logistic regression for patients with OC and ovarian cyst.

- **A formula involving the five proteins to be administered in the preoperative differentiation of OC from an ovarian cyst.**

Menopause Model - The parameter estimates are given in column exp(B) of Table 33. Each of these estimates, which is associated with a quantitative factor variable, indicates how many times (or by what percentage) the chance of Y will change as

the the corresponding factor variable with unity at a controlled (constant) level of the remaining factors.

Table 33. Logistic model of the relationship between **ovarian cyst** and the five quantitative factors in menopausal women.

	B	S.E.	Wald	Sig.	Exp(B)	95% CI за Exp(B)	
						Lower	Upper
Prealbumin	0.002	0.010	0.054	0.816	1.002	0.984	1.021
Apo A1 LP	3.212	2.589	1.539	0.215	24.833	0.155	3969.273
CA125	-0.010	0.005	3.982	0.046	0.990	0.980	1.000
TFR	0.193	0.905	0.045	0.831	1.212	0.206	7.143
β2MG	-0.126	0.462	0.075	0.785	0.881	0.357	2.179
Constant	-5.398	4.956	1.186	0.276	0.005		

The chance of having an **ovarian cyst** in menopausal women will:

• **increased 1.002 times**, respectively by 0.2%, when increasing Prealb (X_1) by 0.01 g/L;

• **increased 24,833 times**, respectively by 2483.3%, when Aro A1 LP (X_2) was increased by 1 g/L;

• **increased 0.01 times**, respectively by 1.0% when CA125 (X_3) decreased by 1 IU/mL;

• **increased 1,212 times**, respectively by 21.2% when increasing TFR (X_4) by 1g/L;

• **increased 0.119 times**, respectively by 11.9% when β2MG (X_5) was reduced by 1mg/L.

The type of model estimated, expressed in terms of the probability of having an ovarian cyst in menopausal women, would look like this:

$$\hat{p} = \frac{1}{1 + e^{-(-5.398 + 0.002X_1 + 3.212X_2 - 0.010X_3 + 0.193X_4 - 0.126X_5)}}$$

f – Non-prime number = 2.718

Premenopausal Model – Parameter estimates are given in the Exp(B) column of Table 34.

Table 34. Logistic model of the relationship between the presence of an **ovarian cyst** and the five quantitative factors in premenopausal women.

	B	S.E.	Wald	Sig.	Exp(B)	95% CI за Exp(B)	
						Lower	Upper
Prealbumin	-0.002	0.010	0.051	0.822	0.998	0.978	1.018
ApoA1 LP	2.155	2.296	0.881	0.348	8.630	0.096	776.384
CA125	-0.013	0.005	8.609	0.003	0.987	0.978	0.996
TFR	0.584	0.674	0.749	0.387	1.792	0.478	6.717
β2MG	-0.279	0.589	0.225	0.635	0.756	0.238	2.401
Constant	-1.933	3.631	0.283	0.595	0.145		

This indicates that the chance of having an **ovarian cyst** in premenopausal women will:

• **increased 0.002 times**, respectively by 0.2%, when Prealb (X_1) decreased by 0.01 g/L;

• **increased 8,630 times**, respectively by 863.0%, when Aro A1 LP (X_2) was increased by 1 g/L;

• **increased 0.013 times**, respectively by 1.3% when CA125 (X_3) was reduced by 1 IU/mL;

• **increased 1,792 times**, respectively by 79.2% when increasing TFR (X_4) by 1 g/L;

• **increased 0.244 times**, respectively by 24.4% when β2MG (X_5) was reduced by 1mg/L.

The type of model evaluated, expressed in terms of the probability of having a cyst in premenopausal women, would look like this:

$$\hat{p} = \frac{1}{1 + e^{-(-1.933 - 0.002X_1 + 2.155X_2 - 0.013 + 0.584X_4 - 0.279X_5)}}$$

General model independent of menopausal status - Parameter estimates are given in column exp(B) of the following table.

Table 35. Logistic model of the relationship between the presence of an **ovarian cyst** and the five quantitative factors regardless of menopausal status.

	B	S.E.	Wald	Sig.	Exp(B)	95% CI за Exp(B)	
						Lower	Upper
Prealbumin	-0.002	0.007	0.089	0.765	0.998	0.985	1.011
ApoA1 LP	2.616	1.633	2.566	0.109	13.676	0.557	335.600
CA125	-0.012	0.003	12.639	0.000	0.988	0.982	0.995
TFR	0.481	0.498	0.934	0.334	1.618	0.609	4.298
β2MG	-0.394	0.322	1.493	0.222	0.675	0.359	1.268
Constant	-2.846	2.714	1.099	0.294	0.058		

This indicates that the chance of having an **ovarian cyst** in a proven ovarian tumor regardless of menopausal status will:

• **increased 0.002 times**, respectively by 0.2%, when Prealb (X_1) decreased by 0.01 g/L;

• **increased 13.676 times**, respectively by 1367.6%, when Aro A1 LP (X_2) was increased by 1 g/L;

• **increased 0.012 times**, correspondingly by 1.2% when CA125 (X_3) decreased by 1 IU/mL;

• **increased 1,618 times**, respectively by 61.8% when increasing TFR (X_4) by 1g/L;

• **increased 0.325 times**, respectively by 32.5% when lowering β2MG (X_5) by 1mg/L.

The type of model evaluated, expressed in terms of the probability of the presence of a cyst, will look like this:

$$\hat{p} = \frac{1}{1 + e^{-(-2.846 - 0.002 + 2.616X_2 - 0.012X_3 + 0.481X_4 - 0.394X_5)}}$$

We used the logistic model regardless of menopausal status and calculated for each patient with OC and an ovarian cyst the probability that the diagnosed ovarian tumor was an ovarian cyst. Through ROC analysis and construction of an ROC curve, we determined the limit value for this probability - **60%**.

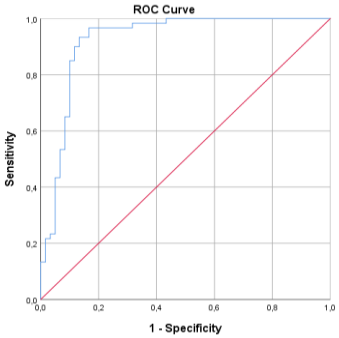


Figure 40. ROC curve of the probability $\hat{p}$.

Table 36. AUC probability coefficient $\hat{p}$.

Area Under the Curve					
				95% CI	
Тест	AUC	Std. Error	p	Lower Bound	Upper Bound
probability $\hat{p}$	**0.923**	0.027	0.000	0.870	0.976

It can be seen that the diagnostic value of our calculated probability is high. Its statistical significance is p = 0.000 - less than the 0.05 significance level. The AUC coefficient was high (AUC=0.923; 95% CI 0.870 ÷ 0.976). The calculated threshold is ≥ 60%, which determines Sn 96.7% and Sp 83.3%.

We also calculated a J index for the probability $\hat{p}$.

J index= 0.800, Sensitivity = 0.967, Specificity = 0.833.

For greater precision of the results, we also applied the logistic models for menopause and premenopause. We calculated for each patient with OC and an ovarian cyst in menopause and in premenopause the probability ($\hat{p}$) that the diagnosed ovarian

tumor was an ovarian cyst. Through ROC analysis and ROC curve construction, we determined the cutoff values for this probability in both models.

Menopausal pattern: The cut-off probability of an ovarian cyst is **30%**.

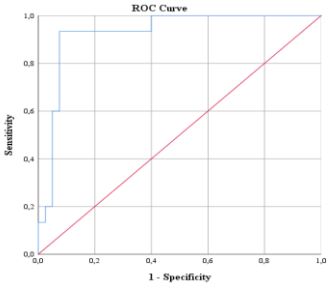


Figure 41. ROC curve of probability $\hat{p}$ in menopausal model.

Table 37. AUC probability coefficient $\hat{p}$ in a menopausal model.

Area Under the Curve					
				95% CI	
Тест	AUC	Std. Error	p	Lower Bound	Upper Bound
probability $\hat{p}$	0.927	0.038	0.000	0.852	1.000

The diagnostic value of our calculated probability is high. Its statistical significance p is 0.000 - less than the 0.05 significance level. The AUC coefficient is high (AUC=0.927; 95% CI 0.852 ÷ 1.000;). The calculated threshold is ≥ 30 %, which determines Sn 93.3 % and Sp 90 %.

We also calculated the J index for the probability ($\hat{p}$) in menopausal women.

J index= 0.833, Sensitivity = 0.933, Specificity = 0.900.

Premenopausal model: The cutoff probability for an ovarian cyst is **78%.**

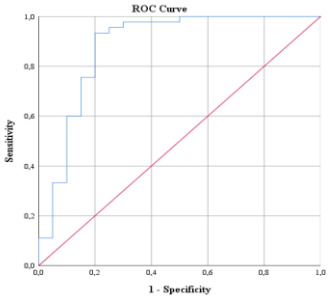


Figure 42. ROC curve of the probability $\hat{p}$ in a premenopausal model.

Table 38. AUC probability coefficient $\hat{p}$ in premenopausal model.

Area Under the Curve					
				95% CI	
Тест	AUC	Std. Error	p	Lower Bound	Upper Bound
probability $\hat{p}$	0.880	0.056	0.000	0.770	0.990

The diagnostic value of our calculated probability is high. Its statistical significance p is 0.000 - less than the 0.05 significance level. The AUC coefficient is high (AUC=0.880; 95% CI 0.770 ÷ 0.990;). The calculated threshold is ≥ 78 %, which determines Sn 93.3 % and Sp 80 %.

We also calculated a J index for the probability ($\hat{p}$) in premenopausal women.

J index= 0.733, Sensitivity = 0.933, Specificity = 0.800.

DISCUSSION

Ovarian tumors are common in women of all age groups. The analyzes of our study confirm these data - the age of the observed patients with ovarian tumor varies from 21 years to 83 years.

Currently, the detection of an ovarian tumor is performed by a gynecological examination with TVUS and the measurement of the tumor marker CA125.

In our study, for the first time in Bulgaria, we investigated the changes that occur in the concentrations of five serum proteins in women with ovarian tumors (n=120) and in healthy women without proven ovarian tumors (n=60).

Through the information obtained from the patients' files and anamnestic data, we were able to exclude other conditions that may increase or decrease the concentrations of the biomarkers we determined.

Menopause is a period of women's life that is characterized by a permanent cessation of ovarian function, and many literature sources describe changes in the concentrations of various laboratory parameters in this period.

To examine how menopause affects the concentrations of the five proteins in the entire patient sample (n=180), i.e. whether it affects biomarker levels, both in healthy women and in women with proven ovarian tumor, we divided by menopausal status.

Our results showed that a statistically significant relationship was observed between menopausal status and four of the biomarkers we studied - transthyretin, transferrin, CA 125 and β_2 microglobulin. There was no statistically significant association between menopausal status alone and ApoA1lipoprotein in the entire group of patients studied by us.

Changes occurring in the concentrations of five serum proteins in women with ovarian tumors.

Comparing the group of women with ovarian tumor and the control group of healthy women, we found that the concentrations of observed biomarkers in healthy women were within reference limits. Through correlation analysis, we found that age and

menopausal status in healthy women **are not factors** that would influence or lead to a change in the plasma concentrations of the measured proteins.

In our study, it was found that the concentrations of four of the investigated biomarkers, namely transthyretin, transferrin, ApoA1lipoprotein and β_2 microglobulin in the group of women with ovarian tumor were within reference limits. The CA 125 level is significantly elevated beyond the reference range. This confirms the fact that CA 125 is a tumor marker related to processes occurring in the ovary and elevated levels of CA125 are observed in both benign and malignant ovarian diseases .

Our comparative statistical analysis between the two working groups of patients - women with ovarian tumor and healthy women proved that the presence of ovarian tumor leads to statistically significant differences in the levels of four of the plasma proteins for both groups. Our results showed that in women with ovarian tumor, the concentrations of transthyretin and transferrin are decreased, and the concentrations of CA125 and β_2 MG are increased. ApoA1LP levels decreased to a very small extent in the group of women with ovarian tumors, and this change was statistically insignificant.

We proved different correlational dependences between the observed proteins, which also confirm the conclusion made above that changes occur in the levels of the plasma proteins studied by us in women with ovarian tumors and these changes are in different correlations between each other.

How does menopause affect the changes occurring in the concentrations of the five serum proteins in women with ovarian tumor?

Dividing the patients according to the menopausal status and analyzing the obtained results, we found that in the menopausal patients with ovarian tumor (n=55), the levels of transthyretin and transferrin were lower, the levels of CA125 and β2MG were significantly higher, and the level of ApoA1LP was marginally lower compared to the levels of these serum biomarkers in premenopausal women with ovarian tumors (n=65).

We can conclude that in women with ovarian tumors, the menopausal status **is a factor** that affects the concentration of the biomarkers we determined. Menopause has minimal effect on the change in ApoA1LP level.

In addition, we found that in menopausal women with ovarian tumor, the levels of three of the proteins - transthyretin, CA 125, and β_2 microglobulin were outside the reference range, while in premenopausal women with ovarian tumor, only CA125 levels were outside the reference range.

We found different statistically significant correlations between different proteins in the group of menopausal and in the group of premenopausal women with proven ovarian tumor. Some of these dependencies are common to both groups:

• transthyretin and ApoA1lipoprotein - positive relationship

• transthyretin and transferrin - positive relationship

• transthyretin and CA125- negative relationship

• CA125 and β_2 microglobulin - positive relationship.

This gives us reason to conclude that the changes in the concentrations of these pairs of serum proteins in women with ovarian tumors correlate with each other regardless of the menopausal status of the patients.

What is the diagnostic sensitivity and specificity of the investigated biomarkers in the diagnosis of ovarian tumor?

Using the limit values (cut off) and reference limits for the investigated indicators according to the recommendations of the manufacturers of the individual kit reagents, we checked their diagnostic qualities. According to the results obtained by us, related to the diagnostic reliability for proving ovarian tumor, the best sensitivity (63.3%), specificity (96.7%), PPV (97.4%) and NPV (56.9%) showed CA 125 (table 14) .

In order to improve the diagnostic qualities of the five proteins, we determined threshold values for our sample of patients that would best distinguish women with ovarian tumors from women without ovarian tumors, namely:

• for CA125 $\geq$ 31.5 mU/L

• for TTR $\leq$ 0.25 g/L

• for TFR ≤1.98 g/L

• for β_2 MG ≥ at 2.06 mg/L

• for ApoA1LP ≤ 1.94 g/L

When we applied the thresholds we determined, the best Sn was ApoA1 LP 77.5%, the best Sp 96.7% were CA125 and TFR, the highest PPV 97.5% and NPV 58% was again CA125 (Table 15).

The terms screening and diagnosis are often used interchangeably, but there is an important distinction between them in terms of both their clinical use and performance requirements. A screening test is used to assess an individual's risk of developing a disease and is usually targeted at asymptomatic individuals, but may be specifically targeted at at-risk populations. Screening tests should be focused on achieving a high sensitivity for disease detection in order to limit the number of false negative results leading to missed diagnoses in the study population. A diagnostic test, on the other hand, is used to determine whether or not a person has a given disease and is targeted at individuals who are symptomatic. As such, the diagnostic test must be highly accurate in determining the disease, in order to achieve high specificity for disease diagnosis.

According to the data from ROC curve analysis and AUC coefficients (table 16), we found that the diagnostic value of TTR, CA125, TFR and β_2 MG for diagnosing ovarian tumor is high. Their p values are less than the 0.05 significance level, their specificity is over 78%, and the PPV is over 85%. This gives reason to believe that these indicators can be used as **diagnostic tests** in the presence of an ovarian tumor. AUC coefficients of CA125, TTR and β_2 MG were the highest, 0.814, respectively; 0.781; 0.694, which gives us reason to believe that these three indicators can correctly distinguish women with ovarian tumors from healthy women in 69%.

Differentiating benign from malignant ovarian tumors is key to success in the successful treatment of ovarian tumors.

OC is the eighth most common cancer in women worldwide and is the leading cause of death among all gynecological malignancies. The lack of specific symptoms and

effective screening programs to detect the disease at an early stage are the reason for the high mortality and that almost 70% of patients are diagnosed with advanced disease (stage III or IV) and their 5-year survival is less than 30 %. Early detection of OC is a long-sought goal and challenge. Many researchers have tried to develop diagnostic models for the diagnosis of ovarian cancer in order to improve its detection.

This led us to investigate whether the five biomarkers we defined could serve in the diagnosis of OC, which could be used in the preoperative differentiation of malignant from benign ovarian tumors.

In our study, from the group of women with ovarian tumors, n=60 had OC and n=60 had ovarian cysts.

Comparing the age distribution of the two types of ovarian tumors in our sample of patients, we can draw the following conclusion: OC predominates in the age range of 51-70 years, while ovarian cysts are more common in the age range of 31-50 years.

According to various literature sources, high-grade epithelial serous ovarian carcinoma accounts for approximately 70–80% of all ovarian cancer subtypes. Our study confirms these data. In our study, this type of OC was diagnosed in 88.7% of patients.

What changes occur in the concentrations of the five serum proteins in women with OC and women with ovarian cysts

Our results showed that in the group of OC patients, the levels of transthyretin, CA125 and β_2 microglobulin were outside the reference limits, while in the group of women with ovarian cysts, only the concentration of CA125 was slightly above the cut-off value of 35 IU/mL.

We found that transthyretin, transferrin, and ApoA1lipoprotein values were significantly lower in women with OC compared to women with ovarian cysts. CA125 and β_2MG have significantly higher concentrations in the group of patients with OC compared to patients with ovarian cysts. These differences in the concentrations of the studied biomarkers are statistically significant.

There have been several prospective studies related to one of the FDA-approved assays, the OVA 1 test, which includes the five proteins we studied and provides an estimate of the likelihood of malignancy for an ovarian tumor. Our results confirmed the data from these studies that, in the presence of OC, the levels of transthyretin, transferrin, and ApoA1lipoprotein are decreased, and the levels of CA125 and β_2MG are increased.

Influence of menopausal status on the five proteins in women with OC and ovarian cysts

Our results show that in menopausal women with OC, TTR and transferrin values were lower than reference values, and CA125 and β_2 MG values were higher than reference values. In premenopausal women with OC, there were changes outside the reference limits in the concentration of TTR - lower than the reference rank and of CA125 - higher than the reference rank.

We found a moderate positive correlation between menopausal status and β_2 microglobulin and a moderate negative correlation between menopausal status and transferrin.

Comparing the mean values of the five proteins in menopausal women with OC (n=40) and premenopausal women with OC (n=20), we found statistically significant differences in TFR and β_2 MG ($p < 0.05$). For the other three proteins - transthyretin, ApoA1LP and CA125, we did not find statistically significant differences ($p > 0.05$).

Comparing the mean values of the five investigated biomarkers in menopausal and premenopausal women with OC, we found lower concentrations of transthyretin, transferrin and ApoA1lipoprotein in menopausal women, while the values of CA125 and β_2 MG in menopausal patients were higher than in women with premenopausal ovarian carcinoma.

Menopausal status in women with OC was a significant factor most strongly associated with changes in TFR and β_2 MG concentrations.

The results of our study show that in patients with ovarian cysts, there is a change outside the reference values only in the CA125 values - they are higher than the reference limits, both in menopausal and premenopausal women.

In the group of women with ovarian cysts, we found a moderate positive correlation between menopausal status and β_2 microglobulin.

Comparing the mean values of the five proteins in the group of menopausal women with ovarian cysts (n=15) and premenopausal women with ovarian cysts (n=45), we found statistically significant differences in β_2 MG and CA125 ($p< 0.05$). For the other three proteins - TTR, ApoA1LP and TFR, we did not find statistically significant differences ($p> 0.05$).

Comparing the mean values of the five proteins in menopausal and premenopausal women with ovarian cysts, we found that transferrin and CA125 concentrations were lower in menopausal patients. TTR and β_2 MG levels in postmenopausal women with ovarian cysts were higher compared to premenopausal women with ovarian cysts. ApoA1lipoprotein concentrations in both menopausal and premenopausal patients with ovarian cysts are almost the same.

Menopausal status in women with ovarian cysts was a significant factor most strongly associated with changes in β_2 MG and CA125 concentrations.

What is the diagnostic sensitivity and diagnostic specificity of each protein for differentiating ovarian carcinoma from ovarian cyst?

When applying a cut-off value for CA125 of 35 IU/mL, data from studies have shown that its sensitivity in distinguishing between benign and malignant tumors ranges from 61 to 90%, while its specificity ranges from 35 to 91%. The positive predictive value varies between 35 and 91%, and the negative predictive value is between 67 and 90%.

Using the same threshold value, our results showed 90% Sn and 63.3 % Sp, 71% PPV and 86.4 % NPV for this tumor marker to distinguish between benign and malignant ovarian tumors.

There are published data that when values below 35 IU/mL are defined as normal, CA125 is elevated in 80% of epithelial ovarian cancers. In our study, we confirm this - CA 125 is above 35 IU/mL in 90% of women with epithelial ovarian carcinoma.

There are literature reports that CA125 levels remain normal in mucinous, endometrioid, and clear cell epithelial carcinomas. Our results confirmed that the serum concentration of CA125 in endometrioid epithelial OCs was within reference limits, but in mucinous and clear cell epithelial OCs, it was significantly elevated. The two patients with histologically proven endometrioid epithelial OC had normal CA125 values, while in the patients with clear cell (n=3) and mucinous (n=2) OC, CA125 values were significantly elevated.

According to other literature sources, CA125 is elevated in approximately 50%–60% of early-stage epithelial ovarian carcinomas and 75%–90% of patients with advanced-stage disease. The data from our study indicate that CA125 is elevated in 89.5% of patients with early (I and II) stage of OC and in 90.9% of patients with late (III and IV) stage of OC.

According to various authors of publications, CA125 levels in women without ovarian cancer remain normal, while levels associated with malignancy tend to increase. We confirm this statement. Our results indicate that 96.7% of healthy women (without ovarian tumor) have normal values of CA 125. Only 2 women (3.33%) have slightly elevated values of the tumor marker, which proves other statements that its concentration can be influenced by additional factors: smoking, obesity, age, ethnicity. From the anamnestic data of the two patients with elevated CA125 values, we received information that they were long-term smokers.

There are literature data suggesting that CA125 concentrations > 95 IU/mL in menopausal women can help differentiate benign from malignant tumors, and our observations support this fact. In menopausal women with OC, 59 (98.3%) had CA125 > 95 IU/mL.

There is evidence that transthyretin is downregulated in epithelial OC. In our study, 40 (66.7%) of the women with OC had decreased TTR values.

The level of ApoA1LP has been reported to decrease in the sera of patients with ovarian cancer. Our results showed that ApoA1LP levels were decreased in only 12 (20%) of the OC patients and its Sn was low as an independent marker.

Some authors reported decreased levels of transferrin in the serum of patients with ovarian cancer. In our study, 31 (51.7%) of women with OC showed decreased TFR values.

TTR, TFR and ApoA1LP are negative reactants of the acute phase of inflammation. Their concentrations decrease in the presence of inflammation in the body. With our results, we confirm the theory that tumorigenesis is closely related to inflammation .

We determined threshold values for our identified biomarkers and performed ROC curve analysis to examine the diagnostic ability of the biomarkers to differentiate OC patients from ovarian cyst patients.

The threshold values we set are as follows:

CA 125- $\geq$ 99.05 IU/mL;

β_2 MG - $\geq$ 1.295 mg/L;

TTR - $\leq$0.205 g/L;

ApoA1LP- $\leq$1.615g/L;

TFR - $\leq$ 2.37g/L,

We found that the diagnostic value of all five serum proteins is high. Their p values are less than the 0.05 significance level (table 29). This gives reason to believe that these indicators can be used as **diagnostic tests** to differentiate OC from ovarian cyst. AUC coefficients are high for all proteins and exceed 0.666. Their threshold values determine very good Sn and Sp (table 28). For CA125 Sn 81.7% Sp 90%; for transthyretin Sn 73.3% Sp 66.7%; for TFR Sn 78.3% Sp 61.7 %; for ApoA1LP Sn 86.7% Sp 45%; for β_2 MG Sn 85% Sp 70%. The high Sn of the five proteins gives reason to believe that they can also be used as **screening tests** to prove OC. We can conclude from the obtained AUC coefficients that these indicators can correctly distinguish OC from ovarian cyst in 66.6% and can be used in preoperative diagnosis.

What is the change of the five proteins depending on the stage of OC – early or late?

According to our results, observing the patients with early and late stage of OC, we found that there was a change outside the reference rank in the concentrations of three of the proteins - transthyretin, CA125 and β_2 MG. But statistically significant differences between the two groups were not observed. We also found no correlation between the stage and the levels of the five studied biomarkers.

This shows that both in early and late stage OC, the levels of the five biomarkers undergo stage-independent changes.

Modeling ovarian tumor associations by logistic regression

Applying logistic regression analysis, we first checked the weight of the six factors: age, TTR, ApoA1, CA125, TFR and β_2MG in determining the risk of ovarian tumor formation. We confirmed the statistical significance for ovarian tumor development of two parameters - TTR and CA125. We reached the following conclusions:

• An increase in TTR of 0.01 g/L will reduce the chance of a tumor being present by 0.8% to 2.8%.

• An increase in CA125 of 1 IU/mL will increase the chance of a tumor being present by 1.9% to 7.6%.

Regarding the chance of ovarian tumor formation, we can make the following conclusion:

The chance of having an ovarian tumor:

• will increase as TTR decreases and as CA125 increases

This could be used in practice as a diagnostic test in symptomatic women. The detection of low TTR values and elevated CA 125 levels and the presence of non-specific symptoms (dyspepsia, bloating, early satiety, flatulence) is a reason to perform a gynecological examination with TVUS.

The development of a test or algorithm to provide an accurate approach to ovarian malignancy risk assessment has been a long-sought goal. A multivariate analysis

including tumor biomarkers along with ultrasound and an appropriate algorithm would be useful in the preoperative diagnosis of ovarian tumors.

The high AUC coefficients from the ROC curve analysis made us to look for some formula including the five proteins and menopausal status for differential diagnosis between OC and ovarian cyst to be applied preoperatively.

A formula with the participation of the five proteins to be used in the preoperative differentiation of OC from ovarian cyst.

The results of our study are similar to the results of the multi-biomarker analysis OVA1, which serves to assess the level of malignancy risk in OC patients and is also based on the five biomarkers - transthyretin, transferrin, ApoA1lipoprotein, CA125 and β_2 microglobulin. This test combines the results of the five protein levels with information about the patient's menopausal status to classify the OC risk group. OVA1 has shown 96% sensitivity, 35% specificity, 40% positive predictive value (PPV) and 95% negative predictive value (NPV) .

Again using logistic regression we analyzed three models: in menopausal women, premenopausal women and an overall model regardless of menopausal status. We created formulas for each model to obtain the **probability** that a given ovarian tumor was an ovarian cyst. Through them, we can say how many times (respectively by how much percentage) the chance of an ovarian cyst will change when each protein increases or decreases by one unit at a constant level of other factors.

Our results showed that the chance of having an **ovarian cyst in menopausal women** will:

• **increased** with an increase in TTR, Apo A1 lipoprotein and TFR and with a decrease in CA125 and β_2MG.

Our results showed that the chance of having an **ovarian cyst in premenopausal women** will:

• **increased** with an increase in TFR and Apo A1 lipoprotein and with a decrease in TTR, CA125 and β_2MG.

The results showed that the chance of having an **ovarian cyst in a proven ovarian tumor regardless of menopausal status** would:

• **increased** with an increase in TFR and Apo A1 lipoprotein and with a decrease in TTR, CA125 and β_2MG.

Using these regression models and ROC analysis, we constructed ROC curves and calculated threshold values for the probability of an ovarian cyst.

In the model regardless of menopausal status, we obtained a probability threshold ≥ 60%. AUC coefficient is high 0.923. This threshold value showed good Sn 96.7% and Sp 83.3%. This gives us reason to believe that it, as an "indicator", can correctly distinguish an ovarian cyst from OC regardless of menopausal status in 92.3%.

In the menopausal model, we obtained a probability threshold of ≥ 30%. This threshold value showed good Sn 93.3 % and Sp 90 %. The AUC coefficient is high 0.927 This gives us reason to believe that it as an "indicator" can correctly distinguish ovarian cyst from OK in menopausal women in 92.7%.

In the premenopausal model, we obtained a probability threshold of ≥ 78%. This threshold value showed good Sn 93.3 % and Sp 80 %. The AUC coefficient is high 0.880 This gives us reason to believe that it as an "indicator" can correctly distinguish ovarian cyst from OC in premenopausal women in 88.0%.

The statistical significance of the probability in all three logistic models is 0.000 - less than the 0.05 significance level. This gives reason to believe that it can be used as a **diagnostic test** in the preoperative differentiation of ovarian cyst from OC.

The high diagnostic Sn we determined for the "indicator" probability $\hat{p}$ in all three logistic models gives us reason to believe that it can also be used as a **screening test** in asymptomatic individuals or patients at risk.

Each of the formulas we have developed can be applied in a computer program and applying the values obtained from the concentrations of each of the five plasma proteins, a result can be obtained for the "probability" of a given ovarian tumor being differentiated as an ovarian cyst or ovarian carcinoma. This could be used in the

preoperative diagnosis, after a gynecological examination has been performed and an ovarian tumor has been identified.

These tests are readily available, noninvasive, adaptable to the majority of analysts we work with in the clinical laboratory, can be routinely screened, and are cost-effective.

We believe that the research and results of this dissertation work would help the diagnosis of ovarian cancer to improve its detection and thus increase the survival of patients with this insidious disease.

CONCLUSIONS

1. In healthy women, the concentrations of the five proteins are within reference limits. Age and menopausal status were not factors that would influence or lead to a change in the plasma concentrations of the measured proteins in women without proven ovarian tumor.

2. In the presence of an ovarian tumor, there are statistically significant changes in the levels of the five observed serum proteins. The concentrations of TTR, TFR and ApoA1LP are decreased, and the concentrations of CA125 and β_2 MG are increased.

3. In women with ovarian tumors, the menopausal status is a factor that affects the concentration of the biomarkers we determined.

a/ The levels of TTR, TFR and ApoA1LP were lower in menopausal women with ovarian tumor than in premenopausal women. CA125 and β2MG levels were significantly higher in menopausal women with ovarian tumors than in premenopausal women.

b/ In menopausal women with ovarian tumor, TTR, CA 125 and β_2 MG levels were outside the reference range, while in premenopausal women with ovarian tumor, only CA125 levels were outside the reference range.

4. We found that the diagnostic ability of transthyretin, CA125 and β_2 MG is relatively good - they can correctly distinguish patients with ovarian tumor from women without ovarian tumor in 69%, and in our opinion these indicators can be used as diagnostic tests in the presence of ovarian tumor.

5. Comparing the two subgroups - women with OC and women with ovarian cysts, we found that:

a/ In the patients with OC, the levels of TTR, CA125 and β_2 MG were outside the reference limits, while in the group of women with ovarian cysts, only the concentration of CA125 was outside the reference values.

b/ There are statistically significant differences in the concentrations of all five plasma proteins. The levels of TTR, TFR and ApoA1 LP were significantly lower in women with OC compared to their levels in women with ovarian cysts. CA125 and

β_2 MG had significantly higher concentrations in the group of patients with OC, compared to patients with ovarian cysts.

6. Comparing menopausal and premenopausal women with OC we found that:

a/ In menopausal women, there are deviations in the reference values for TTR, TFR, CA125 and β_2 MG, while in premenopausal women with OK outside the reference range are TTR and CA125.

b/ There are statistically significant changes in the concentrations of TFR and β_2 MG.

c/ TTR, TFR and ApoA1LP concentrations were lower and CA125 and β_2 MG concentrations were higher in menopausal women with OC compared to premenopausal women with OC.

7. Comparing menopausal and premenopausal women with ovarian cysts, we found that:

a/ in both groups of patients, there are deviations in the reference values only in the CA125 values.

b/ there are statistically significant changes in the concentrations of CA125 and β_2 MG.

c/ TFR and CA125 concentrations were lower and TTR and β_2 MG concentrations were higher in menopausal women with ovarian cysts compared to premenopausal women with ovarian cysts. ApoA1 LP levels were almost identical in the presence of an ovarian cyst, regardless of menopausal status.

8. We found that the changes that occur in biomarker levels in the presence of OC are independent of carcinoma stage.

9. We found that the diagnostic ability of the five proteins to distinguish OK from ovarian cyst is relatively good (AUC $\geq$ 0.666). The high diagnostic Sp ($\geq$ 61.7 %) of four of the proteins: CA125, β2 MG, TFR and TTR give us reason to believe that these indicators can be used as diagnostic tests to differentiate OC from an ovarian cyst.

The high diagnostic Sn (> 73 %) of the five proteins gives us reason to believe that they can also be used as screening tests to prove OC.

We believe that they can correctly distinguish OC from ovarian cyst in 66.6% and can be used in preoperative diagnosis.

10. According to the created logistic models, we determined the probability of a given ovarian tumor being an ovarian cyst in: women in menopause, women in premenopause, women without determination of menopausal status. We also determined how the five proteins affected the chance of having an ovarian cyst.

CONTRIBUTIONS

Contributions of original character

1. For the first time in Bulgaria, a study and analysis of the changes in the concentrations of five serum proteins - TTR, TFR, ApoA1LP, CA125 and β_2MG in women with ovarian tumors was carried out.

2. For the first time in Bulgaria, a study and analysis of the changes in the concentrations of the five serum biomarkers and their possibilities for distinguishing benign from malignant ovarian tumors was carried out.

3. For the first time in Bulgaria, an attempt is made to determine threshold values for the levels of the five serum proteins:

a/ as predictors of ovarian tumor.

b/ as differentiating benign from malignant ovarian tumors

3. For the first time in Bulgaria, an adequate system of statistical methods was used for complex analysis and modeling of biomarker relationships:

a/ in view of their diagnostic value in determining the risk of ovarian tumor

b/ with a view to their application in preoperative diagnostics to distinguish OC from an ovarian cyst.

Contributions with scientific applied and confirmatory analysis

1. A significant volume of literature on the subject was analyzed, confirming the relevance of the problem and proving the need for effective programs for the early detection of OC.

2. The informative value and diagnostic possibilities of biomarkers in the presence of ovarian tumor and in the preoperative differential diagnosis of OC and benign ovarian tumors were analyzed.

2. The changes occurring in the concentrations of the five serum proteins in women with OC subjected to the FDA-recognized OVA1 test were confirmed, namely:

a/ decrease in the concentration of transthyretin, transferrin and ApoA1lipoprotein

b/ increasing the concentration of CA125 and β_2MG.

3. The relationship between biomarker values and menopausal status was investigated and the role of menopausal status as a factor affecting changes in the levels of the five biomarkers in women with OC was confirmed.

5. A software product has been created - a formula for:

a/ probability in % of presence of ovarian tumor according to obtained values of transthyretin and CA125 concentrations

b/ the probability in % that an already diagnosed ovarian tumor will be benign.

6. Our results can serve as an information base for optimizing the diagnostic process in patients with ovarian tumors, with a view to the early detection of ovarian cancer.

APPLICATIONS

Logistic models

1. Logistic model for the probability of the presence of an ovarian tumor in relation to the values of transthyretin and CA125.

$$\hat{p} = \frac{1}{1 + e^{-(-1.309 - 0.019 * \boldsymbol{X_1} + 0.046 * \boldsymbol{X_2})}}$$

Where: transthyretin (X_1)

CA125 (X_2)

2. Logistic model for the probability of the presence of an ovarian cyst in menopausal women:

$$\hat{p} = \frac{1}{1 + e^{-(-5.398 + 0.002\boldsymbol{X_1} + 3.212\boldsymbol{X_2} - 0.010\boldsymbol{X_3} + 0.193\boldsymbol{X_4} - 0.126\boldsymbol{X_5})}}$$

Where: transthyretin (X_1)

Aro A1 lipoprotein (X_2)

CA125 (X_3)

TFR (X_4)

β2MG (X_5)

3. Logistic model for the probability of the presence of an ovarian cyst in women with an ovarian tumor regardless of menopausal status

$$\hat{p} = \frac{1}{1 + e^{-(-2.846 - 0.002 + 2.616\boldsymbol{X_2} - 0.012\boldsymbol{X_3} + 0.481\boldsymbol{X_4} - 0.394\boldsymbol{X_5})}}$$

Where: transthyretin (X_1)

Aro A1 lipoprotein (X_2)

CA125 (X_3)

TFR (X_4)

β2MG (X_5)

DISSERTATION-RELATED PUBLICATIONS AND SCIENTIFIC ANNOUNCEMENTS

I. Publications related to the dissertation work:

1. **Racheva, V.Z.**, Ruseva, A.L., Yordanov, A.D. *The role of five plasma proteins in the diagnosis of ovarian tumors.* Gazzetta Medica Italiana Archivio per le Scienze Mediche, 2023, 182(4): 222-230; ISSN:0393-3660; Web of Science, Scopus
2. **Racheva, V.Z.**, Ruseva, A.L., Gorcheva, Z.V., Yordanov, A.D. *The role of β2 microglobulin, transferin and CA125 in the diagnosis of women with ovarian tumors.* Gazzetta Medica Italiana Archivio per le Scienze Mediche, **in press**; ISSN:0393-3660; Web of Science, Scopus
3. **V. Racheva**, A. Ruseva, S. Mateva, I. Malkodanski. *The Role of Three Plasma Proteins in the Diagnosis of Ovarian Tumors*. Journal of Biomedical and Clinical Research. 2022, 15(1): 41-46; ISSN: 1313-6917; Web of Science (CABI)

II. Participation in research projects at Pleven University of Applied Sciences, related to the dissertation work:

1. Change in the levels of some plasma proteins in women with ovarian tumors.

III. Participation in scientific forums in Bulgaria:

1. Poster on the topic "Change in the concentrations of five plasma proteins in women with ovarian tumors" at the XIV National Conference on Clinical Laboratory 14-16.10.2022.